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AFFINITY CHROMATOGRAPHY OF THE ANION TRANSPORT PROTEIN FROM ERYTHROCYTES AND A NOVEL GLYCOPROTEIN FROM KIDNEY

BY

© SANJAY WAMANRAO PIMPLIKAR

A THESIS

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3. ibid. (1988). Identification, purification and characterization of stilbene disulfonate-binding glycoprotein from canine brush-border membranes: a candidate for a renal anion exchanger. J. Biol. Chem. (in press).

4. ibid. (1988). Interaction of Band 3 in erythrocytes and vesicles with an immobilized anion transport inhibitor. Biochim. Biophys. Acta (submitted for publication).

Yours sincerely

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".....I make the wildest theories, connecting up the test tube reaction with broadest philosophical ideas, but spend most of my time in the laboratory, playing with living matter, keeping my eyes open, observing and pursuing the smallest detail. The theories serve to satisfy the mind, prepare it for an "accident," and keep one going. I must admit that most of the new observations I made were based on wrong theories. My theories collapsed, but something was left afterwards."

Albert Szent-Gyorgy

1893-1986

It doesn't matter if you fall down as long as you pick up something from the floor while you get up.

Oswald T. Avery

1887-1955

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, a thesis entitled AFFINITY CHROMATOGRAPHY OF THE ANION TRANSPORT PROTEIN FROM ERYTHROCYTES AND A NOVEL GLYCOPROTEIN FROM KIDNEY submitted by SANJAY W. PIMPLIKAR in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

Supervisor

Date: Dec 21 '87

To my family, especially to Tatya and Aai

ABSTRACT

Band 3, the major integral membrane glycoprotein of human erythrocytes (M_r = 95,000), catalyzes exchange of chloride and bicarbonate *in vivo*. This anion exchange is specifically inhibited by stilbene disulfonates. When added to intact erythrocytes, these inhibitors bind specifically to Band 3 and bring about complete inhibition of anion exchange when bound at 1:1 stoichiometry.

The present investigations were directed towards designing an affinity resin suitable for Band 3 purification, for studying inhibitor-protein interaction and for identifying Band 3-like proteins from non-erythroid tissues. Of the various ligands and spacer molecules tested, an affinity resin prepared by reacting 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS; *Ki* = 10μM) to Affi-Gel 102 was found to be most effective. The solubilized Band 3 bound to the resin and pure Band 3 was subsequently recovered with 1 mM 4-benzamido-4'aminostilbene-2,2'-disulfonate (BADS; *Ki* = 2μM). Optimal binding of Band 3 was achieved at 4 °C with SITS attached to a 6-atom hydrophilic spacer molecule. At 4 °C, Band 3 was weakly bound to the resin and was eluted by BADS. At 37 °C, it was rapidly converted to a strongly bound state not elutable by BADS. The strongly bound state was also obtained by prolonged incubations at 4 °C. Thus, for successful purification of Band 3 protein by affinity chromatography, the experiment should be carried out quickly at low temperatures.

In order to study ligand-protein interactions, Band 3 was partially labelled with 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS). Band 3 dimers, covalently labelled with DIDS in one monomer, bound to SITS-Affi-Gel 102 but did not achieve the strongly bound state. This suggests that the strongly-bound conformation is achieved only when both monomers are functionally intact. Band 3, covalently modified with citrate at the inhibitor binding site, bound poorly to the resin but was converted to the strongly bound form at 37 °C, thereby suggesting that the residue involved in the initial weak binding was not important in achieving the strong binding. Studies with Band 3 *in situ* showed that

both the intact erythrocytes and the rightside-out vesicles bound to immobilized SITS and therefore the inhibitor binding site was accessible to the immobilized ligand. The binding was mediated via the Band 3 protein and was inhibited in the presence of excess free ligand or when the erythrocytes were covalently labelled with DIDS. Inside-out vesicles showed some degree of non-specific binding to the affinity resin.

Proximal tubules of mammalian kidney exhibit stilbene disulfonate-sensitive anion-exchange systems both on the luminal and contraluminal sides of the epithelium. A single polypeptide (M_r = 130,000) from dog renal brush border membranes was shown to specifically bind to SITS-Affi-Gel 102 and eluted by BADS. This binding was abolished in the presence of free BADS or when the solubilized membrane extract was labelled with DIDS. This polypeptide bound to a number of lectins and was sensitive to endo-β-N-acetylglucosaminidase, suggesting it was a glycoprotein. This glycoprotein was specifically labelled with [^{3}H]-H_2DIDS. The data conclusively shows that the 130-kDa glycoprotein is a stilbene disulfonate binding protein and therefore makes a strong candidate for the putative anion exchanger from kidney.

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I have been very fortunate in having made a number of friends within and without the University. The list of people I have to thank is very long and therefore without mentioning individual names I would like to thank all those people who made me feel at home in Canada. Last, and certainly not least, I would like to thank Heather Good for her special friendship.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5P8 buffer	5 mM sodium phosphate, pH 8.0
ALM	anti-luminal membrane
ATP	adenosine-5'-triphosphate
BADS	4-benzamido-4'-aminostilbene-2,2'-disulfonate
Band 3 extract	supernatant from KI-extracted solubilized ghosts in citrate buffer with 1% $C_{12}E_8$
Band 3-5P8 extract	eluate from aminoethyl column in 5P8 buffer with 0.1% $C_{12}E_8$
Band 3-228C8 extract	supernatant from KI extracted solubilized ghosts in citrate buffer with 1% $C_{12}E_8$
BBM	brush border membrane
$C_{12}E_8$	octaethylene glycol mono-n-dodecyl ether;
citrate buffer	228 mM sodium citrate, pH 8.0
DADS	4,4'-diaminostilbene-2,2'-disulfonate
DBDS	4,4'-dibenzamidostilbene-2,2'-disulfonate
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonate
DNDS	4,4'-dinitrostilbene-2,2'-disulfonate
DTT	dithiothreitol
EAC	1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide
EDC	1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride
EDTA	ethylene diamine tetraacetate
KI-E	membranes extracted with EDTA and KI, solubilized with 1 % C12E8, 228 mM sodium citrate, pH 8.0
LDS	lithium dodecyl sulfate

mol. wt.	molecular weight
PAGE	polyacrylamide gel electrophoresis
phosphate-buffered saline	5 mM sodium phosphate-150 mM NaCl, pH 8.0
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate
SPITC	3-sulfophenyl isothiocyanate.
sucrose-citrate buffer	28.5 mM sodium citrate-205.3 mM sucrose, pH 8.0

I. INTRODUCTION

A. Biological Membranes

Cells, both prokaryotic and eukaryotic, are separated from the environment by a barrier composed of lipid and protein molecules. This barrier, the biological membrane, is not just a rigid physical structure that protects the cellular world from the outside, but it also provides and regulates a viable link of communication between these two worlds. In prokaryotes and in unicellular organisms, biological membranes keep toxic material from entering the cell and allow the specific entry of nutrients. In eukaryotes and in highly evolved organisms, biological membranes in addition to the above functions, maintain a flow of information between various cells. Within the eukaryotic cells, biological membranes also establish discrete compartments, intracellular organelles, and prevent the random mixing of the contents of one compartment with those of another, thereby creating and maintaining order within a cell. In short, biological membranes maintain a healthy intracellular environment enabling the chemistry of life to proceed without obstructions and prepare a cell's response to the changes in its environment. Without the proper functioning of biological membranes the viability of cells can not be supported.

1. Membrane Structure

How do the membranes carry out these complex and sometimes opposite functions (e.g. facilitating entry of certain molecules while preventing the others from entering the cell)? The key to answering these questions lies in understanding the basic structure of the biological membrane. One of the earliest insights into the structure of membranes came from the experiments of Gorter and Grendell (1925) who erroneously came to the correct conclusion that the red blood cell membranes contained sufficient lipid to form a bilayer. The hydrophobic nature of the lipids provided a permeability barrier to water soluble molecules. It was, however, for Danielli and Davson (1935), from permeability, surface

tension and electrical conductivity measurements, to propose the first model of membrane structure that was to have the most impact on the understanding of the organization of biological membranes. They proposed that membranes were made of a phospholipid bilayer sandwiched between two layers of proteins. Later, Robertson (1959) based on interpretation of electron microscopic and X-ray diffraction studies of the membranes, presented the *unit membrane hypothesis*. This model viewed all biological membranes as having the same 'unit' structure composed of three laminae (the outer layer of mucoprotein, the middle layer of phospholipids and the inner layer of unconjugated proteins). The protein molecules were thought to be present in an extended (β) conformation covering the membrane surface. The currently favoured view, however, and which is also most consistent with the present day knowledge, was forwarded by Singer and Nicolson (1972) in their *fluid mosaic model* of the cell membranes.

2. The Fluid Mosaic Model

The *fluid mosaic model* tells us that biological membranes are highly dynamic structures. The phospholipid molecules, together with other lipid components such as cholesterol and glycolipids, are oriented roughly perpendicular to the plane of the membrane with their polar head groups facing the exterior of the membrane and the hydrophobic tails buried within the bilayer. The phospholipid bilayers are fluid. Membrane fluidity is a rather ill-defined term for describing the 'fluid' nature of the lipid bilayer. The measure of membrane-fluidity is determined by the order parameter and the lateral and rotational diffusion coefficients of the lipids. The fluid nature of the bilayer allows rapid lateral diffusion of lipids in the plane of the membranes (McConnell and Kornberg, 1971). The transbilayer movement of lipids, from one leaflet of the bilayer to the other leaflet, also occurs although very slowly (Op den Kamp, 1979). The proteins are globular in nature and often, but not always, inserted into and through the phospholipid bilayer thereby being exposed on both sides of the membrane. The fluid nature of the lipid

matrix allows rotational as well as lateral motions of the proteins in the plane of the membranes (Frye and Edidin, 1970; Bretscher, 1980). The proteins that penetrate the membranes (integral membrane proteins) are associated with the bilayer via hydrophobic interactions. They can be usually, but not always, isolated in the native form from the membranes using nonionic detergents. The proteins that are attached to the surface (peripheral membrane proteins) are associated with the bilayer via electrostatic interactions. They can be easily detached from the membranes by changing the pH or ionic conditions. Finally, the distribution of the individual lipid species and the proteins in the two monolayers of the membrane bilayer is not identical. Thus, the biological membranes are asymmetric with respect to the lipid and the protein distribution across the two halves of the membranes.

B. The Erythrocyte Membrane

The plasma membrane of the human erythrocyte has been extensively studied and is one of the best characterized and best understood eukaryotic cell membranes. The erythrocyte possesses the particular advantage that the only membrane present is the plasma membrane. Also, the erythrocytes are readily available, and the membranes can be obtained easily and rapidly by simple hypotonic lysis and subsequent washes to remove the cytoplasmic contents (especially hemoglobin). This procedure yields a white fraction of pure plasma membrane termed erythrocyte 'ghosts'. Moreover, sealed vesicles of either orientation can easily be prepared from such ghost membranes to study the sidedness of the membranes. No other system, eukaryotic or prokaryotic, provides all these advantages.

1. Proteins

a. Structure

Since the time Weber and Osborn (1968) and later Laemmli (1970) introduced the use of SDS for the electrophoretic separation of proteins, erythrocyte membrane proteins in

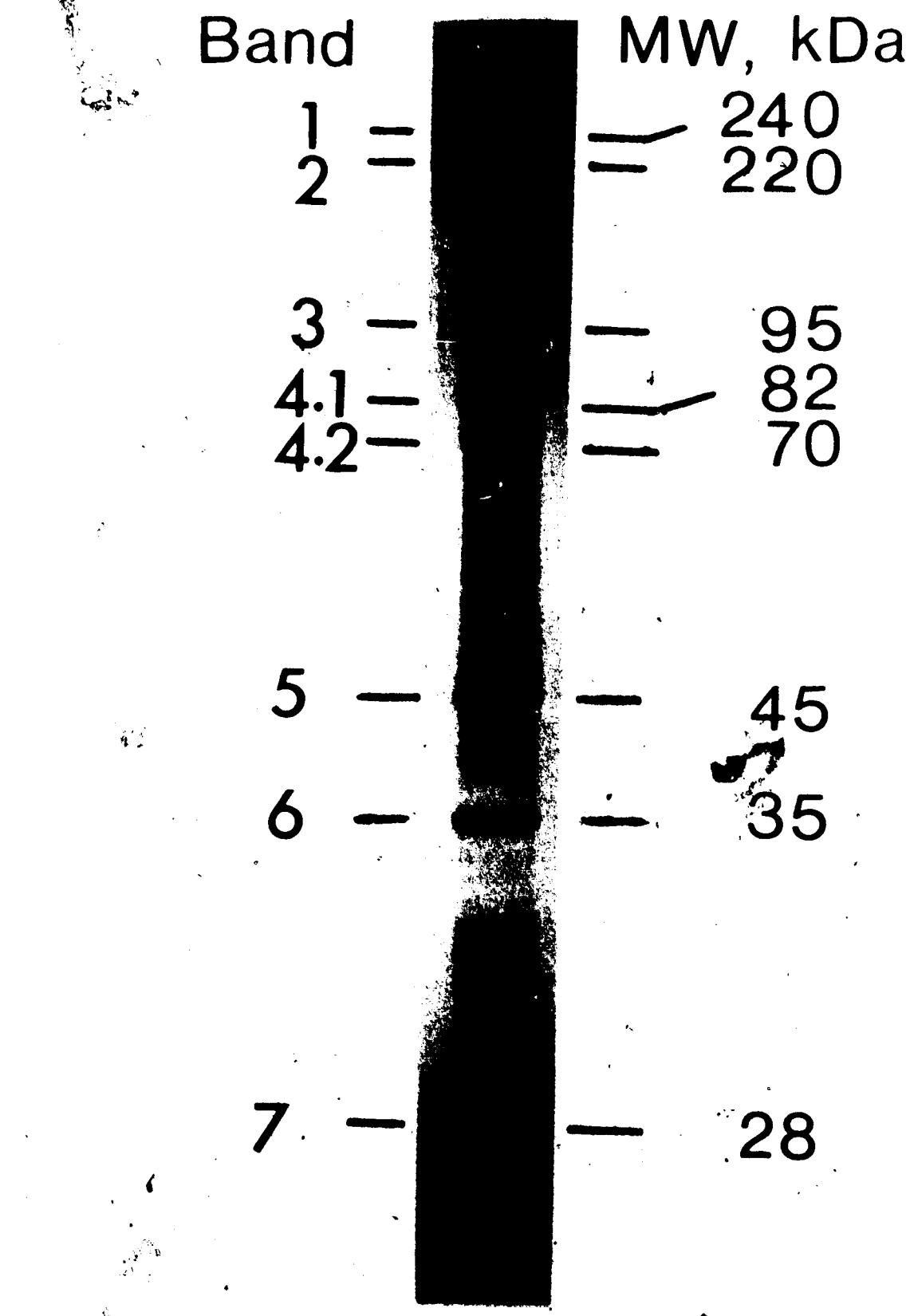
particular and membrane proteins in general, have been extensively investigated. An electrophoretic pattern of erythrocyte membrane proteins, shows the presence of 7 major bands (Fig. I.1). Initially they were classified according to their degree of migration. Today we know that Band 1 and Band 2 proteins are the α and β subunits of spectrin which together with actin, form the bulk of the cytoskeletal network. Band 3, an integral membrane protein, is the anion exchange protein and also a site for the cytoskeletal attachment to the membrane. Band 4.1 and 4.2 proteins are the components of the cytoskeletal network. Proteins migrating in the area of 4.5 are involved in sugar and nucleoside transport. Band 5 is actin, a major component of the cytoskeletal network. Band 6 is glyceraldehyde-3-phosphate dehydrogenase which associates with the erythrocyte membrane via ionic interactions. Little is known about Band 7 except that it is an integral membrane protein (Fairbanks *et al*, 1971; Steck, 1974; Marchesi, 1985; Jay and Cantley, 1986).

1) Spectrin

The main component of the cytoskeletal network, spectrin, exists as a heterodimer (αβ) of about 460,000 daltons. The two subunits associate side-to-side to form a flexible rod of approximately 1,000 Å in length (Branton *et al*, 1981; Speicher 1984). Both subunits consist of 106 amino acid long repeating subsegments. Each subsegment could form a closely packed triple helical unit (Speicher and Marchesi, 1984). Spectrin binds to ankyrin, a component of the cytoskeletal network via β subunit (Weaver *et al*,1984), to protein 4.1 (Tyler *et al*, 1980) and to actin (Fowler *et al*, 1981). Spectrin is phosphorylated in β subunit although the precise role of phosphorylation is unclear (Harris and Lux, 1980). Spectrin, isolated from patients with hereditary blood disorders, shows a decreased capacity to self assemble into higher oligomeric forms and a few other abnormalities (Knowles *et al*, 1983; Zail, 1986; Pothier *et al*, 1987).

Figure I.1

SDS-polyacrylamide gel of the human erythrocyte membrane proteins. Electrophoresis was performed on a 10% gel using the Laemmli buffer system. Numbers on left show the 7 major bands observed when stained with Coomassie-Blue. Numbers on right refer to molecular weights of the major proteins.


Band
MW, kDa
1
2
3
4.1
4.2
5
6
7
240
220
95
82
70
45
35
28

2) Ankyrin

Ankyrin was the first protein to be shown to mediate spectrin binding to the cytoplasmic face of the erythrocyte membrane (Bennett and Stenbuck, 1980). A multi-phosphorylated 210 kDa protein, it binds to β subunit of spectrin and to the cytoplasmic domain of Band 3 , the major integral membrane protein of the erythrocyte membrane (Hargreaves *et al*, 1980). The association between ankyrin and spectrin is sensitive to low ionic conditions whereas ankyrin-Band 3 complex can be dissociated only at high ionic strength (Bennett, 1983). Functional implication of its large size and the phosphorylated state are yet unclear (Marchesi, 1985).

3) Band 3

The major integral membrane protein of the erythrocyte membrane, Band 3 migrates as a diffused band of 95 kDa on a SDS-gel. It has an amino-terminal cytoplasmic domain which acts as an anchor site for binding hemoglobin, ankyrin and other proteins (for review see Low, 1986). The carboxyl-terminal domain of Band 3 protein crosses the membrane bilayer several times and mediates the anion-exchange (for a review see Jay and Cantley, 1986). A detailed discussion of this protein follows at the end of this section.

4) Band 4.1

A dimer of two almost identical proteins (80 kDa and 78 kDa) Band 4.1 is increasingly becoming the focus of many investigations. Recently, these two subunits have been shown to be generated by alternative splicing of mRNA coding for Band 4.1 (Conbody *et al.*, 1987). Besides its association with Band 3, Band 4.1 binds to actin and spectrin and seems to promote associations between these two cytoskeletal elements (Ohanian, *et al*, 1984). It does not bind to the separated subunits of spectrin, but needs the presence of both subunits to form a stable complex (Cohen and Langley, 1984; Coleman *et al*, 1987). Thus, it seems to play a pivotal role in the assembly of the cytoskeletal network.

It has also been shown to associate with glycophorin, an integral membrane protein, and this association is shown to be mediated via a polyphosphoinositide (Anderson and Marchesi, 1985). Functional implications of such associations are exciting since the polyphosphoinositides are known to act as second messengers (Puttney, 1987).

5) Band 4.5

Band 4.5 is comprised of a heterogenous population of glycoproteins of the erythrocyte membrane with a molecular weight of around 55 kDa. They are present in small amounts and their glycoprotein nature makes them migrate on a gel as a diffuse band. The two best characterized functional activities associated with Band 4.5 proteins are the glucose transport and the nucleoside transport (Wheeler and Hinkle, 1985; Plagemann and Wohlhueter, 1980). Glucose transporter from human hepatoma cells has been cloned and the amino acid sequence deduced (Mueckler *et al*, 1985). It is suggested that this protein has 12 membrane-spanning domains and is highly homologous or may be identical with the erythrocyte membrane glucose transporter. Due to extremely low amounts of the nucleoside transporter in the erythrocyte membrane, it has proved a difficult protein to characterize structurally.

6) Actin

A monomer of 45,000 daltons, actin is predominantly present as short oligomers of 15-20 monomers associated with the inner face of the erythrocyte membrane. Less than 10% of the actin is found in monomeric form (Pinder and Gratzer, 1983), while the bulk of the actin attached to the membranes is in a filamentous form. Actin can bind to spectrin in the absence of protein Band 4.1, but the addition of Band 4.1 makes spectrin-actin-protein 4.1 complex far more stable than spectrin-actin complex. Spectrin and actin interact to form a two-dimensional network and protein 4.1 stabilizes and links the network to the inner surface of the erythrocyte membrane (Marchesi, 1985). Besides binding with spectrin

and protein 4.1, actin's association with the membrane also seems to be stabilized by erythrocyte tropomyosin (Fowler and Bennett, 1984) and a new, as yet uncharacterized protein, Band 4.9 (Siegel and Branton, 1985).

Two other major erythrocyte membrane proteins are glyceraldehyde-3-phosphate dehydrogenase (Band 6) and Band 7 protein. Glyceraldehyde-3-phosphate dehydrogenase is a glycolytic enzyme and like several other glycolytic enzymes, it associates with the inner side of the membrane via Band 3 protein (Low, 1986).

7) Glycophorins

Glycoproteins generally do not stain well with Coomassie Blue stain, but are easily visualized by periodic acid-Schiff (PAS) stain or by Stains-All. When the erythrocyte membrane proteins are separated by SDS-PAGE and stained with PAS stain, one major and several minor bands are observed. The major band, also known as sialoglycoprotein or glycophorin A is an integral membrane protein with a molecular weight of 31,000. It spans the membrane only once with a heavily glycosylated (about 60% carbohydrate by weight) amino-terminal domain on the extra-cellular side and a short carboxyl-terminal segment on the cytoplasmic side (Marchesi *et al*, 1976). It contains 131 amino acids and carries the blood group-M and -N antigens of the human erythrocyte (Lisowska and Duk,1975). Recent evidence indicates that it is also linked with the cytoskeletal network via protein 4.1 (Anderson and Marchesi, 1985).

The minor glycoproteins, glycophorin B and glycophorin C, have recently been cloned and their amino acid sequence determined (Colin *et al.*, 1986; Blanchard *et al.*, 1987; Siebert and Fukuda, 1987). Glycophorin B carries the Ss blood group antigens and shows extensive amino acid homology with that of glycophorin A (Blanchard *et al.*, 1987) but these two proteins are encoded by separate single copy genes (Siebert and Fukuda, 198). Glycophorin C is 128 amino acids long and carries the blood group *Gerbich*

antigens (Siebert and Fukuda, 1987). The glycophorins may also be involved in maintaining membrane deformability and mechanical strength (Reid *et al*, 1987).

b. Function and Distribution

The membrane asymmetry with respect to proteins is absolute. All integral membrane proteins are oriented in the membrane in only one direction. Band 3 molecules have both their termini on the cytoplasmic side of the erythrocyte membrane. Glycophorin A has its amino-terminus to the outside and the carboxyl-terminus on the inside of the membrane. All the peripheral proteins are associated with the cytosolic side of the membrane via the cytosolic portions of integral membrane proteins. Glycoproteins have their oligosaccharide chains facing towards the outside of the membrane.

Spectrin, actin, ankyrin, protein 4.1 are all components of the cytoskeletal network and are important in maintaining erythrocyte shape, deformability and mechanical strength during the erythrocytes' turbulent journey through the blood capillaries. Recently, the cytoskeletal network has undergone intense investigation as to its functional role in the normal erythrocytes as well as in various blood disorders. This and other aspects of the cytoskeletal network have been reviewed recently (Marchesi, 1985; Zail, 1986). The integral membrane proteins of the erythrocyte membrane, with the exception of glycophorin A, are mostly involved in transport of various solutes. Glycophorin A is involved in antigenic identification.

C. Band 3 Protein

1. Introduction

The term Band 3 was assigned in 1971, to a protein of unknown function that migrated as a diffuse band with a molecular weight of 95,000 daltons (Fairbanks *et al*, 1971). In 1974-75, it was identified as the protein involved in the transport of anions across the erythrocyte membrane (Cabantchick and Rothstein, 1974; Ho and Guidotti,

1975). From earlier studies it was inferred that the anion exchange in erythrocytes was a protein-mediated process and that it was inhibited by a family of compounds known as stilbene disulfonates (Knauf and Rothstein, 1971). Using non-penetrating stilbene disulfonates, Rothstein's groups demonstrated that in intact erythrocytes these compounds selectively labelled the Band 3 protein. Moreover, there existed a linear relationship between inhibition and binding (Lepke *et al*,1976; Ship *et al*, 1977). Finally, purified Band 3 protein was inserted into liposomes and showed to facilitate anion-exchange (Ross and McConnell, 1977, 1978; Rothstein *et al.*, 1975, Lukacovic *et al.*, 1981).

It was soon realized that exchange of anions was not the only activity performed by this protein. The Band 3 protein was shown to bind several glycolytic enzymes (Yu and Steck, 1975a), hemoglobin (Shaklai *et al*, 1977) and to the cytoskeletal network (Hargreaves *et al*, 1980). It is now believed, based on some recent observations, that Band 3 protein also facilitates water and urea transport across the membrane (Solomon *et al*, 1983), may carry the blood group specificity Rho-D (Victoria *et al*, 1987) but not the specificity for Kell blood group (Redeman *et al*, 1986). The Band 3 protein is also thought to be the senescent antigen that helps in the removal of aged or abnormal erythrocytes (Kay *et al*, 1986; Winograd *et al*, 1987). Recent evidence indicates that the defect in cystic fibrosis involves chloride transport in various epithelial cells. However, the role of Band 3 protein in cystic fibrosis is not clear. Using immunohistochemical techniques, Hazen-Martin *et al*, (1986) found no differences in the pattern or intensity of Band 3 immunoreactivity in sweat duct cell membranes of normal and cystic fibrosis samples. Also, erythrocytes from cystic fibrosis patients showed normal chloride transport activity (R. Reithmeier, unpublished observation).

The natural abu[redacted]of the protein together with its functional diversity has made the Band 3 protein one of the most widely studied membrane proteins in the last decade. Presently, only the anion transport related structural and functional properties of Band 3 will be discussed in detail. A comprehensive review on the interaction of the Band 3

protein with the glycolytic enzymes and the cytoskeletal network has been recently written by Low (1986). Some recent reviews on the other aspects of Band 3 are also available (Jennings, 1984; Jennings, 1985; Passow, 1986; Jay and Cantley, 1986).

2. Isolation

There exist many biochemical purification procedures for preparation of large quantities of Band 3 protein. It is the most abundant protein in the erythrocyte membrane. With 1.2 X 10^6 molecules per cell, it makes up to 25% of the total membrane protein (Fairbanks *et al*, 1971). Band 3 protein is an integral membrane protein and after selective removal of the peripheral membrane proteins (mainly spectrin) by low ionic strength or high pH, the Band 3 protein can be enriched up to 70% of the membrane proteins (Jay and Cantley, 1986). The peripheral membrane proteins can also be stripped by incubation with acetic acid, KI or KBr (Grinstein *et al*, 1979; Bennett, 1983). Further purification is achieved by solubilizing the Band 3 protein in nonionic detergents and separating it from the rest of the proteins by chromatographic techniques such as anion exchange (Yu and Steck, 1975a; 1975b), p-chloromercuribenzamido ethyl agarose (Lukacovic *et al*, 1981) affinity chromatography with Concanavalin A-Sepharose (Findlay, 1974). These procedures generally yield reasonably pure Band 3 protein (at least 95% by SDS-PAGE) in milligram amounts.

3. Size and Composition

When analysed on a SDS-gel, Band 3 protein migrates as a broad diffuse band of 95,000 daltons (Fig. I.1). The diffuse nature of the band is in part due to its glycoprotein nature (Drickamer, 1978), and due to heterogeneity of the oligosaccharide chains (Jenkins and Tanner, 1977).

In vivo organization of Band 3 has been a focus of many studies (Jennings, 1984). Early observations that Band 3 in nonionic detergents sediments as a dimer indicated that

Band 3 might exist as non-covalent dimers (Yu and Steck, 1975a). Reithmeier (1979) suggested that this interaction was probably mediated by the membrane bound domain since the proteolytic removal of the cytoplasmic domain did not affect the dimeric state. Using cross-linking reagents such as DTBP (Wang and Richards, 1975) or Cu^{++} o-phenanthroline at 0 °C, it was shown that Band 3 is exclusively cross-linked to dimers (Reithmeier and Rao, 1979). A photoactivable cross-linker with a half life in millisecond timescale also cross-links Band 3 to dimers suggesting that the dimer formation was not due to random collision (Khiem and Ji, 1977).

More recent experiments by Schubert and coworkers, however, indicate that when solubilized in highly purified nonionic detergents, Band 3 exists in reversible equilibria between monomers, dimers and tetramers (Pappert and Schubert 1982, 1983; Schubert *et al*, 1983). They attributed earlier results suggestive of dimer association to traces of contamination present in the commercially obtained Triton X-100. Other techniques such as fluorescence resonance energy transfer (Macara and Cantley, 1981), freeze fracture electron microscopy (Weinstein *et al*, 1980) have verified the dimeric association but also suggested the presence of tetrameric species in the membrane. Most recent studies, that involve the technique known as target size analysis on the radiation induced-inactivation, have demonstrated that Band 3 exists in the membranes as a dimer of 220,000 daltons (Cuppoletti *et al*, 1985). Thus, although this problem is not totally settled, it seems that in the membranes Band 3 exists predominantly as dimers and to a small degree as higher oligomers (Jennings, 1984).

4. Major Proteolytic Fragments

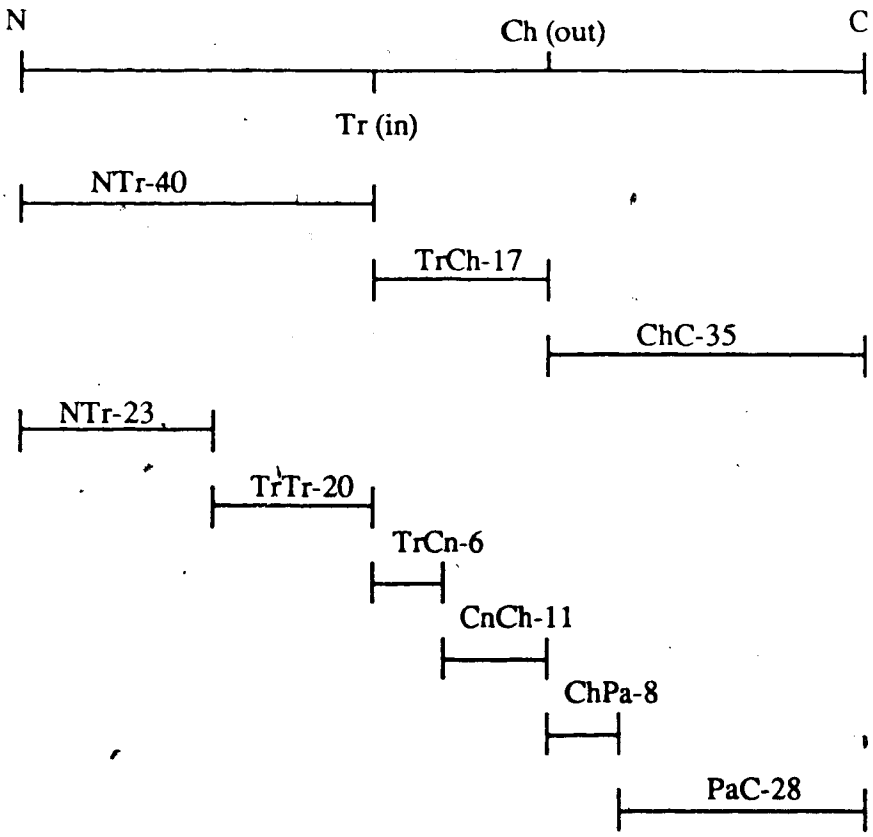
The Band 3 protein can be cleaved *in situ* into a number of well-defined proteolytic fragments. A recently proposed (Jay and Cantley, 1986) nomenclature for proteolytic fragments is used in this presentation. It has a three-unit code and the first unit represents the reagent used to generate the amino terminus. The second unit represents the reagent

used to generate the carboxyl terminus. The third unit represents approximate molecular weight of the fragment in kilodaltons. If the fragment is generated from either termini of the protein, the letter N or C appears as the first or the second unit.

A number of well defined fragments (Fig. I.2) can be obtained by *in situ* proteolytic digestion of Band 3 protein (Drickamer, 1976; 1977). Externally added trypsin (Tr) does not cleave Band 3, whereas on the cytoplasmic face it cleaves Band 3 to form NTr-40 and TrC-55 (Yu and Steck, 1975b; Grinstein *et al*, 1978). The NTr-40 is the soluble cytoplasmic amino-terminal domain and is released from the membrane as a result of the trypsin treatment. The TrC-55 is a hydrophobic membrane bound carboxyl-terminal domain and it remains associated with the membrane after the protease treatment. The NTr-40 has a trypsin sensitive site that results in formation of two fragments, NTr-23 and TrTr-20 (Kaul *et al*, 1983). The amino acid sequence of 201 amino-terminal amino acid residues has been reported (Kaul *et al*, 1983). All the known cytoskeletal elements, glycolytic enzymes and hemoglobin attachments sites reside in this fragment (Low, 1986).

The membrane bound TrC-55 still retains the anion translocation activity of the native Band 3 protein (Grinstein *et al.*, 1978) and can be further cleaved by externally added chymotrypsin (Ch) to form fragments TrCh-17 and ChC-35 (Drickamer, 1976; Jenkins and Tanner, 1977). These two fragments retain a stable interaction in the membrane and also retain the anion translocation competence (Jennings and Passow, 1979; Reithmeier, 1979; Grinstein *et al*, 1978). The TrCh-17 can be further reduced in size by internal chymotrypsin (Ramjeesingh *et al*, 1980a) or cleaved by treatment with cyanogen bromide to form TrCn-6 and CnCh-11 (Mawby and Findlay 1983). The ChC-35 can be further cleaved by external papain to form fragments ChPa-8 and PaC-28 (Jennings *et al*, 1984). The papain treatment results in the loss of anion translocation activity presumably from inhibition of the efflux step in the catalytic cycle (Jennings and Adams, 1981). The proteolytic fragments can be purified and further cleaved by various chemical agents to form peptides as small as 2 kDa (Steck *et al*, 1978; Ramjeesingh *et al*, 1982).

Fig. I.2 Proteolytic Fragments of Band 3



The nomenclature for fragments is a three-subunit code described in text. The first unit represents the amino terminus and the second unit represents the carboxy terminus of the fragment; the third unit is the size of the fragment in kilodaltons. Abbreviations used are: C, carboxy terminus; Ch, chymotryptic cleavage site; Cn, cyanogen bromide cleavage site; N, amino terminus; Pa, papain cleavage site; Tr, trypsin cleavage site.

When intact erythrocytes are treated with chymotrypsin, Band 3 protein is cleaved to form fragments NCh-60 and ChC-35. The NCh-60 can be cleaved by internal trypsin to form fragments NTr-43 and TrCh-17. The ChC-35 fragment carries the total Band 3 carbohydrate linked to a single asparagine residue (Drickamer, 1978). This site has been further localized PaC-28 (Jennings *et al.*,1984). Using a novel technique of end-labelling, Jay (1986) has mapped this site to less than 300 residues from the carboxyl-terminus of Band 3. The oligosaccharide chain contributes to the Band 3 molecular weight by approx. 6,000 and binds to wheat germ agglutinin (Tsuji *et al.*, 1980). The structure of the entire oligosaccharide chain for both adult and fetal forms of Band 3 has been elucidated by Fukuda *et al.* (1984a, b) by methylation, exoglycosidase digestion and fast atom bombardment mass spectrometry. The adult form has two or three polylactosamine side chains attached to the core protein via an asparagine residue. Mouse Band 3 has two potential residues for glycosylation, Asn-611 and Asn-660 (Kopito and Lodish, 1985). Each polylactosamine chain is composed of (Galβ1→4GlcNAcβ1→3) repeating units that terminate with galactose, fucose or sialic acid (Fukuda *et al.*, 1984b).

5. Amino Acid Sequence

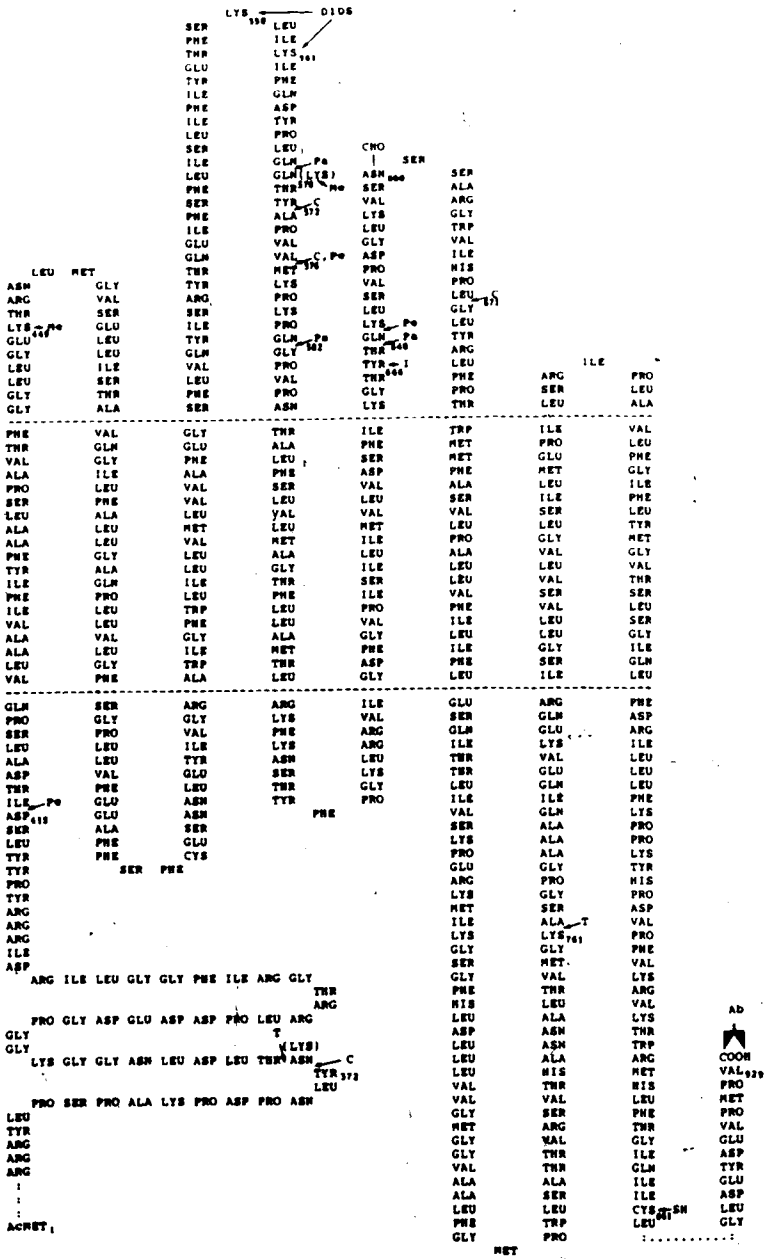
Since the membrane proteins contain long hydrophobic stretches, the classical protein sequencing techniques cannot be applied with great success. However, the amino-terminal cytoplasmic domain being hydrophilic, the first 201 amino acid residues were successfully sequenced using the protein sequencing techniques (Kaul *et al*, 1983). Small stretches of membrane domain such as, the amino-terminal 37 amino acids of the fragment CnCh-11 (Mawby and Findlay 1983) and the 72 amino acids of a papain subfragment of the fragment ChPa-8 (Brock *et al*, 1983; Brock and Tanner, 1986) have also been sequenced.

Kopito and Lodish (1985a) have published complete amino acid sequence of murine Band 3. They isolated murine Band 3 mRNA and constructed a cDNA library in

the expression vector λ-gt 11. The mouse Band 3 is 929 amino acids long and is almost identical or strongly homologous with known sequences of the human Band 3 protein. The amino-terminal 50 amino acids, however, show very little homology. This region of human Band 3 provides the binding sites for the glycolytic enzymes (Murthy *et al*, 1981) and the mouse Band 3 does not seem to bind glycolytic enzymes (Kopito and Lodish, 1985a). The amino-terminal domain of human Band 3 is highly acidic and is suggested to bind to positively charged regions of glycolytic enzymes. The amino-terminal domain of mouse Band 3 is less acidic than that of the human Band 3. It has recently been shown that the murine Band 3 gene is present in a single copy with 19 intervening sequences (Kopito *et al.*, 1987a). There also exists a good correlation between the sites of the intron/exon junctions with the predicted structural features (e.g. the membrane spanning helices, β-turn index) of Band 3.

The hydrophobicity index of murine Band 3 indicates that there are 12 potential membrane spanning domains. The fact that some of the analogous stretches in human Band 3 do indeed span the membrane bilayer has been experimentally verified using various proteolytic and chemical labelling techniques (see Fig. I.3; Jennings *et al.*, 1986). The known protein sequence for two hydrophobic fragments of human Band 3 are almost identical with the respective sequences of the murine Band 3 (Kopito and Lodish, 1985; Brock *et al.*, 1984). This may indicate that these hydrophobic stretches are important in transport site structure. Despite the similarity between the membrane domains of these two proteins, there are also some notable differences. The fragment TrCh-17 from human Band 3 has two histidines (Steck *et al*, 1978), the corresponding region of mouse Band 3 has none. Thus, interpretation of the structural or chemical information obtained for human Band 3 in terms of the murine Band 3 sequence should be made with caution (Jay and Cantley, 1986).

Figure I. 3. A model for the proposed transmembrane orientation of Band 3.



The model is based on the experimentally verified sites of chemical labelling and proteolytic cleavage of human Band 3 and on the hydropathy plot of the murine Band 3 sequence. Arrows show the precise location and numbers refer to the residue number of a site. Abbreviations used are: Ab, anti-carboxy terminal antibodies binding site; C, chymotryptic cleavage site; DIDS, covalent stilbene disulfonate labelling site; I, iodination site; Me, reductive methylation site; Pa, papain cleavage site; Pe, pepsin cleavage site; T, trypsin cleavage site.

6. Chemical Modifications

a. Covalent Modification with Stilbene Disulfonates

The usefulness of chemical modifications in the structural and functional characterization of Band 3 protein cannot be overemphasized. It was this approach (covalent modification of Band 3 protein with stilbene disulfonate and *p*-sulfophenyl isothiocyanate) that identified this protein as the anion transporter of the human erythrocyte membranes (Cabantchick and Rothstein, 1974; Ho and Guidotti, 1975). Indeed the stilbene disulfonates have proved most useful in Band 3 research (Cabantchik *et al*, 1978). When added to intact erythrocytes at neutral pH, DIDS reacts exclusively with Band 3 and completely inhibits anion exchange when bound at a 1:1 stoichiometry (Lepke *et al*, 1976). Using $[^3H_2]H_2$-DIDS, synthesized by catalytic reduction of DIDS by $[^3H_2]$, the reactive site on Band 3 is identified as a lysine residue in the fragment TrCh-17 (Grinstein *et al*, 1978) and has been mapped to 7,000-9,000 daltons from the carboxyl-terminus of the fragment (Ramjeesingh *et al.* (1980). This corresponds to either lysine-558 or 561 in the murine Band 3 sequence (Kopito and Lodish, 1985). At alkaline pH (>9.0), DIDS cross links TrCh-17 and ChC-35 fragments (Jennings and Passow, 1979). The site of cross-linking has been localized to the PaC-28 fragment (Jennings *et al*, 1984). The kinetics of DIDS cross-linking has been studied in detail by Passow and his coworkers (Passow *et al*, 1982, Passow, 1986). Their results suggest that at low pH, DIDS reacts much faster with Lys a (residing in the fragment TrCh-17 or NCh-60) than with Lys b (in the fragment ChC-35 or PaC-28). At neutral pH most of the label is associated with Lys a. At no stage is the inhibitor solely linked to Lys b. They propose that DIDS first binds non-covalently at the stilbene disulfonate binding site then reacts covalently with Lys a, and at a higher pH, finally cross-links to Lys b.

b. Reductive Methylation

Importance of Lys b in the transport activity was shown by reductive methylation by Jennings (1982a). Repeated treatments with formaldehyde and $NaBH_4$ reduces chloride transport by 75% and results in only partial inactivation of Lys a but total inactivation of Lys b on the PaC-28 fragment. The methylation is believed to affect the rate of the conformational changes associated with anion translocation but not anion binding (Jennings,1982a).

c. Dinitrophenylation

Many amino acids including lysine are sensitive to modification by 1-fluoro-2,4-dinitrobenzene. About 80% inhibition of anion transport is observed when two dinitrophenyl residues are incorporated per Band 3 molecule (Rudloff *et al*, 1983). In the presence of a stilbene disulfonate only one dinitrophenyl residue is incorporated in the protein (Passow *et al*, 1975). This lost site has been located to a lysine on the TrCh-17 fragment (Rudloff *et al*, 1983) and is probably identical to Lys a (Ramjeesingh *et al*, 1981). The second lysine has been located to the ChC-35 fragment but it is not identical to Lys b since its modification is not prevented by the presence of DNDS (Rudloff *et al*, 1983). An important conclusion drawn from the dinitrophenylation experiments, in the presence of different substrates and different inhibitors, is that Lys a at the stilbene disulfonate binding site may exist in two different states with different reactivities (Passow, 1986). The rate of dinitrophenylation, which can be used as an indicator of the conformational state of the protein, was different in the presence of different substrates and different inhibitors (Passow, 1986).

d. Lysine-Modifying Reagents

Hydrophilic sulfophenylisothiocyanate is shown to react at the stilbene disulfonate binding site on the Band 3 protein presumably with Lys a (Ho and Guidotti, 1975;

Reithmeier and Rao, 1979). Pyridoxal 5-phosphate treatment, in the presence of $NaBH_4$, causes irreversible inhibition of anion transport. The pyridoxal 5-phosphate binding site partially overlaps the H_2-DIDS site (Cabantchik *et al*, 1975). Recently this site has been located to a small 5,300 dalton peptide derived from the papain sensitive area of the ChC-35 (Matsuyama, 1983; Kawano and Hamasaki, 1986) and could be Lys b. Pyridoxal 5-phosphate does not react with Lys a and sulfophenylisothiocyanate does not react with Lys b (Nanri *et al*, 1983).

e. Arginine-Modifying Reagents

Two arginine specific reagents, 1,2-cyclohexandione (Zaki, 1981) and phenyglyoxal (Weith *et al*, 1982a,c; Bjerrum *et al*, 1983) have been shown to modify two different arginine residues and inhibit anion equilibrium exchange. These arginine residues have been located to the ChC-35 fragment (Bjerrum *et al*, 1983). It is suggested that this site partially overlaps the stilbene disulfonate binding site or is allosterically linked with the stilbene disulfonate binding site. Phenylglyoxylation reduces DBDS binding in proportion to the phenyglyoxal concentration and slows H_2-DIDS binding to erythrocytes (Passow, 1986). Involvement of the arginine residues in anion transport has also been suggested by the pH profile showing titrable groups with pK_As of approximately 11 (Wieth and Bjerrum, 1982). Increasing the Cl^- concentration reduces the rate of glyoxylation, suggesting that the modified arginines could be the part of substrate binding site (Passow, 1986). This conclusion has also been supported by the recent NMR experiments of Chan and coworkers (Falke and Chan, 1986a).

f. Carboxyl-Modifying Reagents

Early indication that a carboxylic group may be involved in the structure of the Band 3 active site came from the titration studies showing inactivation of Cl^- self-exchange with a pK of 5.2-5.4 (Wieth and Bjerrum, 1982). This inference was later experimentally

verified using a nonpenetrating carbodiimide derivative (Craik and Reithmeier, 1984; 1985; Andersen *et al*,1983). Using tyrosine ethyl ester, the modified carboxyls have been located to the fragment ChPa-8 (Bjerrum *et al*, 1983). There are two aspartates in the sequence of the corresponding mouse fragment (Asp 625 and Asp 639). Papain digestion releases the peptide fragment that houses these aspartates (Bjerrum *et al*, 1983). Since the papain treatment leaves the substrate site intact (Jennings and Adams, 1981), it can be concluded that the carboxyl residues are most likely the components of the modifier sites.

Recently Werner and Reithmeier (unpublished data) have observed a novel mode of inhibition with carbodiimide. When Band 3 protein is incubated with carbodiimide in the presence of citrate, the latter is incorporated in the protein and the most likely site is Lys a. Jennings has recently shown, using activation of carboxyl groups on the Band 3 protein by Woodward's reagent K followed by borohydride reduction, that two glutamate residues near the stilbene disulfonate site are necessary for the anion translocation (Jennings and Anderson, 1987). The glutamates are located to the ChC-35 fragment (Jennings, unpublished data).

g. NAP-taurine

The photoactivable reagent NAP-taurine [N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate] when irradiated forms a nitrene which is capable of forming a covalent bond with aliphatic or aromatic amino acid residues. It is also a substrate for transport by Band 3 and can inhibit competitively (Ki = 20 μM) from the cytoplasmic side of the membrane (Cabantchik *et al*, 1976). The binding site has been mapped to the TrCh-17 fragment (Knauf *et al*, 1978) and is shown to overlap the stilbene disulfonate binding site by competition between stilbene disulfonate and NAP-taurine for binding to Band 3 (Macara and Cantley, 1981b). Recent experiments by Frohlich and Gunn (1987) also suggest that these two sites overlap. Covalently attached H_2-DIDS from the outside

reduces NAP-taurine binding to Band 3 at the inner surface (Grinstein *et al*, 1979). Thus, allosteric interaction between these two sites is also possible.

7. A Structural View of the Transport Site

In the absence of a crystal structure for Band 3 protein, one can only predict the tertiary structure of the protein. Based on the structural studies on human Band 3 (discussed above) together with the hydropathy plots from the murine Band 3 (Kopito and Lodish, 1985a), it is possible to construct a model of tertiary structure of Band 3 in membranes. Kopito and Lodish (1985b) presented a model with 12 membrane spanning domains arranged as amphipathic helices (a helix with the polar residues on one side of the helix and the non-polar residues on the other). The hydrophilic face of the helices form the interior of not one but two separate channels, one channel formed by the helices 1-5 and the other by the helices 6-12. They propose that the two states of Band 3 (the inward-facing state and the outward-facing state) are obtained by sliding of the helices common to both channels. Thus, the two channel model may explain the tight coupling of inward and outward translocation of anions (obligatory 1:1 exchange) and the involvement of both TrCh-17 (helices1-5) and ChC-35 (helices 6-12).

Jay and Cantley (1986) have proposed a model with eight helical membrane spanning domains and a hairpin loop into the membrane (see Fig. I.3). The membrane spanning portion of the Band 3 protein is mostly α-helical (Oikawa *et al*, 1985). The polar faces of the helices make up the inner side of a single channel. Both the amino-terminus (Low,1986) and the carboxyl-terminus (Lieberman and Reithmeier, in press) have been experimentally verified to be on the cytoplasmic side of the membrane and therefore the protein must cross the bilayer an even number of times. The presence of at least eight helices has the direct experimental support: three in TrCh-17 (Ramjeesingh *et al.*, 1983), two in ChPa-8 (Jennings *et al.*, 1984) and at least three in PaC-28 (Ramjeesingh *et al.*, 1983; Jennings *et al.*, 1986; Lieberman and Reithmeier, in press). Kopito and Lodish

(1985b) presented a model with 12 membrane spanning domains based on hydropathy plots. The actual number of membrane-spanning domains will only be obtained by knowing the crystal structure.

One feature of the model proposed by Jay and Cantley is that when viewed from either side of the membrane the channel appears as a positively charged funnel that narrows into a small cluster of negative charges. On the extracellular side, the mouth of the 'funnel' is surrounded by 4 arginine and 3 lysine residues. These residues may be involved in the repulsion of cations and the binding of anions. Deeper into the cavity Asp 639 and Glu 554 probably act as part of the gating mechanism. From the inner side of the channel, 3 arginine and 3 lysine residues surround the opening. Towards the middle of the bilayer are present Glu 527 and Asp 625. Such an arrangement would be suitable for an exchange mechanism. The positively charged residues present on the surface may be involved in the binding of anions. A conformational change in the protein would then internalize the bound anions. The negatively charged residues within the cavity of the protein may be involved in forming salt-bridges with the positively charged residues present near the opening of the channel and thus assist in the movement of anions.

8. A Kinetic View of the Transport Site

The anion transport mediated by the Band 3 protein has two major characteristics. One, the rate of anion exchange is relatively rapid (for Cl^-, 10^5 sec^{-1} band 3 molecule^{-1} at 37 °C). Secondly, the transport is electroneutral, there is no net charge movement. The rapid movement of the anions argues against a major conformational change that involves the peptide backbone. The electroneutrality requires an obligatory 1:1 exchange of the anions. The kinetic data available at present, overwhelmingly favours a ping-pong mechanism for anion-exchange (Gunn and Frohlich, 1979; Passow, 1986;). An anion binds the transport site from the outside and is translocated to the inside. The 'empty' carrier then binds an anion on the inside and transports it to the outside. Thus the protein

exists in two different stable conformations, an 'outward-facing' state and an 'inward-facing' state. The change from one state to the other state does not occur in the absence of the substrate, thereby accounting for one-for-one exchange.

In order to explain kinetics of the anion-exchange, a gating mechanism involving formation of two alternative salt bonds at the transport site was proposed (Macara and Cantley, 1981a; 1983). The model proposes that an anion entering the transport site from outside would break the salt bond between a carboxylate and a positive charge. The carboxylate would reform a salt bond with a second nearby cationic group. This conformational change would allow the transport of the anion to the inside. Entry of a second anion from outside to inside is not allowed in this conformation. The original conformation can only be achieved if an anion from the inside enters the transport site, breaks and reverses the process. Most of the data is consistent with such a model. The proposed distribution of the positive charges at the tip of the channel and that of the negative charges in the middle (Jay and Cantley, 1986) is also consistent with the salt bond model. However, assigning the residues that might be involved in the proposed bond formation, in the absence of a crystal structure or without site-specific mutagenesis of the residues in question, will be merely speculative.

D. Noncovalent Interaction of Band 3 with Stilbenedisulfonate

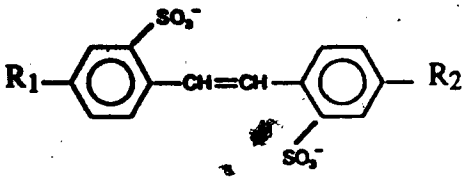
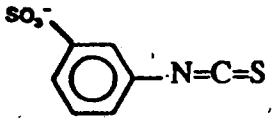
The stilbene disulfonates and other aromatic sulfonates have been extensively used to study mechanism of anion-exchange since, besides their use as covalent labels, they can also be used as reversible competitive inhibitors (Knauf, 1979). The stilbene disulfonate binding site on the protein is accessible to the inhibitors only from the outside. The stilbene disulfonates can also bind non-specifically when added from the inside of the cell but they inhibit the anion-exchange only when bound to the specific stilbene disulfonate binding site on the protein. Some stilbene disulfonate derivatives are incapable of forming covalent bonds. Even for the stilbene disulfonates that are capable of acting as covalent labels,

formation of the covalent bond is not necessary to bring about almost complete reversible inhibition of anion transport (Shami *et al*, 1978). Affinity of various stilbene disulfonate derivatives vary between micromolar to nanomolar range (reviewed by Knauf, 1979; Barzily *et al*, 1979; also see Table I.1). The affinity of these inhibitors seems to increase with increasing hydrophobicity and is dependent on the substituent group of the benzene ring (Barzilay *et al*, 1979).

Evidence from the kinetic studies of stilbene disulfonate inhibition of anion exchange suggest that the stilbene disulfonates binding site overlaps the substrate binding site (Shami *et al*, 1978). Recent NMR studies using $^{35}Cl^-$, provide a direct evidence for the overlapping sites. The chloride bound to the substrate site is shown to be released by addition of the stilbene disulfonate DNDS (Falke *et al*, 1984a). The substrate can approach the binding site from either side of the membrane and addition of stilbene disulfonates from the outside is capable of displacing Cl^- bound from the inside of the membrane (Falke *et al*, 1985). It is also clear, from the effects of papain treatment on anion transport and DNDS binding, that the stilbene disulfonate binding site extends into areas of the protein not directly involved in Cl^- binding (Jennings and Adams, 1981).

Fluorescent derivatives of stilbene disulfonates such as 4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate (BIDS), 4-benzamido-4'-aminostilbene-2,2'-disulfonate (BADS), 4-4'-dibenzamidostilbene-2,2'-disulfonate (DBDS) can be synthesized (Kotaki *et al*, 1971; Rao *et al*, 1979). These compounds have been used to study the kinetics of inhibitor binding (Dix *et al*, 1979; Verkman *et al*, 1983) as well as to determine the location of the inhibitor binding site (Rao *et al*, 1979; Macara *et al*, 1983). The distance between the stilbene disulfonate site and the cytoplasmic domain was measured using the technique of resonance energy transfer. The three sulfhydryl groups on the cytoplasmic domain of Band 3 were linked with fluorescent maleimides. The distance between BIDS and maleimides was determined to be 34-42 Å (Rao *et al*, 1979).

Table I.1 Structure and inhibitory potency of various ligands

Structure	Name	Abbrev.	Ki (μM)[a]
			
R_1 = (-N=C=S), R_2 = (CH_3CONH-)	4-acetamido-4'-isothiocyano-stilbene-2,2'-dislufonate	SITS	~10.0
$R_1 = R_2$ = ($-NH_2$)	4,4'-diaminostilbene-2,2'-dislufonate	DADS	1,300.0
$R_1 = R_2$ = ($-NO_2$)	4,4'-dinitrostilbene-2,2'-dislufonate	DNDS	2.0
$R_1 = R_2$ = (-N=C=S)	4,4'-diisothiocyanostilbene-2,2'-dislufonate	DIDS	0.04
R_1 = ($-NH_2$), R_2 = (-NHCO-⬡)	4-benzamido-4'-aminostilbene 2,2'-dislufonate	BADS	2.0
$R_1 = R_2$ = (-NHCO-⬡)	4,4'-dibenzamidostilbene-2,2'-dislufonate	DBDS	1.3
	3-sulfophenylisothiocyanate	SPITC	~4,000.0[b]

[a]Concentration for 50% inhibition of chloride or sulfate flux (Knauf, 1979)
[b]value is for isothiocyanate derivative of sulfanilic acid (Ho and Guidotti, 1975)

This suggests that, since this distance is less than the width of the bilayer, the stilbene disulfonate site is probably buried within the membrane.

The first indication that the Band 3 protein undergoes a conformational change following an inhibitor or substrate binding came from the intrinsic tryptophan flourescence studies of Macara *et al* (1983). They studied changes in membrane tryptophan flourescence upon inhibitor binding to the Band 3 protein. They showed that DBDS caused a 7% quenching of tryptophan fluorescence. Covalent labelling of red cells by BIDS caused an increase in the susceptibility of protein fluorescence quenching by CsCl. The tryptophan residues that were quenched by CsCl were different from those quenched by BIDS and were probably located on the cytoplasmic domain of Band 3. They also observed that eosin maleimide could be covalently reacted with the extracellular transport site of the Band 3 protein. Surprisingly, its fluorescence was not quenched by the externally added Cs^+ but was quenched by Cs^+ added to the cytosolic side. Since both the probe and Cs^+ are impermeable to the membrane, these results suggested that the maleimide inhibitor first bound the protein from the outside and then Band 3 protein had partially translocated the bulky anion. Such a translocation was accompanied by increase in the Cs^+ induced quenching of tryptophan residues of the cytoplasmic domain of Band 3 indicating a conformational change in the protein. It is believed that such a conformational change in the protein is accopanied with the translocation of the substrate anions. The conformational change observed following the inhibtor binidng to the Band 3 protein represents internalization of the inhibitors.

Solomon's group performed detailed studies on the kinetics of inhibitor binding and measured thermodynamic parameters associated with the conformational change. They studied DBDS interaction with Band 3 by stopped-flow and temperature-jump experiments (Dix *et al*, 1979; Verkman *et al*, 1983). They observed that DBDS initially binds rapidly to one monomer of Band 3 dimer. The rapid binding step is followed by a slow (4 s^{-1}) conformational change that locks the inhibitor in place. The initial rapid binding step is

associated with decrease in enthalpy whereas the conformational change is associated with increase in entropy. The overall net enthalpy change, however, is large enough (-13.1 kcal/mol) to compensate for the net entropy decrease equivalent to 3.4 kcal/mol (Verkman *et al*, 1983). Their results indicate that binding of a second DBDS molecule to its site on the second monomer is, however, different from the binding of the first DBDS molecule to the first monomer. The energy for binding of the second molecule is derived from an increase in entropy, unlike the binding of the first molecule which is mostly enthalpy driven. If both the monomers were to bind DBDS independently, then the thermodynamic parameters in both cases would have been identical. Solomon and coworkers (Verkman *et al*, 1983) interpret these results to suggest that the second DBDS molecule binds to its site only after the first DBDS molecule has been partially internalized by the conformational change in the protein.

Macara and Cantley (1981a) also reported a negative cooperativity in the inhibitor binding to Band 3. They observed that the affinity of stilbene disulfonates binding to dimers containing one subunit already occupied by BIDS is an order of magnitude lower than to unoccupied dimers. The binding of substrate to the unmodified subunit is, however, not affected by blocking one subunit with inhibitor. They suggest that the negative interaction is due to steric hindrance between the two bulky inhibitor molecules rather than an allosteric effect. An implication of this observation is that the stilbene disulfonate binding site is formed by both monomers. Strong evidence supporting this view is presented by Boodhoo and Reithmeier (1983). They observed that Band 3 in monomeric state (obtained by immobilizing Band 3 on a resin via one monomer and removing the second monomer with low SDS or urea treatment) does not bind BADS. The inhibitor binding is, however, regained when the monomers are removed from the matrix and allowed to dimerize in solution. This observation strongly suggests that the stilbene disulfonates binding site requires a dimeric structure. The binding site may be present in

between two monomers and the site is lost when the dimers are dissociated to form monomers.

Covalent labelling of Band 3 with DIDS is shown to have a stabilizing effect on the membrane domain (TrC-55) of the protein. Using a calorimetric technique, Appell and Low (1982) showed that under normal conditions, thermal denaturation of the membrane domain occurs prior to that of the cytoplasmic domain. However, in DIDS-labelled Band 3, the thermal transition of the membrane domain was shifted up ~10 °C and occurred following that of the cytoplasmic domain. Oikawa *et al.* (1985) also showed that covalent attachment of DIDS had little effect on circular dichroism spectra of Band 3 or the membrane domain (TrC-55), but resulted in stabilization of Band 3 to heat denaturation. Binding of the bulky stilbene disulfonates to the stilbene disulfonates binding site also affects the cytoplasmic domain as well as the whole Band 3 molecule. Labelling the intact erythrocytes with H_2-DIDS results in decreased adenosine induced Concanavalin A agglutination of the cells (Singer and Morrison, 1980). Salhaney *et al* (1980) reported that DIDS bound to the external surface of Band 3, altered hemoglobin binding to the cytoplasmic domain. They observed that the hemoglobin binding isotherm changed from being noncooperative to apparent negatively cooperative as a result of DIDS treatment. Hsu and Morrison (1983) showed that following DIDS binding to the membrane-spanning domain of Band 3, the latter's association with the cytoskeletal network was affected. As a result, ankyrin and spectrin become less susceptible to extraction by NaOH. While some believe that this demonstrates a stilbene disulfonate-induced transmembrane conformational change (Salhaney *et al*, 1980), others believe that such effects may be mediated by another protein (Hsu and Morrison, 1983) or via an altered dissociation of Band 3 oligomers (Low, 1986). Whatever the mechanism may be, it is clear that binding of these bulky inhibitors to the stilbene disulfonate binding site on Band 3 results in profound intramolecular and intermolecular structural alterations in the protein.

E. Affinity Chromatography

Affinity chromatography, as the name indicates, is a purification technique that separates macromolecules based on their biospecific affinity towards a ligand. Primarily, a ligand that binds specifically and reversibly to the protein of interest is covalently immobilized to a solid support. Then a crude mixture containing the protein of interest and the contaminating material is passed over the 'affinity-matrix'. The protein binds to the ligand and is retained while the contaminating substances are removed by washing. Finally, the protein is eluted from the column with free ligand to obtain purified protein in solution.

Early studies on isolation of proteins based on the principle of biospecific affinity were performed by Lerman to isolate antibodies and enzyme tyrosinase (Campbell *et al*, 1951; Lerman, 1953). However, a widespread application of this technique to isolate and purify proteins and nucleic acid did not begin until Cuatrecasas and others developed simple basic chemical strategies for attaching various ligands to activated solid supports (Cuatrecasas and Anfinsen, 1971; Parikh *et al*, 1974). Since then the technique of affinity chromatography has been used to isolate and purify a number of enzymes, receptors and even specialized cell populations (for reviews see Venter, 1982; Strosberg, 1984; Dean *et al*, 1985). However, the usefulness of this technique is not limited only to isolation and purification procedures. Immobilization of a ligand has also been explored to study ligand-protein interaction (Nichol *et al*, 1981; Winzor, 1985), protein-protein interactions (Low *et al*, 1987) and mechanisms of action for various drugs, hormones and neurotransmitters (Venter, 1982).

1. Theory

Although the technique of affinity chromatography has been used widely in the past and a number of new affinity matrices, spacer molecules and new activation and coupling procedures have been developed, it has received only a marginal theoretical treatment

(Nichol *et al*, 1974, 1981; Graves and Wu, 1974). Most of the successful attempts at affinity chromatography are based on a trial-and-error basis. The critical factor on which success or failure of affinity chromatography depends is the proper choice of ligand and spacer molecules and suitable binding and elution conditions.

Graves and Wu (1974) have applied a theoretical treatment to get a better understanding of the results and to predict degree of success (purity plus yields) in achieving final purification of a protein. Their treatment is based on kinetic and equilibrium models of the affinity adsorption and desorption between the protein and the ligand. They examine the binding-phase and the elution-phase of affinity chromatography separately and assume that the chromatography is carried out in a batch experiment. In a batch procedure, the affinity resin is incubated with the crude mixture in a container and the unbound material (and the washes) are removed by pouring off the solution. Thus at any given time all the volumes (gel-phase, solution-phase and trapped volume) are known. In a column procedure, the resin is packed in a column and the process of binding, washing and elution are carried out in a continuous stream of buffer. The mathematics for a column process becomes quite complicated.

From the mathematical analysis of the batch process and applying those principles to a simulated column procedure, they draw the following conclusions.

1) In order to produce adequate affinity, the immobilized ligand concentration should be more than 10 times the ligand-protein dissociation constant (*Ki*). For example, if the ligand concentration is 10 mM then it can be predicted that the critical *Ki* would be 1 mM. Ligands with lower affinity ($Ki > 1$ mM) will make poor adsorbent and the ligands with higher affinity ($Ki < 1$ mM) will make good adsorbents. It should be noted, however, that increasing the ligand concentration to compensate for weak affinity does not always produce desired effects. At a higher ligand concentration nonspecific interactions (such as ionic and hydrophobic interactions) may become dominant over biospecific interactions (Ikeda *et al*, 1984).

2) In order to achieve saturation of the immobilized ligands with the protein, the mixture to be incubated with the resin should be as concentrated as possible. This way, more protein can be recovered from the same volume of the affinity resin than the amount recovered after loading a dilute mixture. An interesting and important result is that in most cases less than 1% of the ligand bound can be saturated with the protein. Such an observation is usually attributed to factors such as steric hindrance or ionic repulsion. Besides these factors, equilibrium effects alone can be totally responsible for such a phenomenon.

3) The strength of interaction between the ligand and the protein can affect the washing and elution procedure. For example, if *Ki* of the ligand is poor, then specifically bound protein will be lost through washing. For a ligand with *Ki* value of 1 mM only column procedures may be effective while for values of 1 μM, batch procedures may be a better choice. With such higher affinity, very little specifically bound protein will be lost even with a large amount of washing. Interactions with very high affinities may make it difficult to elute the bound protein without resorting to denaturing conditions.

4) In order to achieve effective elution of the protein from the resin, the *Ki* value should be increased at least two orders of magnitude (e.g. by changing the pH, or the ionic strength). Elution by the addition of a free ligand can be achieved effectively if affinity of the free ligand is known. If *Ki* values of both the the immobilized and the free ligand are similar, then the concentration of the free ligand should be at least 10 times higher than that of the bound ligand. The concentration of the free ligand needed varies inversely with its affinity towards the protein.

2. Application

a. Matrix

The ideal matrix support is expected to have many properties and many of the supports available today fulfill many of these expectations. Some of the more important

properties are the following: the matrix should have a porous network; beads should be rigid, be able to withstand moderate pressures and be uniform in porosity and size; more importantly, the beads should be chemically and biologically inert but at the same time easily activable for a facile ligand coupling; the activation procedure should be easy to perform, preferably at 4 °C or room temperatures and in aqueous media and is not too harsh for the support or for the ligand to be coupled; and finally the ligand-matrix complex should be stable and reusable. Obviously, no one matrix support available today fulfills all these requirements.

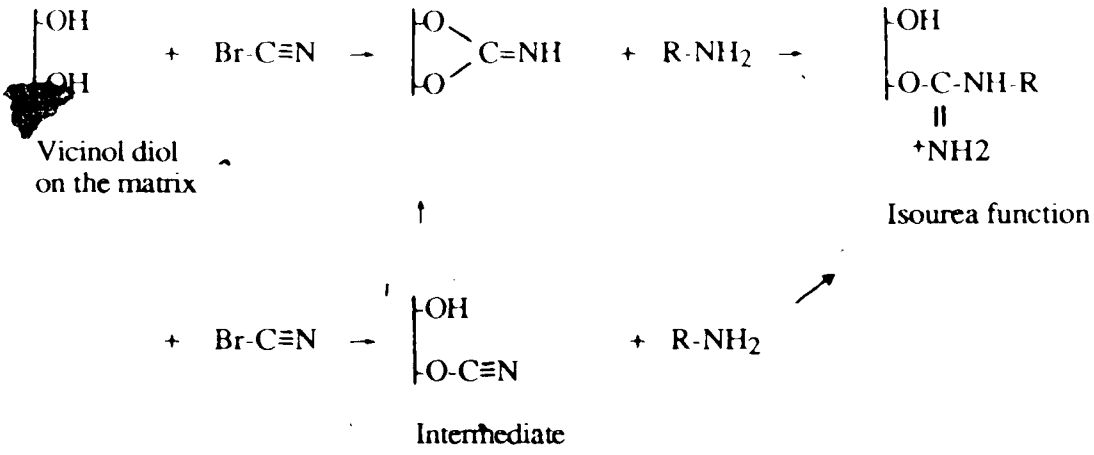
One of the most widely used matrix supports is agarose. It is a linear polymer of galactose containing aerogel-xerogel hybrid colloid. Many varieties of activated agarose, ready for ligand coupling and derivatized agarose are commercially available from several sources. Other popular supports are cellulose (mostly linear polymers of β-1,4-linked D-glucose units), dextran (α-1,6-linked glucose polymer) and polyacrylamide (polyhydrocarbon chains with carboxyamide group).

b. Activation procedures

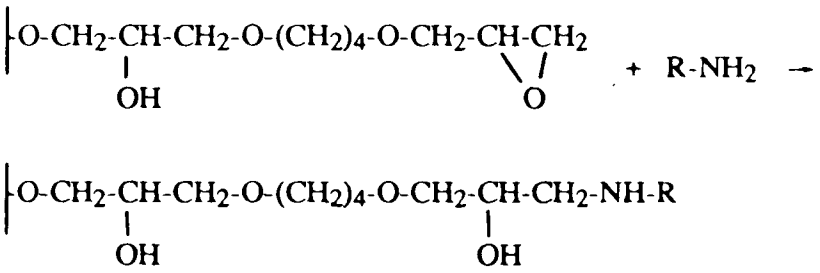
Cyanogen bromide activation of Sepharose resin has been one of the most widely used of all activation procedures (Cuatrecasas and Anfinsen, 1971). Cyanogen bromide reacts with two neighboring hydroxyl groups of agarose, dextran or cellulose and forms an active intermediate (Figure I.4). It can be subsequently derivatized with spacer molecules or ligands with nucleophilic groups, especially primary amino groups. This procedure, however, has two major drawbacks. For one, during the activation and the coupling stages, charged groups are formed which tend to increase nonspecific interactions. Secondly, bonds formed between the activated support and ligands are not very stable and consequently significant leakage of coupled ligand occurs during storage. Also, cyanogen bromide is highly toxic and special care is needed during handling.

Figure I.4. The Reaction Sequences for Matrix Activation and Ligand Coupling.

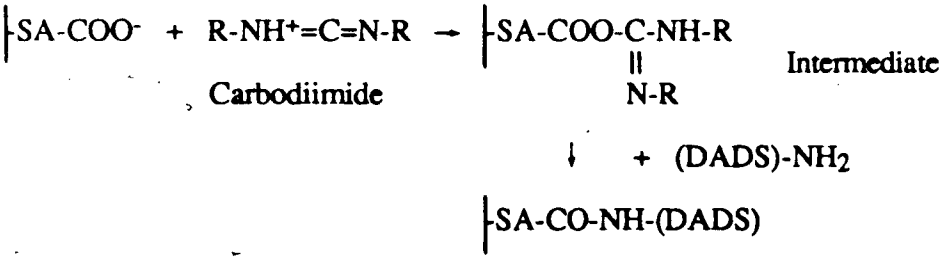
A) The Cyanogen Bromide Activation of Matrix.



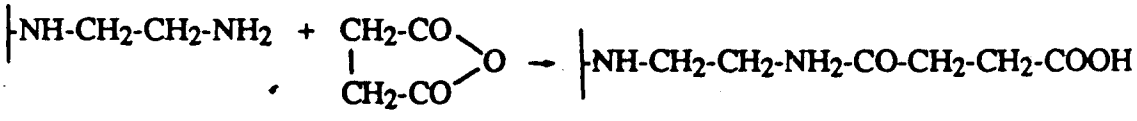
B) Oxirane coupling (epoxy-activated matrix).



C) Carbodiimide coupling of DADS to Carboxyl group of a Spacer Arm.



D) Amino-ethyl Succinyl Spacer Arm.



Several alternate procedures for activation of solid supports have been developed. Bis-oxiranes react readily with hydroxyl groups of matrix. Ligands with nucleophilic groups can then be attached to the long hydrophilic spacers formed by oxiranes. Oxirane based methods produce extremely stable linkages with a minimum number of hydrophobic and ionic groups. A procedure based on carbonyldiimidazole activation is also popular especially since it does not form any charged groups on the matrix (Bethell *et al*, 1979). Periodate oxidation involves oxidation of the vicinal diol groups to form aldehydes. The aldehyde groups are then reacted with ligands containing primary amines to generate Schiff's bases. These are subsequently reduced with borohydride to form a stable linkage.

c. Spacer Molecules

The presence of a spacer molecule is often needed to overcome steric hindrance of the protein-ligand interaction. Sometimes spacer molecules are needed because the ligand can not be coupled directly to the matrix. The length and chemical nature of the spacer molecules may significantly affect interaction of a protein (binding and elution properties) with the immobilized ligand. Although there is no way of predicting how a given spacer molecule will affect the ligand-protein interaction, hydrophobic and charged spacer molecules tend to increase nonspecific interaction. A very short spacer molecule may be unable to overcome steric hindrance and a very long spacer molecule may increase non-specific interaction. Thus, a very short or very long spacer may not be desirable.

Lately, a number of affinity resins with spacers of different chemical nature and length have been available commercially. These resins are convenient to use and have spacers usually terminating in an amino- or carboxyl-group (or their activated derivative) for facile coupling with a ligand. In this way a number of spacer-ligand can be tested in a short period of time. However, one drawback of the commercially available resins is that they usually have a fixed number of reactive groups per unit of resin. Thus, it is usually difficult to vary ligand density on these resins. Also, in some cases better results are

obtained if activation of matrix and coupling of spacer molecules are performed fresh in the lab. This is especially true if the activated resin is not stable or when the ligand density on the support needs to be varied.

d. Ligand

As discussed earlier, the proper choice of a ligand is crucial to the success of affinity chromatography. The affinity of the ligand for the protein should be strong enough to retain the protein specifically and yet it should be possible to recover the protein without denaturation. Weak affinity results in poor yields and nonspecific retention of the contaminating material by the immobilized ligand. If a spacer molecule is to be used, it should be remembered that choice of a spacer can significantly alter the ligand-protein interaction. Besides choosing the proper ligand, the chemistry of ligand coupling is also important. If the ligand is a small molecule, it binds to the protein via certain groups and in a certain orientation. Following the coupling procedure, these groups need to remain unmodified and accessible to the binding site on the protein. If the ligand itself is a macromolecule, say a protein or a peptide, then the coupling conditions should be such that the ligand is not denatured or modified significantly.

e. Experimental Variables

Experimental conditions of pH, ionic strength and temperature may profoundly affect the strength of binding and therefore the outcome of affinity chromatography. In fact, the bound protein can be effectively eluted by changing one of these factors. Other factors that affect the procedure of affinity chromatography are flow-rate and incubation time, column size, and sample size (Lowe *et al*, 1974a, b; Harvey *et al*, 1974a,b). It is not possible to accurately predict how these factors will affect a particular ligand-protein system. For example, increasing the salt concentration strengthens the hydrophobic interactions between a ligand and a protein but weakens the electrostatic interactions. Thus,

increasing the ionic strength can have opposite effects on different systems (reviewed by Dean *et al*, 1985).

Since membrane proteins have a large hydrophobic surface, affinity chromatography of the integral membrane proteins becomes more complicated than that of the soluble proteins. The membrane proteins need to be solubilized with a proper detergent. The solubilized proteins should retain their normal ligand binding properties. Moreover, associations of the membrane proteins with lipids and also with other proteins make purification difficult. These problems generally do not arise when dealing with soluble cytoplasmic proteins.

Ligand density on the matrix significantly affects binding characteristics of a protein. If the ligand molecule is small and bears a charge, then at higher concentrations of ligand, nonspecific electrostatic interactions may take predominance over the biospecific interactions (Ikeda *et al*, 1984). If the ligand is a macromolecule, then at higher densities the amount of protein bound to the resin may actually decrease due to steric hindrance (Low *et al*, 1987). Thus as a general rule, optimal density of the ligand should be determined in preliminary experiments.

F. Renal Anion Exchange

The primary role of the kidney is to maintain the volume and composition of extracellular fluid. As blood passes through the glomerulus, ions due to their small size are freely filtered. Almost all of the ions are later reabsorbed by the kidney.

The two major anions of the ultrafilterate, bicarbonate ion and chloride ion are reabsorbed in proximal tubules as well as in thick ascending limb and collecting tubule segments. Bicarbonate ion is reabsorbed more rapidly in the early proximal tubule while chloride ion is reabsorbed in the middle and late proximal tubule. Carbon dioxide from the lumen enters the cell by rapid diffusion across the apical membrane. It then combines with water to generate hydrogen and bicarbonate ion. The resulting proton is then secreted into

the lumen in exchange for sodium by a Na^+-H^+ antiporter. Chloride ion enters the cell via a Cl^--formate antiporter located in the apical membrane. The anions exit the cell from the basolateral side; bicarbonate ion leaves by a rheogenic Na^+-HCO_3^- cotransporter whereas chloride ion leaves via a Cl^--base antiporter. In collecting tubule segments, chloride ion leaves via a Band 3-like Cl^--HCO_3^- exchanger located on the basolateral side of intercalated cells.

Many studies have demonstrated that anion exchange takes place in proximal tubules of mammalian kidney (for a review see Kokko and Jacobson, 1985). Like erythrocyte anion-exchange, renal anion-exchange has been shown to be sensitive to stilbene disulfonates. Renal epithelial cells, unlike the erythrocytes, are polarized and have two structurally and functionally distinct domains. The membrane facing the lumen is called luminal or brush border membrane whereas the membrane facing the capillaries is called antiluminal or contraluminal or basolateral membrane. Structures called 'tight-junctions' keep the contents of these membranes from intermixing. Anion transport across the basolateral or contraluminal surface has shown to be inhibited by SITS or DIDS in rat, rabbit and dog proximal tubules (Grinstein *et al.*, 1980; Ullrich and Murer, 1982; Brazy and Dennis, 1981). Data on the brush border or luminal anion transport is, however, conflicting. The luminal anion transport is shown not to be inhibited by stilbene disulfonates by some workers (Grinstein *et al.*, 1980; Ullrich *et al.*, 1980), whereas others found it stilbene disulfonate inhibitable (Brazy and Dennis, 1981; Pritchard, 1987). Unlike erythrocyte anion-exchange, renal anion-exchange is more complicated in terms of substrate requirements, dependence on other counter-anions, dependence on Na^+, and the site of ion-translocation (luminal or contraluminal). These and other characteristics (e.g. inhibitor sensitivity, distribution within a nephron) suggest that probably more than one transport system contributes to the renal anion-exchange.

The transport of ions at both ends of epithelial cells (luminal and antiluminal) seem to regulate a variety of cellular functions. These involve active NaCl reabsorption,

intracellular pH regulation, volume reabsorption etc. These transport systems are also functionally linked with each other since they share some of the substrates e.g. Cl^-/HCO_3^- exchange, Na^+/HCO_3^- exchange, Na^+/H^+ exchange, etc. Most of the information on the renal anion-exchange is obtained by using three approaches.

1) Microperfusion studies *in situ* ;

2) Transport studies on cell lines derived from kidney;

3) Transport studies on isolated membrane vesicles.

1. Perfusion Studies

Alpern and Chambers (1987) measured effects of varying substrate concentrations and inhibitors on the intracellular pH in the microperfused proximal tubule using a pH-sensitive dye. Their results suggest presence of Cl^-/HCO_3^- exchange and Na^+/HCO_3^- cotransport on the basolateral membrane. The Cl^-/HCO_3^- exchange is electroneutral whereas the Na^+/HCO_3^- cotransport occurs with a stoichiometry of 3 HCO_3^- : 1 Na^+ (Sasaki, *et al.*, 1987; Baum, 1987). Both these processes are inhibitable by SITS added from the peritubular side (Biagi and Sothell, 1986). Presence of a luminal Na^+/H^+ exchange and a basolateral Na^+/HCO_3^- cotransport has also been suggested by other studies (Alpern and Chambers, 1986). Other perfusion studies have indicated presence of a sodium-independent exchange of bases (Yoshitomi *et al.*, 1985), a chloride/formate exchange (Schild *et al.*, 1987) and a basolateral Cl^-/HCO_3^- exchange (Brisolla *et al.*, 1985).

2. Studies on Cell Lines

Kurtz and Golchini (1987) have shown the presence of Cl^-/HCO_3^- exchange in MDCK cells that plays an important role in intracellular pH regulation following alkalization. The Na^+/H^+ exchanger seems to regulate the intracellular pH following acidification. In BSC-1 cells (monkey kidney epithelial cells) the presence of Na^+/HCO_3^- cotransport, Na^+ independent Cl^-/HCO_3^- exchange and Na^+/H^+ exchange has been

demonstrated by Jentsch *et al.* (1985). They show that in these cells also the intracellular pH is mainly regulated by the amiloride-sensitive Na^+/H^+ exchange system at acidic pH, whereas at alkaline pH, the DIDS-sensitive Na^+/HCO_3^- cotransport and the DIDS-sensitive Cl^-/HCO_3^- exchange processes become dominant in pH regulation (Jentsch *et al.*, 1986a,b,c). In Vero cells, uptake of chloride was shown to occur via a SITS inhibitable exchange system (Olsnes and Sandvig, 1986). Recently, DIDS-inhibitable bicarbonate uptake by Vero cells has been shown to occur both in a Na^+-linked and in a Na^+-independent manner (Tonnessen *et al.*, 1987; Olsnes *et al.*, 1987). They found that at acid pH, the internal pH is regulated by the Na^+/HCO_3^- cotransport while upon alkalization, the Na^+ independent Cl^-/HCO_3^- becomes predominant. A sodium independent Cl^-/HCO_3^- exchanger that regulates intracellular pH was also shown to be present in LLC-PK1 cells (Chaillet *et al.*, 1986).

3. Studies on Membrane Vesicles

Akiba *et al.* (1986) have shown the presence of an electrogenic Na^+/HCO_3^- cotransport in basolateral membrane vesicles prepared from rabbit renal cortex. This process occurred with a stoichiometry of at least two HCO_3^- for each Na^+ and was inhibited by stilbene disulfonates. A similar electrogenic Na^+/HCO_3^- cotransport has recently been shown to occur in basolateral but not in brush border membrane vesicles prepared from rat renal proximal tubules (Grass *et al.*, 1987). Presence of a DIDS sensitive and Na^+ independent sulphate/bicarbonate transport has been demonstrated in rat proximal tubule basolateral membrane vesicles (Hagenbuch *et al.*, 1985). Recently Pritchard (1987) demonstrated sulphate uptake by rat renal brush border membrane vesicles in the presence of an outwardly directed HCO_3^- gradient. This exchange was cis-inhibited by stilbene disulfonates and was not affected by Na^+. The presence of a separate Na^+/sulphate cotransporter, that was not inhibited by SITS, was also demonstrated in the same brush border membrane vesicles. In contrast, however, Bastlein and Burckhardt

(1986) have shown that the brush border membrane Na^+/sulphate cotransport is inhibited by higher concentrations of DIDS (Ki = 350 μM). They have also shown the presence of a proton driven sulfate transport system in the basolateral membranes from rat renal proximal tubules. The presence of stilbene disulfonate-inhibitable anion exchangers from both the brush border membranes and the basolateral membranes from bovine kidney proximal tubules has recently been demonstrated by Talor *et al.*, (1987). They also show that the brush border membrane sulfate uptake is both Na^+ dependent and independent while the basolateral sulfate exchange is Na^+ independent. All these processes are inhibited by DIDS.

From the literature reviewed above it becomes clear that stilbene disulfonate-inhibitable anion transport in renal proximal tubules is carried out by more than one separate transporter. In the mammalian kidney, anions such as sulfateand chloride are freely filtered and subsequently reabsorbed in the proximal tubule. The anions enter epithelial cells across the luminal membrane and this uptake is often but not always coupled to the movement of sodium ions. The anions exit across the basolateral membrane by a sodium-independent anion-exchanger. All these processes are inhibited by stilbene disulfonates. Also, the presence of Band 3-like proteins in mammalian kidney has been recently shown by both immunological (Schuster *et al.*, 1986) and molecular biological techniques (Kopito *et al.*, 1987b).

G. Thesis Problem

Band 3 protein from human erythrocyte membrane has been extensively characterized with respect to its structural and functional properties. Various protocols for isolation and purification have been developed and improved over the years. The anion transport characteristics have been examined in detail in order to understand molecular mechanism of the ion translocation. In this respect various inhibitors of the anion transport, especially the stilbene disulfonates, have proved very useful. The nature of

Band 3-stilbene disulfonate interaction has also been investigated in some detail. The stilbene disulfonate binding site on the protein has been examined with respect to its enviornment and location on the peptide backbone.

This project was mainly aimed at preparing an affinity resin for Band 3 protein using stilbene disulfonate derivatives as ligand. Some stilbene disulfonate derivatives that bind noncovalently to Band 3, when immobilized, could be used as a ligand for affinity chromatography. Such an affinity matrix was to be used mainly for three purposes:

1) for the purification of Band 3 from erythrocytes;

2) for studying protein-ligand interaction and;

3) for identification and isolation of Band 3-like proteins from non-erythroid sources.

Thus, the ongoing presentation is mainly divided into three sections, each dealing with one of the above aspects.

The reasons for trying to design an affinity resin using a stilbene disulfonate as a ligand were many. For one, although the technique of affinity chromatography had some spectacular successes in the purification of soluble proteins, its success with membrane proteins has been somewhat limited. Procedures for purification of membrane glycoproteins on lectin affinity resins were developed (Findlay, 1974). Many receptor proteins were also successfully identified and purified using ligand affinity resins (Cuatrecasas and Anfinsen 1971). However, no successful attempts for affinity purification of a transport protein, using either a substrate or an inhibitor as an immobilized ligand were reported in the literature. Many laboratories had tried to achieve this goal with no or only a moderate success (Weber *et al.*, 1985). Being the first one to achieve this goal with the Band 3 protein was important since the lessons learned from one transport system could assist in designing a strategy for the purification of other transport proteins.

Designing an affinity resin solely for the purpose of purification of Band 3 from human erythrocyte membrane was a challenging but not very attractive idea. Band 3

purification protocols using other types of chromatographic techniques already existed and had a few or no drawbacks. What made the idea of immobilizing a stilbene disulfonate attractive was the potential use of such a system in studying stilbene disulfonate-Band 3 interactions. Also, many nonerythroid tissues exhibit electroneutral exchange of anions that is inhibited by stilbene disulfonates. With the help of such an affinity resin it would be possible to isolate and purify such putative anion transporters.

Chapter II describes experiments to find a proper combination of a spacer molecule and a stilbene disulfonate derivative suitable for affinity chromatography. Initially DADS was used as a ligand in combination with various spacer molecules. However, due to its low affinity (Ki = 1.3 mM), DADS retained low amounts of Band 3 and bound other proteins non-specifically. This was followed by a search for a better ligand than DADS. Of the various ligands and spacer molecules tested, SITS-Affi-Gel 102 affinity resin was found to be most suitable. With this affinity resin experimental conditions were standardized to obtain optimal yields of pure Band 3. Affinity chromatography was performed at various temperatures and the optimal temperature was found to be 4 °C. Effects of ionic strength and the nature and the length of the spacer molecule on protein binding were studied. Finally, it was shown that Band 3 from nonhuman erythrocytes was also capable of binding to the affinity resin.

Experiments on the interaction of Band 3 with the resin are summarized in Chapter III. It was shown that the solubilized Band 3 protein binds to the immobilized ligand as it would to a free ligand. The two stages of Band 3 binding to the resin, namely the weakly-bound stage and the strongly-bound stage were characterized in more detail and conversion of Band 3 from one form to the other form was studied. The effect of DIDS labelling on the interaction of Band 3 with the resin was studied. These studies revealed some interesting aspects of the inhibitor-protein interaction. Evidence was provided for the intersubunit interaction between two monomers of the Band 3 dimer. Interaction of chemically modified protein with the resin was also studied. Studies on the interaction of

Band 3 in membrane (either intact erythrocytes or sealed vesicles of either orientation) with the resin are reported in Chapter IV. It was shown that the stilbene disulfonate binding site of Band 3 in intact erythrocyte was accessible to immobilized ligand. Rightside-out vesicles also bound to the resin.

The kidney plays a crucial role in maintaining and regulating levels of various electrolytes (Wieth and Brahm, 1985). This is done either by excretion or reabsorption of these electrolytes in different parts of the kidneys. A number of studies, based on physiological approaches have suggested that an electroneutral exchange of anions occurs in the proximal tubules of kidney (Warnock and Eveloff, 1982). This transport activity is inhibited by SITS and DIDS. Therefore, the affinity resin was used to identify and isolate a polypeptide that might be involved in the anion transport. These experiments are summarized in Chapter V. A 130-kDa glycoprotein from canine renal brush border membranes specifically bound to SITS-Affi-Gel 102 resin. This polypeptide was also specifically labelled with [^{3}H]-H_2DIDS. Both, the binding to the affinity resin and labelling with [^{3}H]-H_2DIDS was prevented in the presence of 1 mM BADS. This suggests that this polypeptide specifically binds stilbene disulfonates. This integral membrane glycoprotein is present in brush border membranes and not in basolateral membranes and does not cross-react with antibodies raised against dog or human erythrocyte Band 3.

Information obtained from these experiments shows the usefulness of immobilizing a ligand for the purpose of purification, characterization and identification of membrane proteins involved in transport functions. A general strategy for successful application of this technique to other transport systems is suggested. A potential use of affinity resins in studying ligand-protein interaction is illustrated. Finally, the observation that a single polypeptide from kidney membranes can be specifically isolated using the affinity resin proves the vast potential of technique affinity chromatography for the purification of membrane transport proteins.

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II. Design and Synthesis of an Affinity Matrix for Chromatography of Band 3[1]

A. INTRODUCTION

Band 3 protein is a major protein of the erythrocyte membrane and catalyzes the exchange of chloride and bicarbonate ions in vivo (Knauf, 1979). It is an integral membrane protein (M_r=95,000) and is composed of two domains (Steck *et al.*, 1976; Appell and Low, 1982). The amino-terminal domain (M_r=41,000) is involved in binding to the cytoskeletal proteins (Bennett and Stenbuck, 1980) and various enzymes (Tsai *et al.*, 1982) whereas the carboxyl-terminal half of Band 3 (M_r=55,000) spans the membrane bilayer several times and contains the site for ion translocation (Steck *et al.*, 1976; Grinstein *et al.*, 1978). Anion exchange is inhibited either reversibly or irreversibly by stilbene disulfonate derivatives (Knauf, 1979; Ramjeesingh *et al.*, 1981). They presumably bind to a single hydrophobic site on the protein in or near the substrate translocation site and thereby compete with substrates. The affinities of stilbene disulfonates for Band 3 range from 40 nM to ~1 mM (for a review, see Knauf, 1979; Macara and Cantley, 1983).

Generally Band 3 is purified from hemoglobin-free erythrocyte membranes by "stripping" the membranes by addition of EDTA (Fairbanks *et al.*, 1971), or by exposure to extremes of pH (Grinstein *et al.*, 1979), followed by selective extraction with nonionic detergents (Lukacovic *et al.*, 1981). Further purification may be achieved by anion-exchange chromatography (Lukacovic *et al.*, 1981; Yu and Steck, 1975) followed by column chromatography using an activated thiol gel (Fukuda *et al.*, 1978) or a p-(chloromercuri)-benzamido-ethyl agarose gel (Lukacovic *et al.*, 1981). This yields at least 95% pure Band 3.

Affinity chromatography has been used to purify various enzymes and receptors (for a review, see Venter, 1982). The advantage of using immobilized ligand to purify a

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protein of interest over the other procedures is the specificity and rapidity of the procedure. Often, several-fold purification is achieved in a single step. However, no attempts to purify Band 3 using an inhibitor-affinity resin have been reported. The difficulty arises due to the hydrophobic nature of Band 3 and its extensive association with the cytoskeletal network and other proteins. We describe here the design of an inhibitor-affinity resin and experimental conditions of chromatography that allow biospecific purification of Band 3. The affinity resin has a high binding capacity and allows the rapid, convenient isolation of Band 3. We also provide novel evidence for the two-stage binding of stilbene disulfonate inhibitors to Band 3 protein.

B. MATERIALS and METHODS

Affi Gel-102 was purchased from Bio-Rad. Shaltiel hydrophobic chromatography kit with 4, 6 and 8-atom spacer arms was from Miles Scientific. SITS was obtained from Pierce Chemical Co. while 4,4'-diaminostilbene-2,2'-disulfonate (DADS) was obtained from Eastman. BADS was synthesized according to Kotaki *et al.* (1971) as previously described (Rao *et al.*, 1979). Aminoethyl-Sepharose 4B was synthesized according to Shaltiel and Er-El (1973). Lithium dodecyl sulfate was a product of Boehringer Mannheim while $C_{12}E_8$ was from Nikko Chemical Co., Tokyo. All other chemicals were reagent grade or better.

1. Erythrocyte membrane preparation

Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Canadian Red Cross) by hypotonic lysis in 5 mM sodium phosphate, pH 8.0 (Dodge *et al.*, 1963). All steps were carried out at 0-4°C. The cells were washed at least three times with 10 volumes of 0.9% NaCl and were recovered by centrifugation at 5,000 rpm in an SS-34 rotor. Each time the white layer of leucocytes and cell debris was carefully aspirated off from the top of the pellet. The cells were lysed on ice by 5 mM sodium

phosphate, pH 8.0, in the presence of 1 mM EDTA and 1 mM phenylmethylsulfonylfluoride to minimize proteolysis and membranes were recovered by centrifugation at 15,000 rpm in an SS-34 rotor in a Sorvall RC-5B centrifuge. The washing procedure was repeated 3-4 times until creamy white ghosts were obtained.

2. Solubilization of Band 3

Ghost membranes were stripped of cytoskeleton by suspending in at least 10 volumes of 0.2 mM sodium EDTA, pH 7.5, containing 20 µg/ml PMSF and 0.2 mM dithiothreitol at 37 °C for 30 min. (Bennett, 1983). Membranes were centrifuged at 17,000 rpm for 20 min. and washed again with the same buffer at 4 °C. The stripped membranes were then extracted with 10 volumes 1 M KI, 7.5 mM sodium phosphate, 1 mM sodium EDTA, 1 mM dithiothreitol and 20 µg/ml PMSF at 37 °C for 30 min. to remove other extrinsic membrane proteins. Membranes were recovered by centrifugation at 19,000 rpm for 25 min. and washed again with the same buffer at 4 °C. Finally, the pellet was washed with 5 mM sodium phosphate, pH 8.0, centrifuged at 19,000 rpm for 25 min. and suspended in minimal volume of 5 mM sodium phosphate, pH 8.0. (~3-4 mg protein/ml). Stable heteromers of Band 3 with extrinsic proteins are observed if unstripped membranes were solubilized (Bennett, 1983). The KI-extracted membranes were then solubilized (at protein concentrations of 1.5-2 mg/ml) in 1% $C_{12}E_8$ in 228 mM citrate buffer pH 8, containing 1 mM dithiothreitol and 20 µg/ml PMSF (final concentration). Following a 20 min. incubation on ice, the solubilized membranes were centrifuged at 19,000 rpm for 30 min. in an SS-34 rotor. The supernatant (Band 3 - 228C8 extract) was stored at 4°C.

Partially purified Band 3 (Band 3 - 5P8 extract) was prepared as described earlier (Lieberman and Reithmeier, 1983) with a few modifications. Briefly, the ghost membranes were solubilized in 5 mM sodium phosphate, pH 8.0, containing 1% $C_{12}E_8$. The extract was applied to an aminoethyl-Sepharose column and Band 3 was eluted with

20 mM sodium phosphate, pH 8.0, containing 0.1% $C_{12}E_8$. The elute was then dialyzed against 5P8 buffer containing 0.1% $C_{12}E_8$ overnight with at least two changes.

3. Labeling of cells with DIDS

Erythrocytes were washed extensively with 5 mM sodium phosphate, pH 8, and 150 mM NaCl (PBS) to remove medium and cell debris. Erythocytes were then suspended in PBS at a 25% hematocrit and were reacted with 50 µM DIDS at 37°C for 1 h. Cells were washed once with the above buffer containing 0.5% bovine serum albumin (fatty acid free) and twice with buffer alone. Ghosts and Band 3 - 228C8 extract were prepared as above.

4. Affinity resins

1 ml Affi Gel-102 resin (15 µmole -NH_2/ml of settled gel) was washed with water followed by 100 mM sodium bicarbonate buffer, pH 8.5, and suspended in 1 ml bicarbonate buffer. To this suspension was added 16.56 mg SITS (30 µmoles) dissolved in 2 ml bicarbonate buffer. After adjusting the pH to 8.5 the suspension was shaken at 37 °C for 1 hr. The resin was then washed with bicarbonate buffer followed by water and stored at 4° C with 0.1% sodium azide.

Affinity resins not directly purchased from commercial sources were prepared by conjugating various spacer arms to cyanogen-bromide activated Sepharose 6B according to Lukacovic *et al.* (1981). Using radioactive glycine, the cocentration of spacer molecules on the resin was determined to be 3.1 µmole/ml resin. The inhibitor ligand was then coupled to 1 ml of these resins using 3 mmoles DADS with 8 mmoles EDC (1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride) (Lukacovic *et al.*, 1981).

5. Affinity-Purification

Unless stated otherwise, all steps were carried out at 0-4° C. Protein binding assays were performed in 1.5 ml microfuge tubes. 25 µl of packed resin was washed twice with 250 µl of 228 mM sodium citrate- 0.1% $C_{12}E_8$, pH 8.0, (citrate buffer) and was incubated with 1 ml of solubilized membrane supernatant (1.5-2 mg/ml protein) for 15 min. After removing the supernatant, the gel was washed at least three times with 250 µl of citrate buffer and the bound material was then eluted by shaking the resin for 10 min. with 110 µl of 1 mM BADS in 5 mM sodium phosphate-0.1% $C_{12}E_8$. Finally, the resin was again washed at least twice with 10 volumes of citrate buffer and then extracted with 110 µl of 1% LDS in 5 mM phosphate for 10 min.

6. Protein assay

Protein was determined according to Lowry *et al.* (1951). Bovine serum albumin fraction V from Sigma Chemicals was used as protein standard. All samples were solubilized with 1% sodium dodecyl sulfate prior to the assay.

7. Sodium dodecyl sulfate gel electrophoresis

Gel electrophoresis was performed on 10% polyacrylamide slab gels according to Laemmli (1970). Electrophoresis was usually performed at a constant current of 20 mA for gels of .75 mm thickness or 40 mA for 1.5 mm gels. Gels were stained in 0.1% Coomassie Blue in 25% isopropanol, 10% acetic acid for 1-3 h and destained in 25% methanol, 10% acetic acid overnight. Protein bands stained with Coomassie Blue were scanned in Joyce-Loebl Chromoscan 3 densitometer at 530 nm.

When electrophoresis was performed using the Bio-Rad mini apparatus the following modification was adopted. Electrophoresis was performed at a constant voltage of 200 volts for 45 min. regardless of the thickness of gels. Gels were stained for 1 h and destained for 4-5 h.

C. RESULTS

1. Selection of Spacer and Ligand for Affinity Chromatography

For successful affinity chromatography, the choice of the ligand and the spacer molecule is of critical importance. The presence of a spacer arm sometimes becomes necessary to eliminate the steric hindrance of the matrix. Table II.1 describes the structures of various spacer molecules used in the present studies. Table II.2 summarizes the initial attempts to find a suitable spacer arm and ligand. For these studies Band 3 - 5P8 extract was used. 1 ml affinity resin was packed in a 10 cm x 1.5 cm glass column equilibrated with 5P8 buffer containing 0.1% $C_{12}E_8$. 0.5 ml of Band 3 - 5P8 extract was loaded on the column at 4°C and washed with 5P8-0.1% $C_{12}E_8$. Band 3 bound to the column was then eluted with 0.3 M NaCl in 5P8-0.1% $C_{12}E_8$ and subsequently with 1% LDS in 5P8. Binding and elution of Band 3 was monitored on a chart recorder by ultraviolet absorption at 280 nm.

a. Hydrophobic spacer arms with DADS

Band 3 did not bind either to the Sepharose 6B matrix itself or to matrices with any of the hydrophobic spacer arms (listed in Table II.2) alone. Band 3 also did not bind to the column when DADS was either attached directly to the matrix or via a short 2-atom spacer arm. With longer hydrophobic spacer arms (4-atom and 6-atom in length), Band 3 did bind to the column. Band 3 bound to these columns could be eluted by increasing ionic strength of the buffer (up to 0.5 M NaCl) but not by 100 mM DADS or 10 mM DNDS or 1 mM BADS in 5P8. Moreover, Band 3 covalently labelled with DIDS also bound to the column suggesting that the binding was probably not entirely mediated via the inhibitor binding site. Attempts to minimize the non-specific interaction by increasing $C_{12}E_8$ concentration from 0.1% to 1% in the buffer did not meet with success. Adding 20% ethylene glycol or 10% glycerol to the buffer did not improve specific binding.

Table II.1 Chemical Structures of Affinity Resins

Spacer Molecule	Length	Inhibitor ligand[a]	Structure
	atoms		
Hydrophobic			
Glycine[b]	2	DADS	-$NHCH_2CO$-DADS
4-Aminobutanoic acid	4	DADS	-$NH(CH_2)_3CO$-DADS
6-Aminocaproic acid	6	DADS	-$NH(CH_2)_5CO$-DADS
Hydrophilic			
Glycyl-glycine	6	DADS	-$NHCH_2CONHCH_2CO$-DADS
Glycyl-glycyl-glycine	8	DADS	-$NHCH_2CONHCH_2CONHCH_2CO$-DADS
Epoxy-Sepharose[c]	~12	DADS	-$OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2$-DADS
Aminoethyl succinyl[d]	8	DADS	-$NHCH_2CH_2NHCOCH_2CH_2CO$-DADS
Affi-Gel 102[e]	6	SPITC	-$OCH_2CONHCH_2CH_2NH$-SPITC
Affi-Gel 102	6	SITS	-$OCH_2CONHCH_2CH_2NH$-SITS

Spacer arms were coupled to CNBr activated Sepharose 6B through their amino groups. DADS was ligated to the carboxyl group of the spacer arms using carbodiimide.

[a]Structures of the inhibitor ligands are given in Table I.1.

[b]Glycine is neither hydrophobic nor hydrophilic. However, for conveinience it is listed as hydrophobic.

[c]Epoxy-activated Sepharose 6B was obtained from Pharmacia.

[d]Amino-ethyl succinyl spacer arm was prepared according to Ikeda *et al.* (1984).

[e]Affi-Gel 102 was obtained from Bio-Rad.

Table II.2 Inhibitor affinity resins and their interaction with human erythrocyte Band 3

Spacer Molecule	Length	Inhibitor ligand	Protein binding[a]	Specificity[b]	Comments
	atoms				
None[c]	-	DADS	-	NA[d]	
Hydrophobic[e]					
Glycine	2	DADS	-	NA	
4-Aminobutanoic acid	4	DADS	+	-	Band 3 eluted by chloride but not by citrate
5-Aminocaproic acid	5	DADS	++	-	Band 3 eluted by chloride but not by citrate
6-Aminocaproic acid	6	BADS	++	-	
Hydrophilic					
Glycyl-glycine[e]	6	DADS	-	NA	
Glycyl-glycyl-glycine[e]	8	DADS	-	NA	
Epoxy-Sepharose[f]	~12	DADS	+/-	+/-	Band 3 binding not always observed
Aminoethyl succinyl[g]	8	DADS	+/-	+/-	Band 3 binding not always observed
Affi-Gel 102	6	SPITC	-	NA	
Affi-Gel 102	6	SITS	+++	+	Band 3 eluted by BADS in 5P8 buffer

Affinity chromatography was performed on 1 ml columns at 4°C. 0.5 ml of Band 3-5P8 extract (1mg/ml protein in 5P8-0.1% $C_{12}E_8$ or 28.5 mM sodium citrate -0.1% $C_{12}E_8$ buffer) was applied to columns at a flow rate of 10 ml/hr. Columns were washed with 5 mls respective buffers (either phosphate or citrate buffer) and eluted with 0.3 M NaCl-0.1% $C_{12}E_8$ followed by 1% LDS. Appearance of protein (monitored at 280 nm) in either of these two elutes was a criterion of binding.

[a]Plus sign denotes relative amount of protein that bound the coumns and was eluted by chloride.

[b]Biospecificity of the binding was determined by loading the protein sample on the column in presence of excess free ligand or by covalently labelling Band 3 with DIDS. Plus sign denotes specific binding; Minus sign denotes non-specific binding.

[c]DADS was ligated directly to CNBr activated Sepharose 6B.

[d]NA, not applicable.

[e]Spacer arms were coupled to CNBr activated Sepharose 6B through their amino groups. DADS was ligated to the carboxyl group of the spacer arms using carbodiimide.

[f]Epoxy-activated Sepharose 6B was obtained from Pharmacia.

[g]Amino-ethyl succinyl spacer arm was prepared according to Ikeda *et al.* (1984).

b. Hydrophilic spacer arms with DADS

Since the use of hydrophobic spacer arms resulted in non-specific interactions, hydrophilic spacer arms of equal or longer lengths were used in an attempt to minimize these interactions. Band 3 did not bind to the hydrophilic spacer arms alone. When DADS was attached to either Gly-Gly or Gly-Gly-Gly spacer arms, Band 3 did not bind to the columns. When DADS was attached either to epoxy-Sepharose (oxirane spacer arm) or to aminoethyl-succinyl spacer arm then Band 3 did bind to the column. Interactions of Band 3 with these two affinity columns was more specific, since the binding could be prevented by covalently reacting Band 3 with DIDS. However, Band 3 binding to these columns was not always successful. One of the reasons for the irreproducibility could be a structural limitation of DADS itself. DADS has two amino groups (Table II.2) and it is possible that during the carbodiimide coupling reaction (Lukacovic *et al.*, 1981), both ends of the DADS molecule couple to spacer arms.

c. Spacer arms with different ligands

To avoid the possibility of the ligand being coupled to the matrix through two sites, BADS instead of DADS was coupled to a hydrophobic 6-atom spacer arm. BADS has a benzoyl group that blocks one amino group of DADS. However, this makes BADS more hydrophobic and a stronger inhibitor than DADS. Band 3 did bind to this column but the binding was not prevented by covalently labelled DIDS. Another inhibitor, SPITC, has only one benzene ring and is a weak inhibitor as compared to DADS. Affinity resin prepared by linking SPITC to the hydrophilic 6-atom long spacer arm of Affi Gel-102 was unable to bind Band 3 when Band 3 - 228C8 extract was shaken with the affinity resin at 4°C (see Materials and Methods). Finally, when affinity resin was prepared by linking SITS to Affi Gel-102, Band 3 did bind to the column and the binding was prevented by covalently labelling Band 3 with DIDS.

Figure II.1

Purification of human erythrocyte Band 3 using SITS-Affi-Gel 102 resin. The experiment was performed as described in "Materials and Methods". SDS-polyacrylamide gel electrophoresis of human erythrocyte membrane proteins was performed on a 10% gel prepared according to the procedure of Laemmli. Lane 1, erythrocyte ghosts; lane 2, 1 mM EDTA extracted ghosts, pH 7.5; lane 3, stripped ghost membrane (Band 3 - 228C extract); lane 4, protein fraction eluted from SITS-Affi Gel-102 resin by 1 mM BADS; lane 5, protein fraction subsequently eluted by 1% LDS.

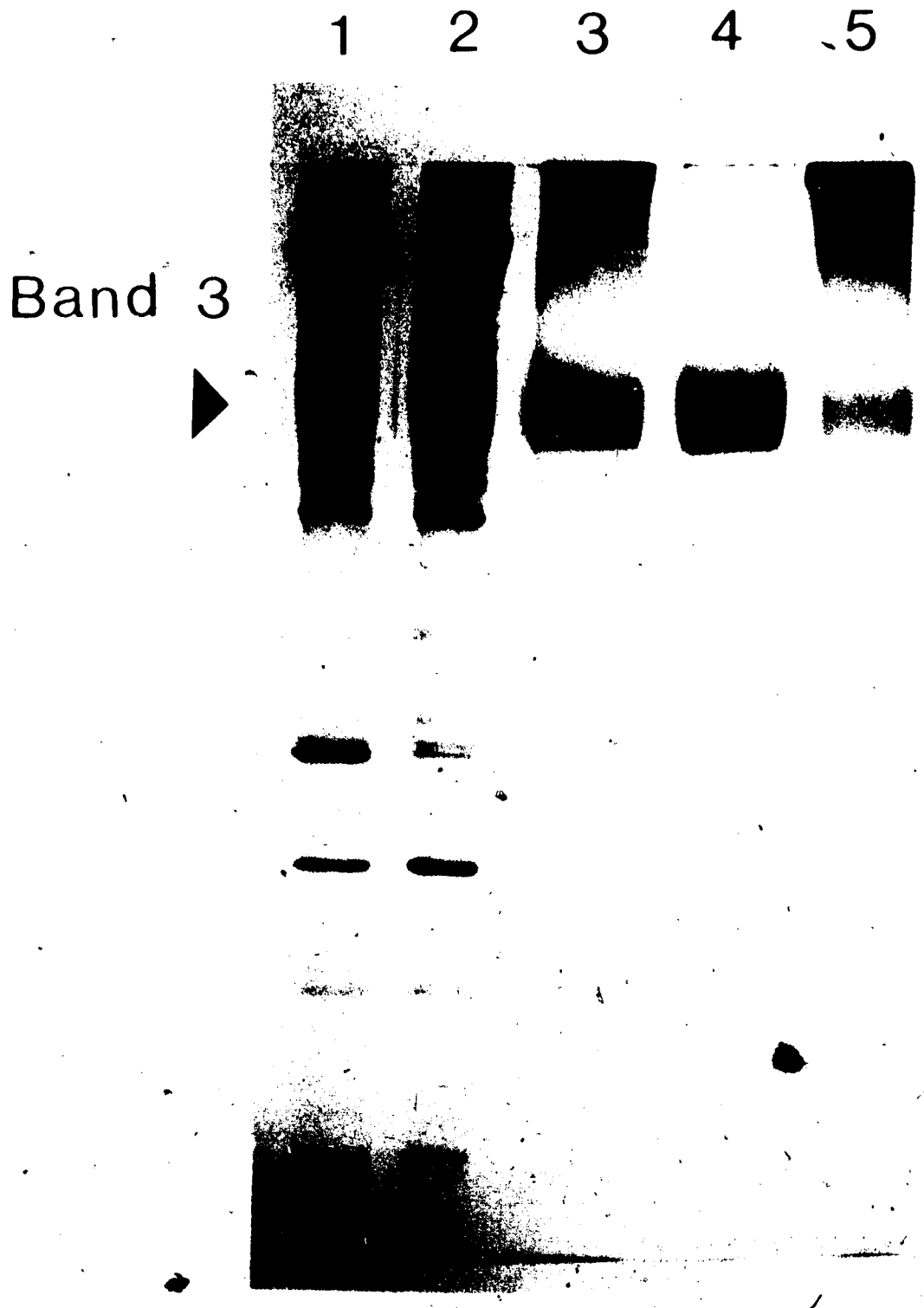
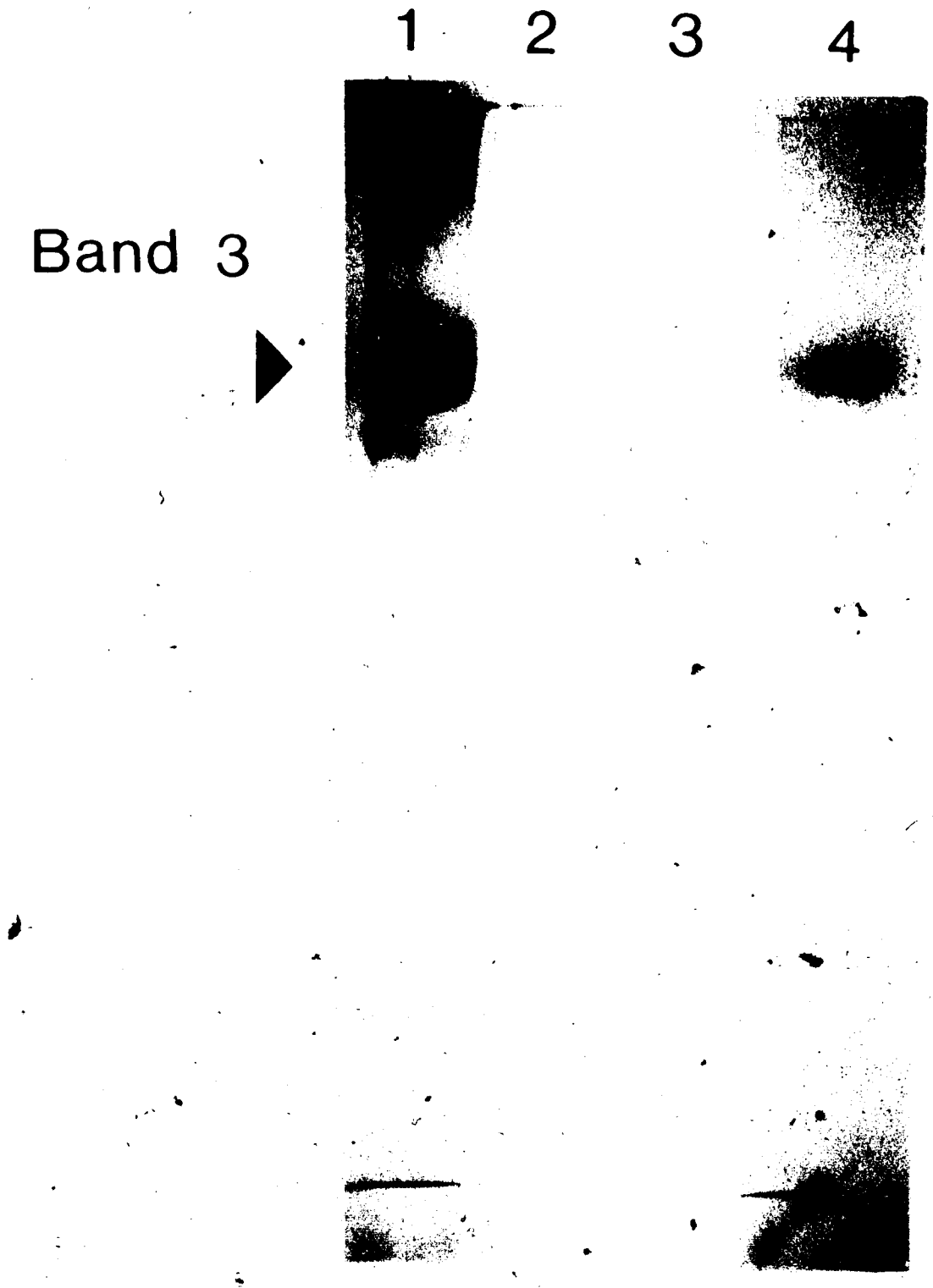

1
2
3
4
5
Band 3

Figure II.2

Specificty of Band 3 binding to SITS-Affi-Gel 102 resin. In a microfuge tube, 100 µl of SITS-Affi Gel-102 resin was incubated with 100 µl of stripped membrane preparation (protein concentration 1 mg/ml) in 1% $C_{12}E_8$, 114 mM sodium citrate buffer, pH 8, for 30 min at 4 °C. Supernatant was then collected and electrophoresed. SDS-polyacrylamide gel electrophoresis was performed on a 10% gel prepared according to the procedure of Laemmli. Lane 1, Band 3 sample; lane 2, incubation in absence of DNDS; lane 3, incubation in presence of 200 µM DNDS; lane 4, incubation with DIDS-labelled sample.


1
2
3
4
Band 3

2. Interaction of Band 3 with SITS-Affi Gel-102 Resin

To establish optimal conditions for Band 3 binding to the SITS-Affi Gel-102 affinity matrix and its subsequent elution in pure form, a small scale assay was developed (see Materials and Methods). This enabled us to examine several different conditions simultaneously. One ml of the Band 3 - 228C8 extract; (Fig. II.1 lane 3) was shaken with 25 µl of SITS-Affi Gel-102 for 15 min. at 4°C and the affinity matrix was washed with citrate buffer containing 0.1% $C_{12}E_8$. The bound protein was then eluted with 1 mM BADS in 5P8 buffer containing 1 mM DTT (lane 4). The affinity matrix was subsequently washed with 10 vol. citrate buffer and eluted with 1% LDS in 5P8 buffer to elute the rest of the bound protein (lane 5).

a. Purity of BADS elute

The protein fraction eluted by 1 mM BADS when resolved on SDS-PAGE showed a single Coomassie blue stained band of about 95,000. Occasionally, a minor band (M_r=70,000) was also seen, especially if the Band 3 - 228C8 extract was more than a week old. When the SDS-PAGE gels were stained with Stains-all to detect glycoproteins, a small amount of glycophorin was observed together with Band 3 in the BADS elute (data not shown).

b. Specificity of the affinity matrix

A specific interaction between the protein and the immobilized ligand is desirable in affinity chromatography. The specificity of Band 3 interaction with immobilized SITS was examined in two ways. When the solubilized membrane proteins (Band 3 - 228C8 extract) were shaken with SITS - Affi Gel-102 in presence of 200µM DNDS, Band 3 did not bind to the matrix and remained in the supernatant (Fig. II.2, lane 2 vs lane 3). Also when the inhibitor binding site on the Band 3 molecules was blocked irreversibly by reacting cells with DIDS at alkaline pH, (DIDS labeled Band 3 - 228C8 extract) Band 3 did not bind to the resin (lane 4).

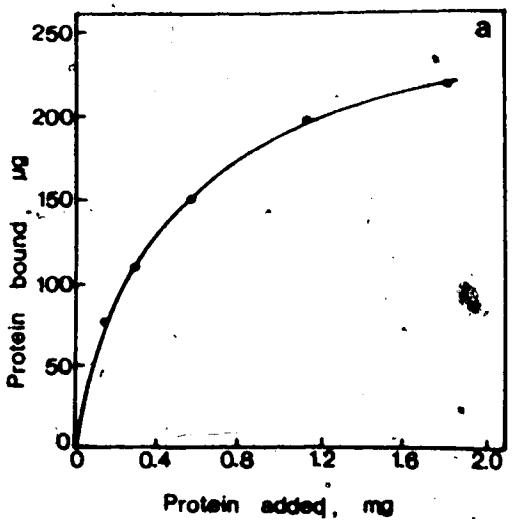
Figure II.3

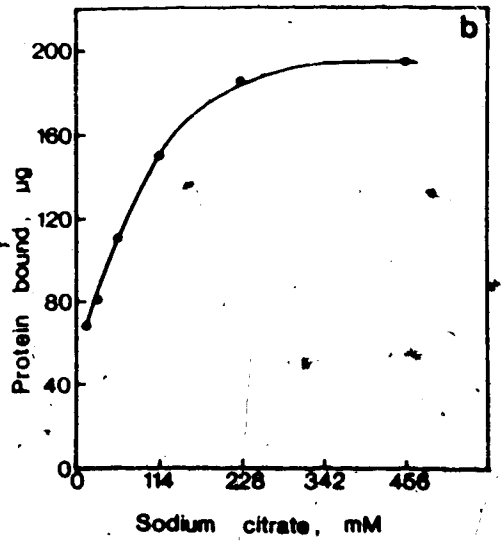
Protein binding characteristics of SITS-Affi Gel-102 affinity resin.

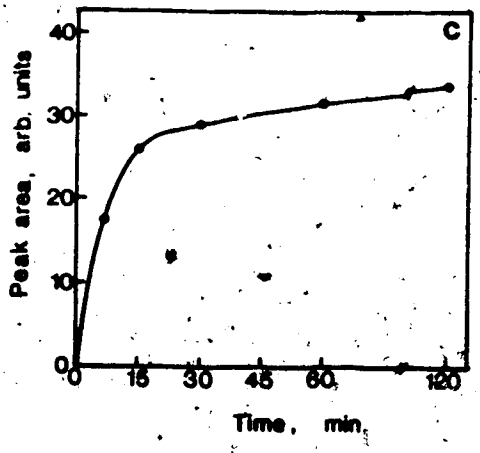
a) Increasing amount of stripped membrane protein in 228 mM citrate buffer, pH 8, with 0.1% $C_{12}E_8$ was incubated with 50 µl of affinity resin. The amount of protein removed from the supernatant is defined as the amount of protein bound to the resin.

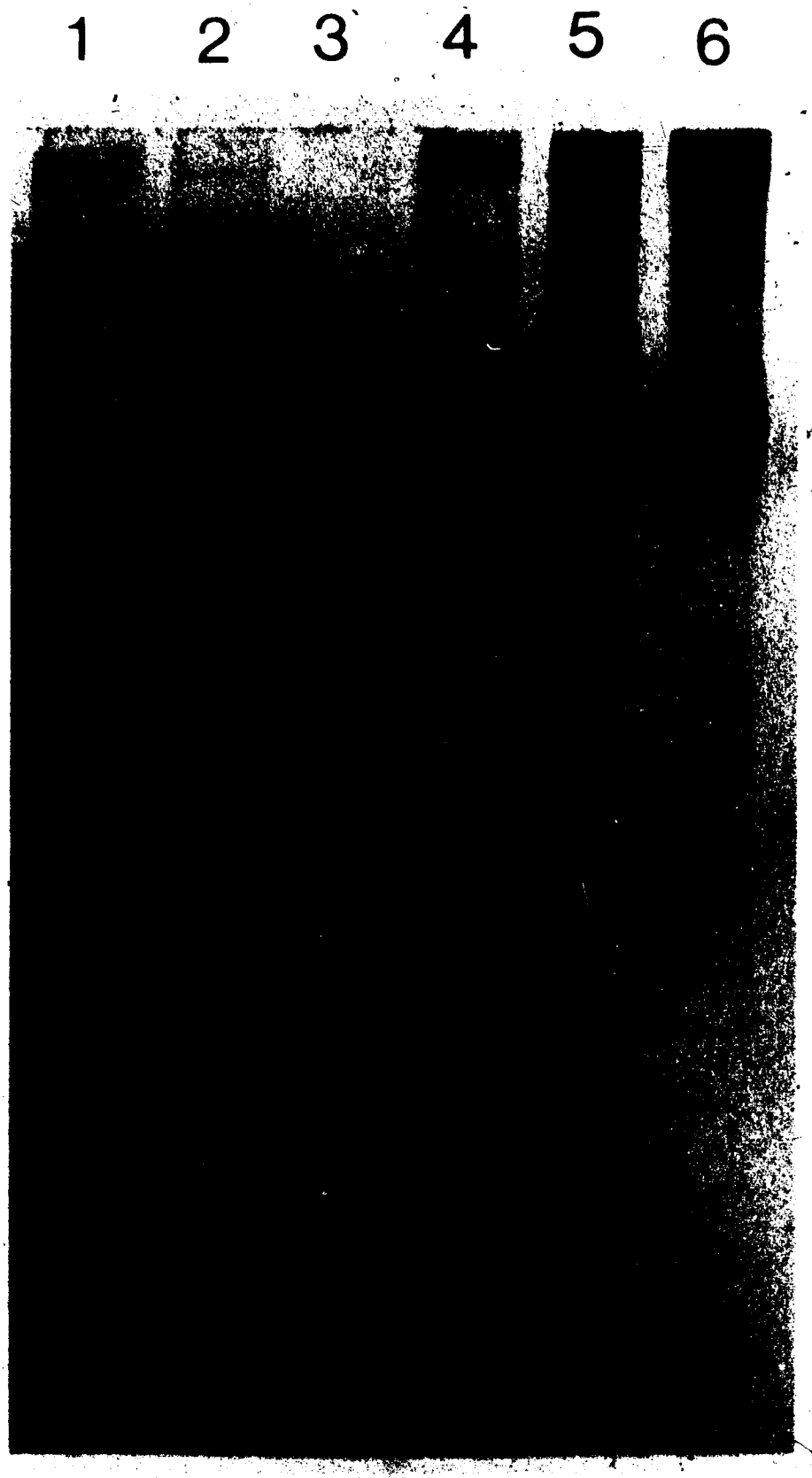
b) Experimental details as described above except that solubilized stripped membranes (1 mg protein) were added to the affinity resin in citrate buffer of increasing molarity, pH 8 at 4 °C for 30 min. The amount of protein removed from the supernatant is defined as the amount of protein bound to the resin.

c) Protein binding was performed as described under "Materials and Methods". Incubation was carried out for varied lengths of time. Fraction eluted with 1 mM BADS was electrophoresed on a 10% Laemmli gel, stained with Coomassie Blue and the gel was scanned on a Joyce Loebl Chromoscan 3 densitometer at 530 nm. The area under the Band 3 peak is defined as amount of Band 3 bound to the resin.


a
Protein bound, µg
250
200
150
100
50
0
0
0.4
0.8
1.2
1.6
2.0
Protein added, mg


b
Protein bound, µg
200
160
120
80
40
0
0
114
228
342
456
Sodium citrate, mM


c
Peak area, arb. units
40
30
20
10
0
0
15
30
45
60
120
Time, min


1
2
3
4
5
6

c. Characterization of binding

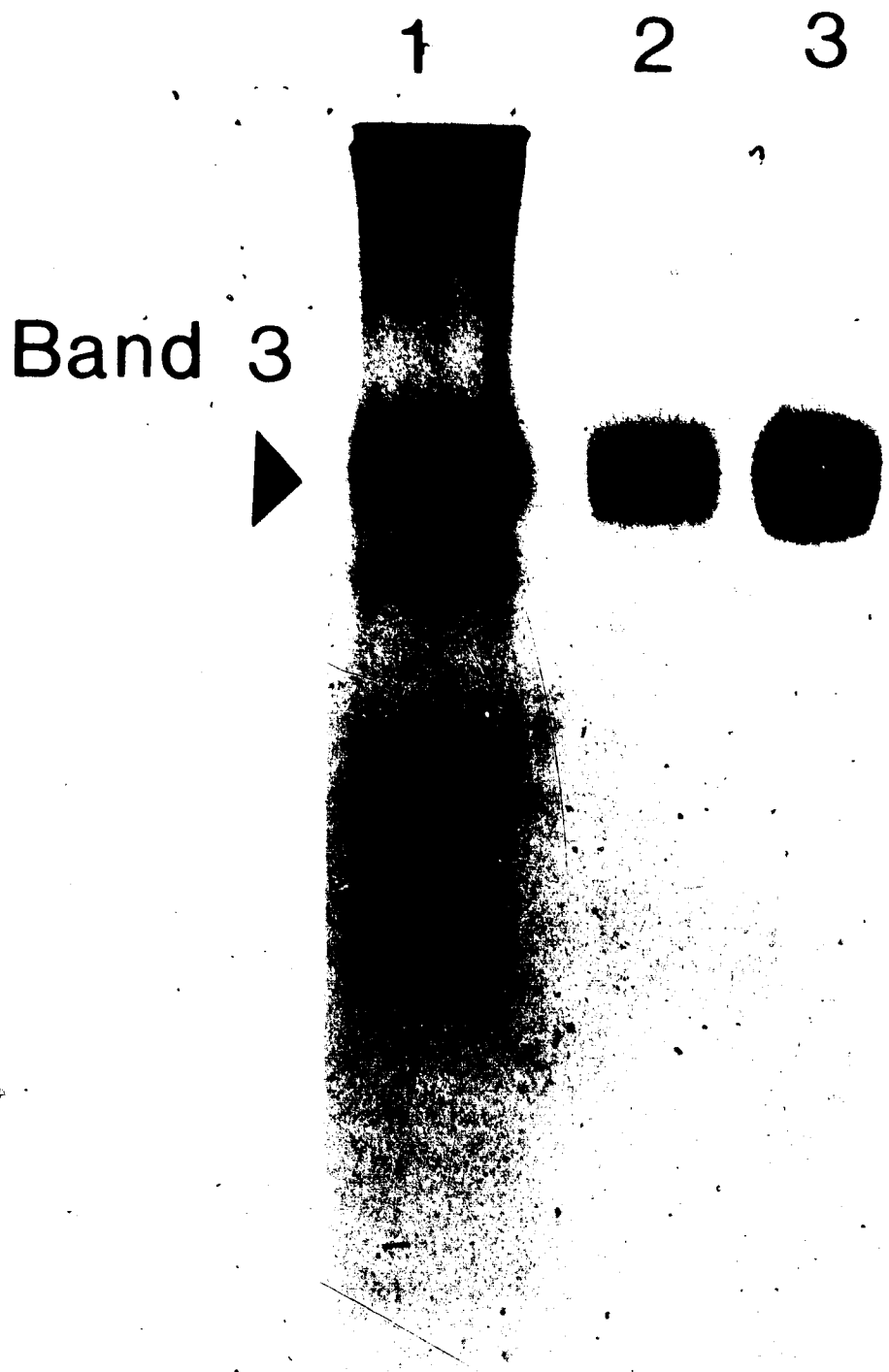
In order to achieve optimal binding, the SITS-Affi Gel-102 matrix must be shaken with membrane proteins at an amount equal to or slightly below its maximal binding capacity (Fig. II.3A). If low amounts of protein were used for binding, then Band 3 eluted with BADS was not detected on a Coomassie Blue-stained SDS-PAGE gel. Reducing the amount of SITS ligated to Affi Gel-102 to 1/5 and 1/10 of the normal amount (15 μmole $-NH_2$/ml resin) resulted in the loss of Band 3 binding. Figure II.3B shows the dependence of protein - affinity matrix interaction on the ionic strength of the buffer. Optimal binding was achieved only at fairly high ionic strength (228 mM sodium citrate, equivalent to about 0.7 M NaCl). In these studies citrate buffer of increasing molarity was used rather than using NaCl because chloride ion is a substrate for the anion transport and is known to compete with stilbene disulfonates (Knauf, 1979). Under the typical assay conditions, optimal binding of Band 3 was obtained when the affinity matrix was shaken with the protein sample for 30 min. at 4°C (Fig. II.3C). Specific and optimal binding was obtained at pH 8. Excess free DNDS did not inhibit Band 3 binding to the matrix at pH 6 whereas the binding capacity of the matrix was reduced at pH 10 (data not shown). Band 3 - 228C8 extract, prepared from ghost membranes that were stripped at pH 12 with 2 mM EDTA rather than at pH 7.5, did not bind to the affinity resin.

d. Characterization of elution

Band 3 bound to the affinity matrix could be eluted by various stilbene disulfonate derivatives dissolved in 5P8 buffer containing 0.1% $C_{12}E_8$ and 1 mM DTT, pH 8 (Fig. II.4). 1 mM BADS (lane 3) was a better eluant than 10 mM DNDS (lane 1) or 0.1 mM DIDS (lane 2). Band 3 was more effectively eluted by BADS at low ionic strength (5 mM phosphate) than at higher ionic strength (228 mM citrate) (data not shown). Band 3 bound to the affinity resin could partially be eluted by lowering the ionic strength of the buffer and addition of a stilbene disulfonate to the low ionic buffer improved the yield of Band 3.

Figure II.4

Elution efficiencies of various stilbene disulfonate derivatives. SDS-polyacrylamide gel electrophoresis of human erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli. Protein binding was performed as described under "Materials and Methods". Following incubation resins were washed with 228 mM citrate buffer, pH 8, and eluted with 5 mM phosphate-0.1% $C_{12}E_8$ buffer, pH 8, containing either 10 mM DNDS (lane 1) or 50 μM DIDS (lane 2) or 1 mM BADS (lane 3). Gels were stained with Coomassie Blue.


1
2
3
Band 3

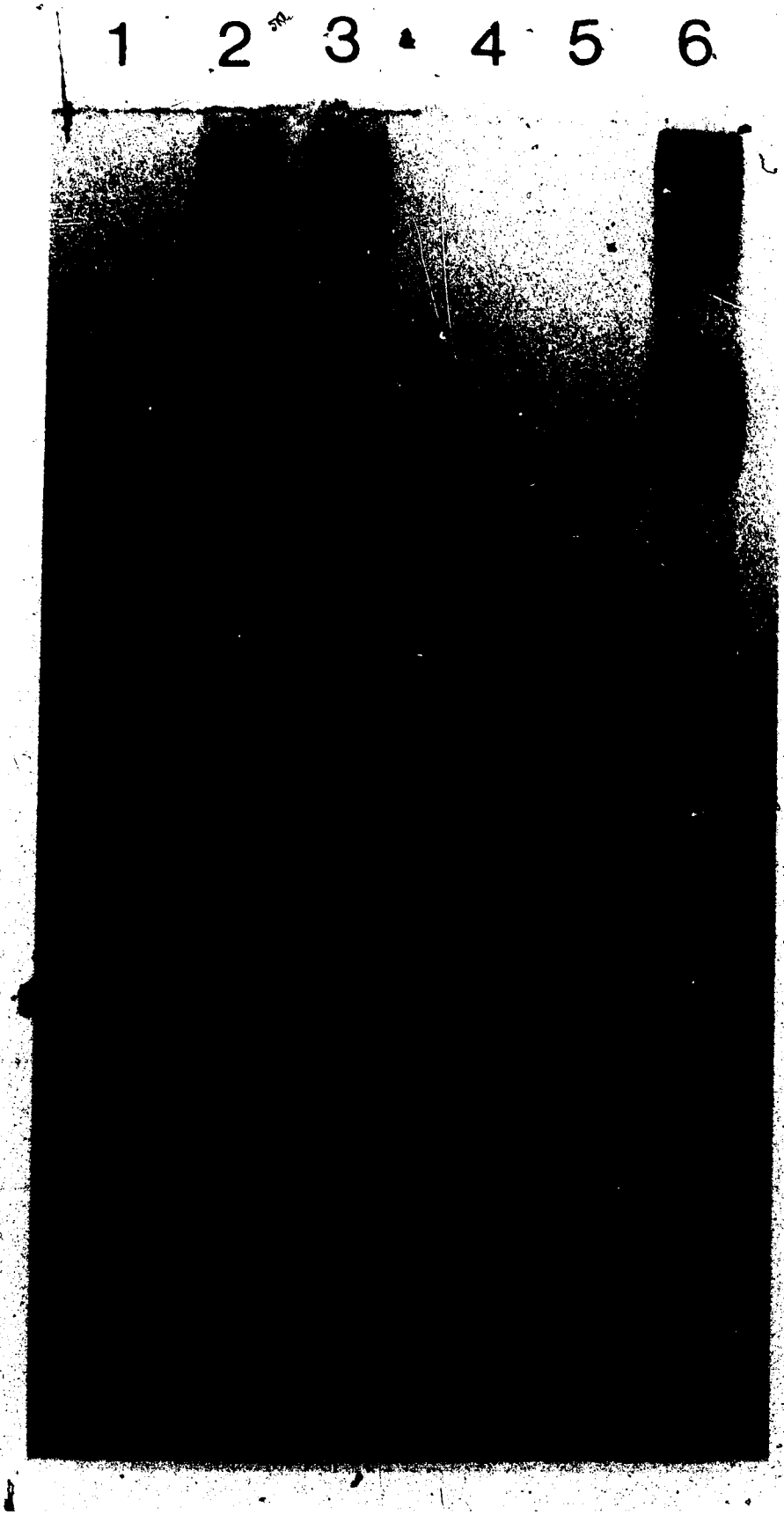

1 2 3 4 5 6

Figure II.5

Effect of increasing amounts of BADS on Band 3 elution from SITS-Affi-Gel 102 resin. Protein binding was performed as described under "Materials and Methods". Following incubation resins were washed with 228 mM citrate buffer, pH 8, and eluted with 5 mM phosphate-0.1% $C_{12}E_8$ buffer, pH 8, containing increasing amounts BADS. Other details as given under figure 3C.

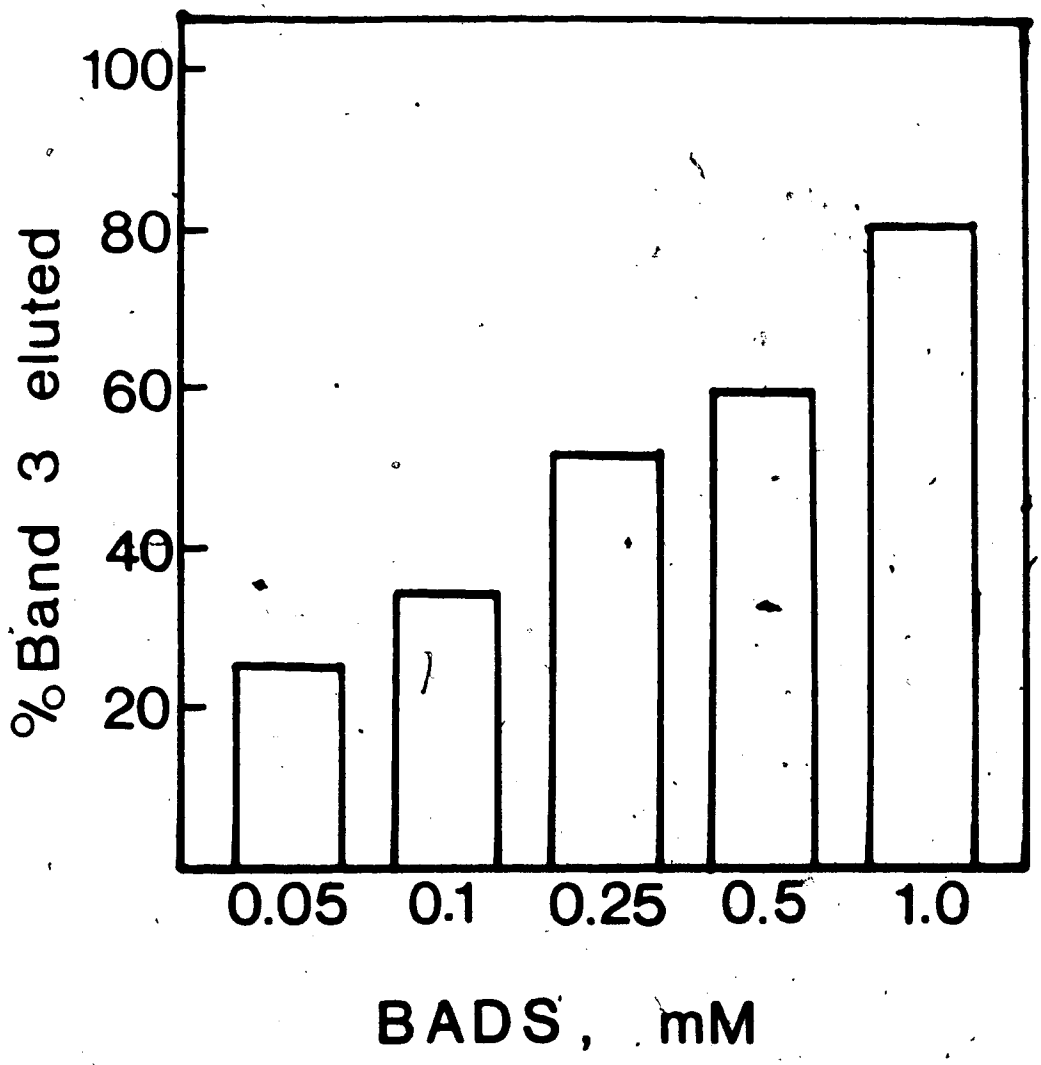

100
80
60
40
20
% Band 3 eluted
0.05
0.1
0.25
0.5
1.0
BADS, mM

Figure II.6

Effect of the spacer length on Band 3 binding to the affinity resin. Panel a: 25 µl of SITS-affinity resin with spacer arms of various lengths was shaken with 1 ml Band 3-228C8 extract at 4 °C for 15 min and the bound protein was eluted with 1 mM BADS. Other details as given under figure 3C. Panel b: Amount of Band 3 eluted with 1 mM BADS from SITS-affinity resin with a 6-atom hydrophobic spacer molecule (a) or with a 6-atom hydrophilic spacer molecule (b).

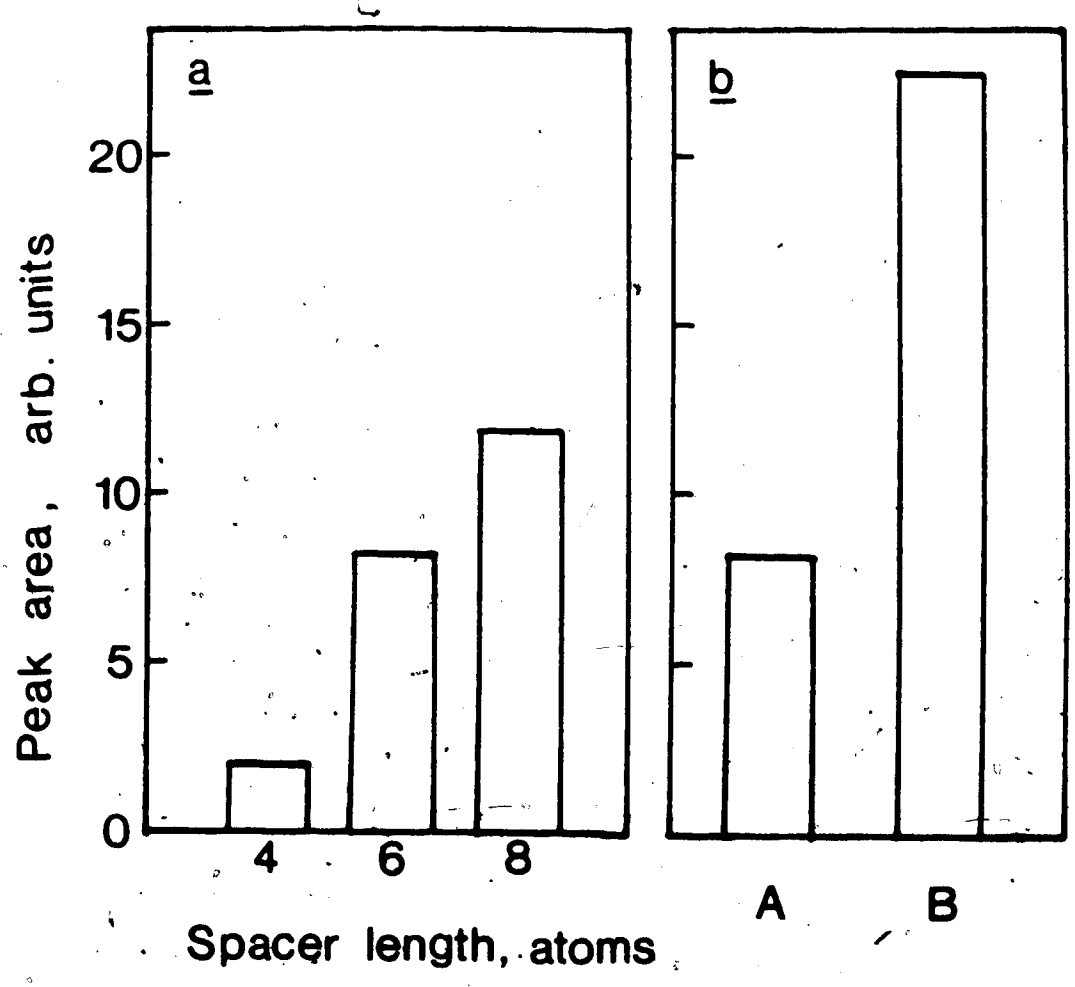

a
b
20
15
10
5
0
Peak area, arb. units
4
6
8
A
B
Spacer length, atoms

Figure II.7

Effect of temperature on affinity chromatography of Band 3. In a microfuge tube, 25 µl of SITS-Affi Gel-102 resin was incubated with 1 ml of Band 3 extract (protein concentration ~1.5 mg/ml) in 1% $C_{12}E_8$, 228 mM citrate buffer, pH 8, for 20 min at 4 °C or 22 °C or 37°C. After washing off the unbound protein, bound protein was eluted first with 1 mM BADS-5P8 (lanes 1-3) and subsequently with 1% LDS-5P8 (lanes 4-6) at respective temperatures. Lanes 1,4- 4 °C; lanes 2,5- 22°C and lanes 3,6- 37°C. SDS-polyacrylamide gel electrophoresis was performed on a 10% gel prepared according to the procedure of Laemmli.

For example, 0.1 mM BADS in 5P8 buffer eluted 40% of Band 3 while 1 mM BADS in 5P8 buffer eluted 80% of Band 3 that was bound to the affinity resin (Fig. II.5). Subsequent washing with 1% LDS eluted the remainder of Band 3 together with other proteins that may be bound to the resin nonspecifically.

3. Effect of Spacer Length on Band 3 Binding

Affi Gel-102 used in these studies is an agarose gel with a 6-atom hydrophilic spacer arm. Under the assay conditions, Band 3 did not bind to Affi Gel-102 alone. The binding capacity of the affinity matrix is dependent on the length of the spacer arm used. The amount of Band 3 that is eluted by BADS decreased with reducing spacer length from 8-atom to 4-atom (Fig. II.6a). Also, the chemical nature of the spacer molecule is important since affinity matrix with a hydrophilic 6-atom spacer binds more Band 3 than the one with a hydrophobic spacer of equal length (Fig. II.6b). Considering that both Affi Gel-102(hydrophilic 6-atom spacer) and omega-amino hexyl Agarose (hydrophobic 6-atom spacer) have equal amounts of spacer molecules (15 μmole/ml beads) and that equal amounts of ligand (30 mM) were used during the coupling reaction, the data suggests that an affinity-matrix with hydrophilic spacer arms interacts more favourably with Band 3 and therefore resulted in increased binding capacity.

4. Optimal Temperature for Protein Binding and Elution is 4°C

In order to establish optimal temperature conditions, protein binding and subsequent elution was carried out at different temperatures. When the experiment was carried out at 4°C, most of the bound Band 3 could be eluted off the resin by 1 mM BADS and the rest of Band 3 with 1% LDS (Fig. II.7). At room temperature, however, the amount of Band 3 in the 1% LDS elute was increased. When the experiment was carried out at 37°C, 1 mM BADS eluted very little Band 3, most of which could be eluted only

with 1% LDS. These data suggest that at higher temperatures Band 3 binds more tightly to the resin and therefore cannot be eluted with 1 mM BADS effectively.

5. At 37°C Immobilized SITS-Band 3 complex is Rapidly Converted to a Tightly-bound Complex

The process of affinity chromatography has two components. A binding component where Band 3 binds to the immobilized SITS and an elution component where free BADS competes with the immobilized SITS for the inhibitor binding site on the Band 3 molecule and elutes the bound protein from the resin. A change in temperature may affect the equilibrium of these two processes in such a way that at higher temperatures Band 3 may exhibit a stronger affinity for the immobilized ligand, resulting in the inability of BADS to elute the protein from the affinity resin. To rule out this possibility the following experiment was carried out. 1 ml Band 3 - 228C8 extract was shaken with 25 µl of SITS-Affi Gel-102 resin for 15 min at 4°C and the unbound protein was removed by washing the resin 3 times with 250 µl citrate buffer-0.1% $C_{12}E_8$. The affinity resin (with the bound protein) was then incubated at 37°C for up to 60 min. At the end of the incubation the microfuge tubes were returned to 4°C. During this step Band 3 was not released from the affinity resin (Fig. II.8a). After 10 min. at 4°C the bound protein was eluted with 1 mM BADS in 5P8 buffer at 4°C. The resin was finally eluted with 1% LDS in 5P8 buffer after 2 washes of 250 µl citrate buffer. Except for the incubation period of 37°C, all other steps were carried out at 4°C. As a result of incubation of 37°C for 20 min, the BADS-elutable Band 3 was converted to BADS-unelutable form. Preincubation of the Band 3 - 228C8 extract alone at 37°C did not alter its binding properties suggesting that the conversion to the BADS-unelutable form is not due to thermal denaturation and can occur only when Band 3 is already bound to SITS. Figure II.8b shows that the amount of Band 3 eluted by 1 mM BADS decreases and that eluted by 1% LDS increases in a time-

Figure II.8-A

Effect of temperature shift on the elution properties of Band 3 bound to the SITS-Affi Gel-102 affinity resin. Binding assay was performed as described under "Materials and Methods" with the following modification. After removing the unbound protein with at least three washes, 100 µl of citrate buffer was added to the resin-bound protein and incubated at 37 °C for up to 60 min. The supernatant (lanes 1 and 4) was removed and the bound protein was then eluted with 1 mM BADS-5P8 (lanes 2 and 5) followed by 1% LDS-5P8 (lanes 3 and 6). Lanes 1-3 samples were incubated at 4 °C after the initial binding and lanes 4-6 samples were incubated at 37 °C for 5 min. Gels of samples incubated up to 60 min. showed identical pattern as in lanes 4-6. SDS-polyacrylamide gel electrophoresis was performed on a 10% gel prepared according to the procedure of Laemmli.

Figure II.8-B

Effect of temperature shift on the elution properties of Band 3 bound to the SITS-Affi Gel-102 affinity resin. Details of experiment are given under Fig. II.8-A except that incubation at 37°C was carried out only up to 8 min. 1 mM BADS-5P8 eluates and 1% LDS-5P8 eluates were electrophoresed on SDS-polyacrylamide gel, stained with the Coomassie Blue stain and scanned in a Joyce Loebl Chromoscan 3 densitometer at 530 nm. ●——● - Band 3 eluted with 1 mM BADS-5P8; ○——○- Band 3 subsequently eluted with 1% LDS-5P8.

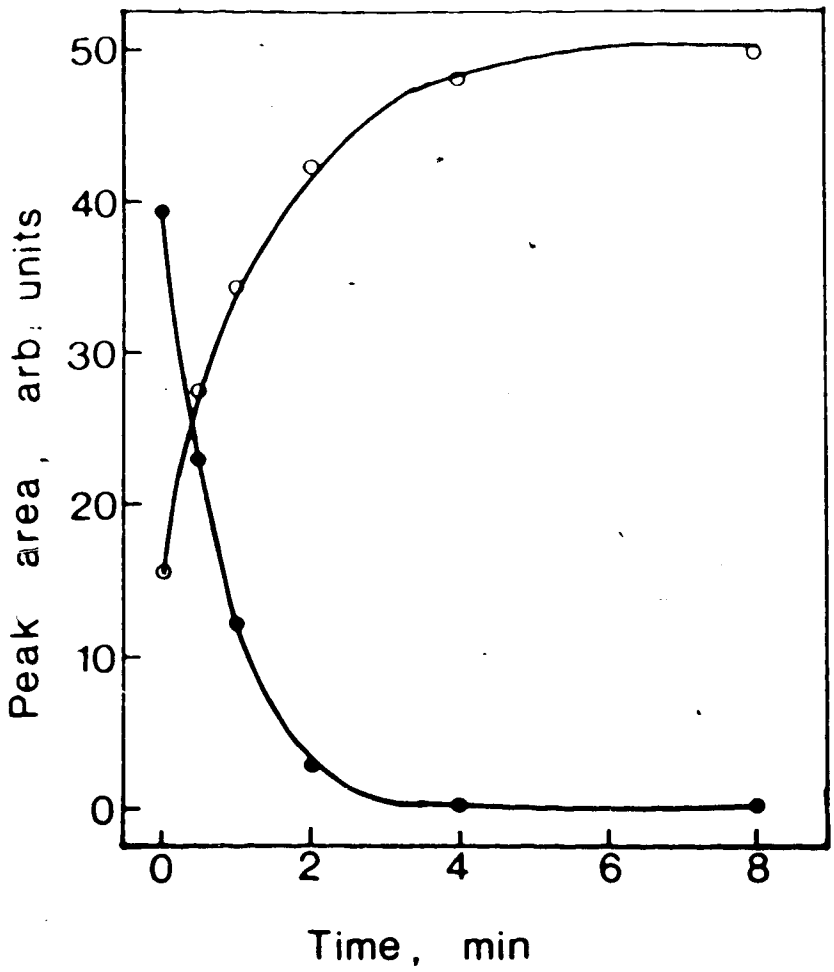

50
40
30
20
10
0
Peak area, arb. units
0
2
4
6
8
Time, min

Table II.3 Binding capacity of SITS-Affi-Gel 102 resin

	Resin
mg/ml	
Protein adsorbed[a]	5.1
Protein eluted by 1 mM BADS[b]	1.5
Protein eluted by 1% LDS	3.6

Protein assay was performed according to Lowry *et al.* (1951). Binding assay was performed as described under "Methods".

[a]Amount of protein adsorbed was calculated from the amount of protein disappeared from the supernatant after 15 min incubation at 4 °C.

[b]BADS itself reacts with Lowry reagent and gives color. Appropriate BADS blanks were included in the assay, and the amount of protein in BADS elute was recalculated.

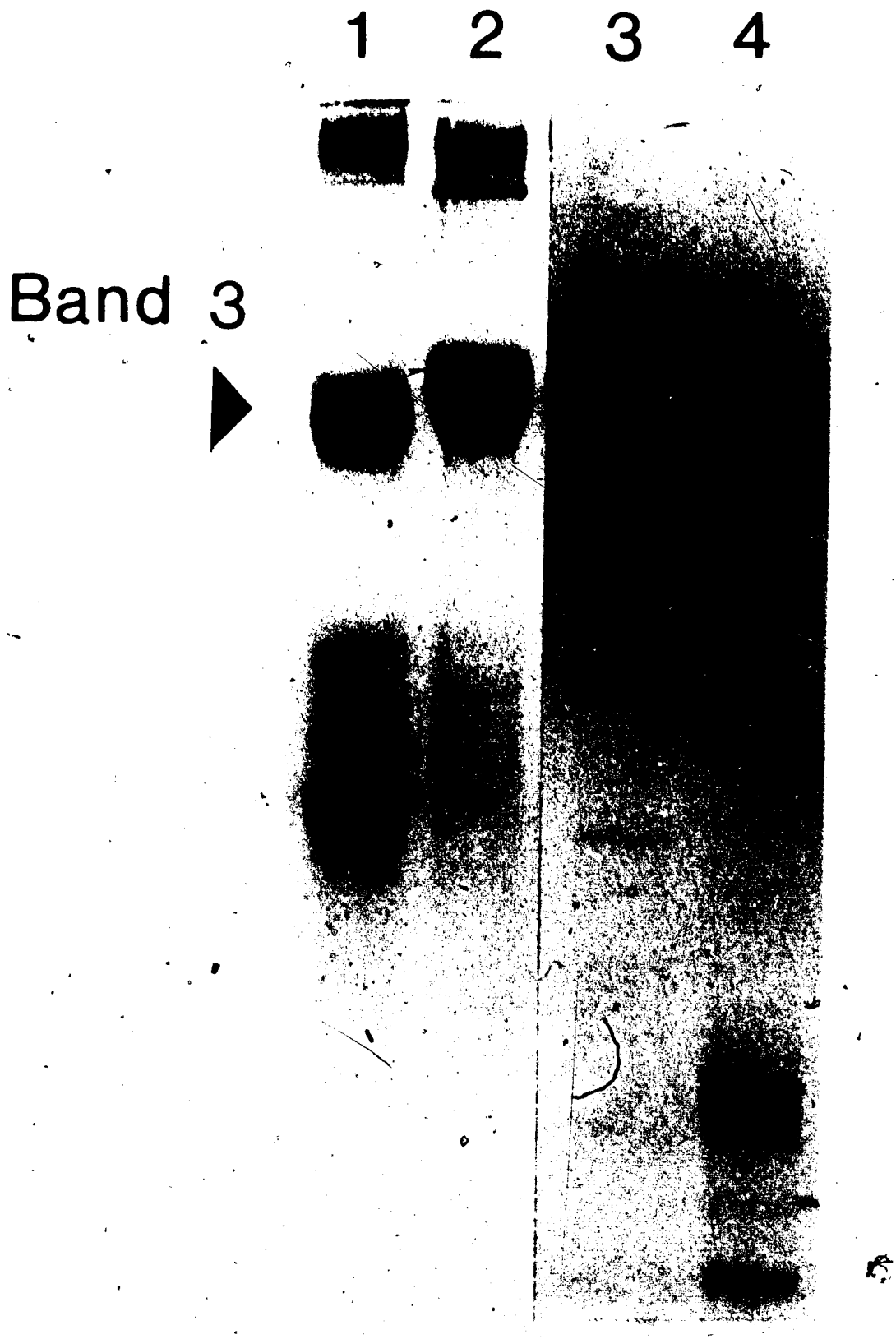
dependent manner as the resin-bound protein is incubated at 37°C. Within 45 sec, 50% of BADS elutable Band 3 is converted to BADS unelutable Band 3 (Fig. II.8b). Since both the binding step and the elution step were performed at 4°C, this observation suggests that upon exposure to 37°C, ligand-bound Band 3 becomes more tightly bound to the resin. It seems likely that the Band 3 eluted by 1 mM BADS is present in the initial weak binding conformation whereas the Band 3 that is not eluted by 1 mM BADS but by 1% LDS is present in the tight binding conformation. These data also show that this change is slow at 4°C but very fast at 37°C.

6. Binding Capacity and Stability of SITS-Affi Gel-102 resin

The binding capacity of SITS-Affi Gel-102 resin was estimated from the amount of Band 3 protein eluted from the resin after it was saturated with Band 3 - 228C8 extract at 4°C for 15 min. Table II.3 shows that the resin has a binding capacity of 1.5 mg Band 3 per ml resin. When stored in citrate buffer with 0.1% sodium azide at 4°C, SITS-Affi Gel-102 affinity resin retained its full binding capacity over at least 3 months. When the same resin samples were repeatedly used, however, the binding capacity of the resin

Figure II.9

Purification of Band 3 from non-human erythrocytes. SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins was performed on a 10% gel prepared according to the procedure of Laemmli. 1 mM BADS elute from SITS-Affi Gel resin shaken with 1% $C_{12}E_8$ extract from chicken ghosts (lane 1), mouse ghosts (lane 2), pig ghosts (lane 3) and horse ghosts (lane 4).


1
2
3
4
Band 3

decreased slightly with each use. This is probably due to a small degree of irreversible adsorption of proteins to the resin, a phenomenon also observed with other affinity resins (Lowe, 1979a).

7. Affinity purification of Band 3 from other sources

Ghost membranes were prepared from horse, chicken, pig and mouse erythrocytes and a crude extract was prepared by solubilizing the membranes in citrate buffer containing 1% $C_{12}E_8$. The solubilized membranes were then centrifuged in a SS-34 rotor at 19,000 rpm for 20 min. The supernatant was shaken with SITS-Affi Gel-102 under the same experimental conditions used for purification of human Band 3. A major polypeptide of mol. wt. between 90,000 to 100,000 was eluted by 1 mM BADS from the affinity matrix shaken with crude extracts from these different sources (Fig. II.9). Subsequent washing of the matrix with 1% LDS eluted the remainder of Band 3 and other non-specifically bound proteins (data not shown).

D. DISCUSSION

Band 3 is an integral membrane protein and spans the erythrocyte membrane several times (Steck *et al.*, 1976; Kopito and Lodish, 1985). No successful attempts of purifying this protein employing the affinity chromatography technique have been reported. One of the difficulties is undoubtedly the hydrophobic nature of Band 3 that results in a high degree of non-specific interactions with the affinity matrix. Stilbene disulfonates being hydrophobic in nature aggravate this problem. Also, stilbene disulfonates bear two negative charges which will promote ionic interactions at low ionic strength. Therefore the binding conditions should be such that the non-specific hydrophobic and ionic interactions be minimized and the biospecific interactions be maximized.

The choice of ligand is very important since the most critical factor in successful affinity chromatography is the strength of interaction between the immobilized ligand and the protein (Ikeda *et al.*, 1984). As a general observation, affinity resins with ligands with relatively high Ki values (>10 mM) do not adsorb proteins whereas with ligands with very low Ki values (<10 nM) may bind proteins so tightly that their elution in the native state becomes impossible. The apparent dissociation constant (Ki) exhibited by a protein for a free ligand may change once the ligand is bound onto a matrix surface. Insertion of a spacer arm and change of functional group due to immobilization could also change the effective equilibrium constant. There is no empirical way to predict and calculate these changes and therefore the suitability of a ligand as an affinity adsorbent has to be determined for individual cases. Our results show that DADS (Ki=1.3 mM) is not a suitable ligand for chromatography probably due to its relatively low affinity for Band 3. It is also possible that during the coupling step, DADS gets ligated to the carboxyl groups of the spacer arms through both its amino groups. This may result in a restricted access to the binding site or in an orientation in which the ligand cannot bind the protein. This may explain our inability to reproduce protein binding when different batches of DADS-hydrophilic spacer affinity matrices were used. BADS (Ki=2μM) has a higher affinity for Band 3 but its use as an immobilized ligand also resulted in non-specific binding, presumably due to an additional benzene ring in its structure that increases the hydrophobicity of the ligand. Band 3 did not bind to immobilized SPITC (Ki=~4 mM). Based on simple kinetic and equilibrium models of the affinity adsorption and desorption events, Graves and Wu (1974) have predicted that in order to produce adequate affinity, the immobilized ligand concentration should be more than 10 times the ligand-protein dissociation constant (Ki). Considering that concentration of immobilized SPITC was not more than 15 mM, it would not have adequate affinity to bind Band 3. We did not attempt to increase the concentration of immobilized SPITC to compensate for weak affinity because SPITC has a net negative charge and it has been observed that with high ligand

concentration, ionic interactions become dominant over biospecific interactions (Ikeda *et al.*, 1984). We observed similar results upon increasing concentrations of immobilized DADS (data not shown).

SITS (Ki = 10 μm) immobilized at a concentration of 15 mM has proved to be satisfactory. Unlike DADS, one amino group of SITS is acetylated and therefore it cannot form 'cross-bridges' on the solid support and thus the ligand is probably present in a proper orientation for the binding. In this case the acetylated end of SITS must enter the protein. When the concentration of immobilized SITS was decreased from 15 mM to 3 mM or 1.5 mM, Band 3 did not bind to the affinity resin (data not shown). The lack of binding could be due to an increased Ki caused by immobilization of SITS and therefore the lower ligand concentration results in inadequate affinity. It is also possible that at lower ligand concentration, the excess free charged spacer arms interfere with the protein binding.

Steric hindrance is a commonly held rational for the need of spacer arm extension (Lowe and Dean, 1974). Thus a spacer arm would be needed if the ligand binding site is recessed deep within the interior of the protein. The present studies indicate that the stilbene binding site is not present on the surface of Band 3 but is recessed deep inside the protein molecule, since Band 3 does not bind to the affinity matrix without a spacer arm or with short spacer arms. In order to achieve binding, a spacer arm of minimum 4-atom length is needed and further elongation of the spacer arm results in an increased binding capacity of the affinity matrix. Increase in binding capacity as a result of increasing spacer length has also been observed earlier (Pantoliano *et al.*, 1984).

It is conceivable that upon binding to an immobilized ligand, the protein will also interact with the spacer arm, especially if the ligand binding site is recessed within the interior of the protein. This means that the chemical nature of the spacer arm is likely to influence protein-ligand interaction in such a case. It is generally observed that hydrophobic spacer arms increase non-specific interactions whereas hydrophilic spacer

arms minimize this problem. The present studies with DADS attached to hydrophobic and hydrophilic spacer arms support the above view. It is interesting to note, however, that Band 3 does not bind to the spacer arms alone and therefore binding of Band 3 to DADS-spacer-Sepharose column cannot be completely non-specific. This is also supported by the fact that Band 3 bound to DADS-affinity columns can be eluted by increasing concentration of Cl^- (a substrate which presumably binds at or near the inhibitor binding site) but not by citrate ions which are not transported by Band 3 (see Table II.2).

Interaction of a protein with a series of ligand derivatives yields information about the chemical nature of the binding site. Similarly, studying protein interaction with a ligand immobilized via different spacer arms can give information about the microenvironment that surrounds the ligand binding site. For example, our results show that keeping the spacer length equal (to 6 atoms), introduction of hydrophilic groups on an otherwise hydrophobic spacer arm dramatically improves Band 3 binding to the affinity matrix and its subsequent recovery in the pure form. This indicates that the region that surrounds the stilbene binding site interacts more favourably with a hydrophilic rather than with a hydrophobic spacer arm. This suggests that the area surrounding the stilbene binding site has a fair degree of hydrophilicity although the binding site itself is probably a hydrophobic cavity (Knauf, 1979; Ramjeesingh *et al.*, 1981; Macara and Cantley, 1983). Our results are particularly interesting since it has very recently been shown that although the haem pocket of myoglobin is lined with non polar amino acid residues, the binding site is strongly polar (Macgregor and Weber, 1986). On the basis of minor changes in acid dissociation constants of ionized groups inside certain proteins, it has been deduced that many protein cavities have a high effective polarity despite the lining of nonpolar amino acid residues (Warshel *et al.*, 1984).

The temperature-shift experiment demonstrates that upon binding to the inhibitor ligand, Band 3 protein becomes tightly bound presumably through a conformational change (Verkman *et al.*, 1983). Macara *et al.* (1983) have suggested that the slow

conformational change may represent a partial translocation of the inhibitor and that it occurs when the protein attempts to transport the inhibitor as it would a substrate and switches from an outward- to an inward-facing state. Thus, prior to the conformational change the inhibitor binding site of the bound Band 3 is accessible to free ligand and therefore the protein could be eluted with BADS. Upon conformational change the site becomes inaccessible (inward-facing state) to the free ligand and consequently cannot be eluted with BADS.

Our results have indicated two important points. Firstly, when using SITS-affinity column for purification of Band 3 it is imperative to carry out the experiment quickly at 4°C. Many workers perform affinity chromatography at room temperature (Pantoliano *et al.*, 1984) or load the sample very slowly on the column (Lowe, 1979b). Obviously under these conditions, isolation and purification of Band 3 will not be very successful. This is important since it has recently been reported that the red cell glucose transporter can be bound to an inhibitor-affinity column but cannot be eluted in the native conformation (Weber *et al.*, 1985) when the binding was performed at 4°C for 2 hrs. Thus, our finding may be of general importance in cases where a transport protein is being purified on an inhibitor-affinity column. Secondly, from the reports published so far (Verkman *et al.*, 1983; Macara *et al.*, 1983), it has only been inferred that upon stilbene disulfonate binding to Band 3 the conformational change locks the inhibitor in place. We believe that our results provide direct evidence that such a phenomenon does occur.

The SITS-Affi Gel-102 affinity matrix was also useful in purifying Band 3 protein from erythrocytes from different species. Further processing of the crude extracts of chicken, mouse, pig and horse ghosts prior to affinity chromatography should yield Band 3 proteins of the same high purity as in the case of human Band 3 protein. We believe that this procedure will provide a quick and easy way to identify and isolate anion-exchange proteins from various sources.

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III. Interaction of Solubilized Band 3 with Immobilized SITS[1]

A. INTRODUCTION

Band 3 is the major integral membrane protein of the erythrocyte and catalyzes the rapid exchange of anions across the membrane (Passow, 1986; Steck *et al.*, 1976; Appel and Low, 1982). The anion-translocation site is located in the membrane-bound carboxyl-terminal domain (M_r=55,000) of Band 3. Anion exchange is thought to occur via a conformational change that translocates the anion binding site between external and cytoplasmic membrane interfaces (Passow, 1986; Macara and Cantley, 1983). Anion exchange is specifically inhibited by stilbene disulfonates which bind at the external transport site of Band 3 (Passow, 1986; Ramjeesingh *et al.*,1981). This site is buried within the membrane (Grinstein *et al.*, 1978; Macara *et al.*, 1983) and has been located 35-40 Å from sulfhydryl residues on the cytoplasmic domain (Macara *et al.*, 1983). The kinetics of 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS) binding studied both by stopped-flow and temperature-jump experiments have suggested that a rapid initial binding step is followed by a slower conformational change that locks the inhibitor in place (Verkman *et al.*, 1983). Intrinsic fluorescence quenching studies also suggest that a conformational change in the Band 3 protein accompanies the binding of BIDS (Macara *et al.*, 1983). There also have been some suggestions that the inhibitor binding sites on two adjacent monomers of a Band 3 dimer may interact with each other (Zaki, 1981; Bjerrum *et al.*, 1983).

We have designed and synthesized an affinity resin for purification of Band 3 protein (Chapter II; Pimplikar and Reithmeier, 1986). Band 3 binds specifically to SITS-Affi-Gel 102 resin via its inhibitor binding site and the bound protein can be eluted specifically by free inhibitor ligands. We have also shown that upon initial binding to

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[1]A version of this chapter has been published. Pimplikar, S.W. and Reithmeier, R.A.F. Biochim. Biophys. Acta (in press).

immobilized SITS, Band 3 becomes more tightly bound, presumably through a conformational change in the protein (Pimplikar and Reithmeier, 1986) as has been suggested from the previous kinetic studies (Verkman *et al.*, 1983; Macara *et al.*, 1983).

The SITS-Affi-Gel 102 can be used to probe the binding characteristics of stilbene disulfonates to Band 3 protein. Immobilization of ligand in a fixed orientation onto a solid matrix makes separation of free protein from protein-ligand complex very easy, and the elution profile of the bound protein gives a direct indication of the protein's affinity for the immobilized ligand. Thus, effects of chemical modification on the affinity of Band 3 towards stilbene disulfonate inhibitors can be easily studied. We here show that citrate modification of the inhibitor binding site on Band 3 affects protein binding to the affinity resin such that the bound protein is more easily eluted from the resin. Moreover, we provide evidence for intersubunit interaction between inhibitor binding sites of adjacent monomers and that citrate modification of the binding sites affects the initial binding of inhibitor but not the translocation of the inhibitor to the tightly-bound state.

B. MATERIALS and METHODS

Affi-Gel 102 was purchased from Bio-Rad. SITS was obtained from Pierce Chemical Co. and U.S. Biochemical Corporation, Cleveland, Ohio. BADS was synthesized according to Kotaki *et al.* (1971). Lithium dodecyl sulfate was a product of Boehringer Mannheim while $C_{12}E_8$ was from Nikko Chemical Co., Tokyo. All other chemicals were reagent grade or better. EAC was prepared as previously described (Craik and Reithmeier, 1985) from the free base of 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (purchased from Pierce Chemical Co., Rockford, Illinois) in anhydrous diethyl ether solution by the method of Sheehan *et al.* (1961). The white cystalline product was extensively washed with anhydrous ether to remove unreacted carbodiimide and methyl iodide. [^{3}H]-H_2DIDS was purchased from Research Development Corporation, Hospital for Sick Children, Toronto, Ontario.

Details of erythrocyte ghost membrane preparation, solubllization of Band 3 and SITS-Affi-Gel 102 affinity resin preparatión are given in the previous chapter. Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Canadian Red Cross) by hypotonic lysis in 5 mM sodium phosphate, pH 8.0.

1. Treatment of Intact Erythrocytes with EAC

Erythrocytes were washed four times with 10-15 volumes of 0.9% w/v NaCl at 4 °C to remove storage medium and white cells. In order to obtain chloride-free external medium, cells were further washed 3 times with 10 volumes of 28.5 mM sodium citrate, pH 7.4, 205.3 mM sucrose. Cells were then incubated with 8.7 mM EAC in 28.5 mM sodium citrate, pH 6.7, 205.3 mM sucrose, for 5 min at 37 °C. Cells were washed three times with 28.5 mM sodium citrate, pH 7.4, 205.3 mM sucrose prior to ghost membrane preparation. This procedure produced ~60% inhibition of anion transport (Craik and Reithmeier, 1985) (data not shown).

2. DIDS Treatment of Erythrocytes

Erythrocytes were washed 3 times with 150 mM NaCl and 5 mM sodium phosphate, pH 8.0, at 4 °C. Cells were suspended at a 25% hematocrit in the same buffer. [^{3}H]-H_2DIDS or DIDS was added at varying concentrations and the suspension was incubated at 37 °C for 1 h. The cells were washed with the same buffer containing 0.5% bovine serum albumin (fatty acid-free) at 4 °C, followed by two washes at 4 °C with the same buffer.

3. Binding assay

Unless stated otherwise, all steps were carried out at 0-4 °C. Protein binding assays were performed in 1.5 ml microfuge tubes. 25 µl of packed resin was washed twice with 250 µl of 228 mM sodium citrate, pH 8.0, 0.1% $C_{12}E_8$, 1 mM DTT (citrate

buffer) and was incubated with 1 ml of Band 3 extract (1.5-2 mg/ml protein) for 15 min. After removing the supernatant, the gel was washed at least three times with 250 µl of citrate buffer and the bound protein was then eluted by shaking the resin for 10 min with 110 µl of 1 mM BADS in 5 mM sodium phosphate, 0.1% $C_{12}E_8$. Finally, the resin was washed at least twice with 10 volumes of citrate buffer and then extracted with 110 µl of 1% LDS, 5 mM sodium phosphate for 10 min.

4. Analytical techniques

Protein assay was according to Lowry *et al.* (1951). Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (1970). Protein bands were stained with Coomassie blue. Coomassie blue-stained gels were scanned in Joyce-Loebl Chromoscan 3 densitometer at 530 nm.

C. RESULTS

1. Prolonged Incubation of Immobilized SITS-Band 3 Complex at 4 °C Results in a Tightly-bound Complex

As observed earlier (Chapter II; Pimplikar and Reithmeier, 1986), when Band 3 extract was shaken with the affinity resin at 4 °C for 15 min, most of Band 3 was bound in the weakly-bound form and was eluted by BADS (Fig. III.1a). When incubated for 18 h, the total amount of Band 3 bound to the resin was increased. However, the amount of Band 3 eluted by 1 mM BADS remained unchanged (Fig. III.1a) and the amount of the strongly-bound form continued to increase and was 3-fold higher than the amount of the weakly-bound Band 3 at 18 h (Fig. III.1b). This observation indicates that Band 3 bound to the resin in the initial weak binding state (BADS-elutable) slowly converts into the tight binding state (BADS-unelutable). Not surprisingly, attempts to purify Band 3 by applying membrane extracts overnight on a SITS-Affi-Gel 102 column gave poor yields of purified

Figure III.1-A

Effect of prolonged incubation on Band 3 binding and elution. The binding assay was performed as described under "Materials and Methods" either for 15 min (lanes 2 and 4) or for 18 h (lanes 1 and 3) at 4 °C. BADS and LDS eluates were electrophoresed on a 10% SDS-polyacrylamide gel prepared according to the procedure of Laemmli. Lanes 1,2- 1 mM BADS-5P8 eluate; lanes 3,4- 1% LDS-5P8 eluate.

Figure III.1-B

Quantification of gel (Fig. III.1-A) by densitometry. Experimental details as given under Fig. III.1-A. 1 mM BADS-5P8 eluates and 1% LDS-5P8 eluates were electrophoresed on a SDS-polyacrylamide gel, stained with Coomassie Blue stain and scanned in a Joyce Loebl Chromoscan 3 densitometer at 530 nm. Open columns- 1 mM BADS-5P8 eluate; hatched columns- subsequent eluate with 1% LDS-5P8.


1
2
3
4

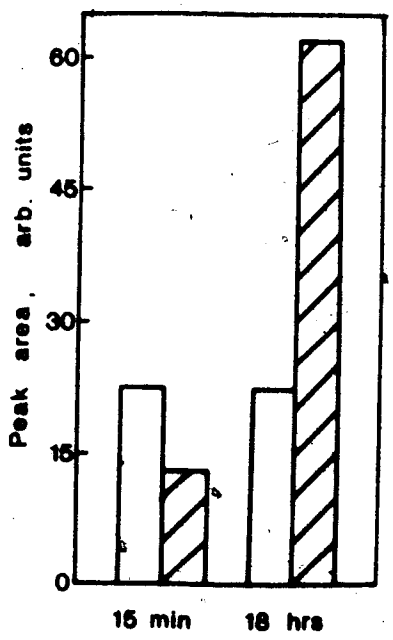
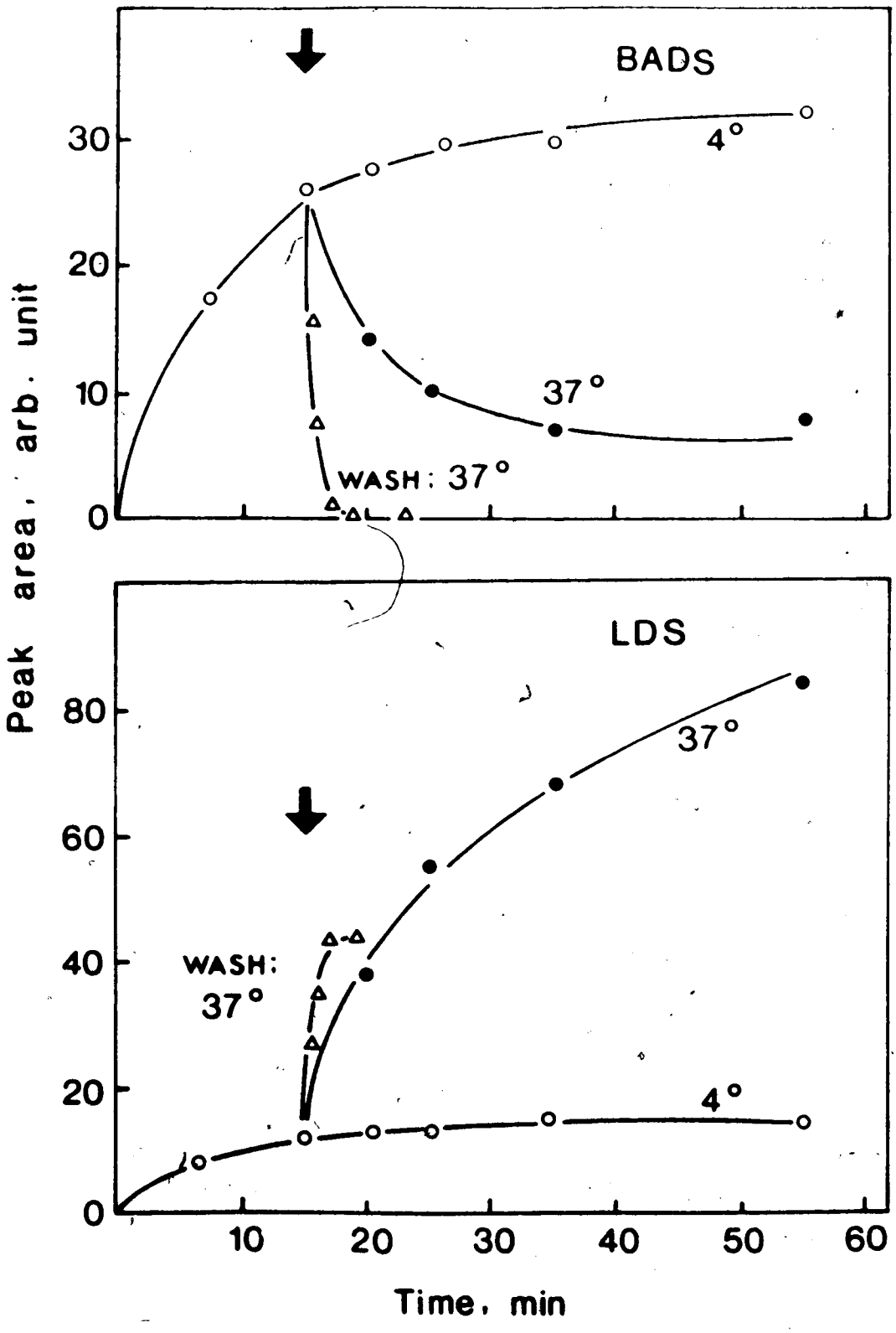

Peak area, arb. units
60
45
30
15
0
15 min
18 hrs

Figure III.2

Effect of temperature shift on the elution properties of Band 3 bound to the SITS-Affigel-102 affinity resin. Details of experiment are given under "Materials and Methods" except that following 15 min at 4 °C, incubation was continued at 4 °C ○——○ or incubated at 37 °C with ▽——▽ or without ●——● washing off the unbound protein. 1 mM BADS-5P8 eluates (top panel) and 1% LDS-5P8 eluates (bottom panel) were electrophoresed on SDS-polyacrylamide gel, stained with Coomassie Blue stain and scanned in a Joyce Loebl Chromoscan 3 densitometer at 530 nm. The arrow shows the time incubation was shifted to 37 °C.


BADS
4°
37°
WASH: 37°
LDS
37°
WASH:
37°
4°
Peak area, arb. unit
Time, min
0
10
20
30
40
50
60
80

Band 3, although large amounts of protein were bound to the resin (data not shown). It is important to note that even after 18 hrs of incubation at 4 °C, a part of the Band 3 population can still be eluted by BADS and therefore remains in the initial conformation.

2. Interaction of Band 3 with SITS-Affi-Gel 102

The time course of Band 3 binding to immobilized SITS was studied at 4 °C and 37 °C. The amount of Band 3 weakly-bound to the resin at 4 °C increased in a time-dependent manner and reached a maximal binding after ~30 min (Fig. III.2 top panel). The proportion of Band 3 in the tightly-bound form was much lower at 4 °C up to 1 h (Fig. III.2 bottom panel).

In a parallel set of experiments, after the initial 15 min at 4 °C, the resin was either incubated at 37 °C or washed to remove free protein and then incubated at 37 °C. As shown previously in Chapter II (Pimplikar and Reithmeier, 1986) removal of unbound protein and incubation of matrix-bound protein at 37 °C resulted in rapid conversion of Band 3 to the tightly-bound form. This is shown by the loss of BADS-elutable protein after washing away free protein and incubation at 37 °C, with a concomitant increase in Band 3 in the LDS eluate (Fig. III.2). In the presence of free protein, incubation at 37 °C resulted in a decrease in BADS-elutable Band 3 with a concomitant increase in LDS-elutable Band 3 (Fig. III.2). Even after a 40 min incubation at 37 °C, however, a part of Band 3 was still present in the BADS-elutable state.

Figure III.2 also shows that a maximum amount of BADS elutable Band 3 is reached within 30 min (saturation state) when Band 3 is shaken with SITS-Affigel-102 resin at 4 °C. To see if all the available ligand sites were occupied at this stage by Band 3, the following experiment was performed. 25 µl SITS-Affigel-102 resin was shaken with 1 ml Band 3 extract for 30 min. The free protein was removed and the resin-bound Band 3 was incubated at 37 °C for 10 min to 'lock' those ligand sites with bound Band 3. No BADS-elutable Band 3 is present on the resin after this procedure (Fig. III.2). However,

Figure III.3-A

Effect of 'locking' the protein to the affinity resin by temperature shift on the binding of subsequently added Band 3 to SITS-Affigel-102 affinity resin. Initial binding was performed as described under "Materials and Methods" for 30 min at 4 °C. The unbound protein was removed and the bound protein was locked by incubating the resin-protein complex at 37 °C for 10 min. One ml of fresh Band 3 extract was added (Addi.) or not added (No Addi.) to the resin and the binding was performed as described under "Methods" for 15 min at 4 °C. Details of elution, electrophoresis and densitometry as given under Fig. III.1-B.

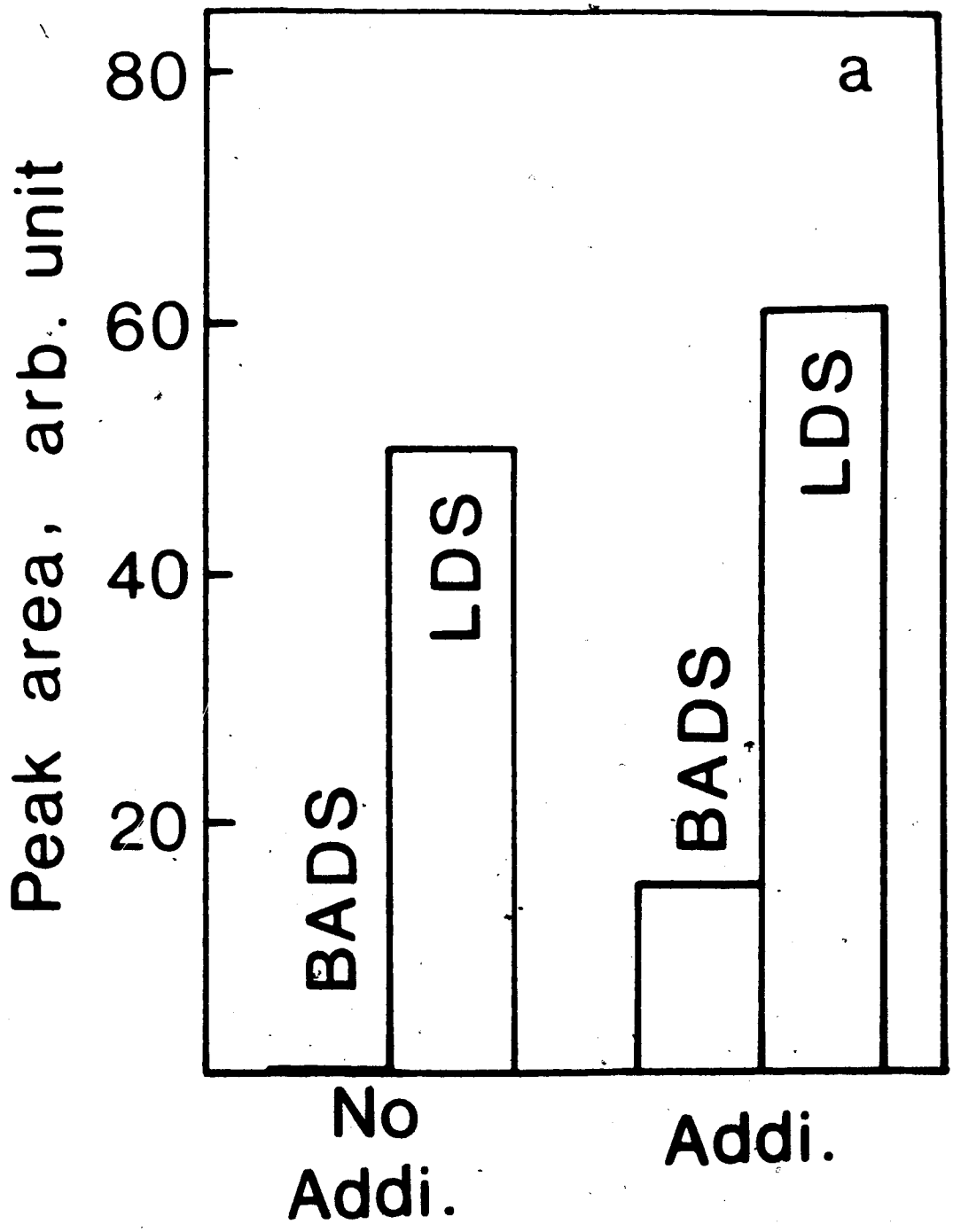
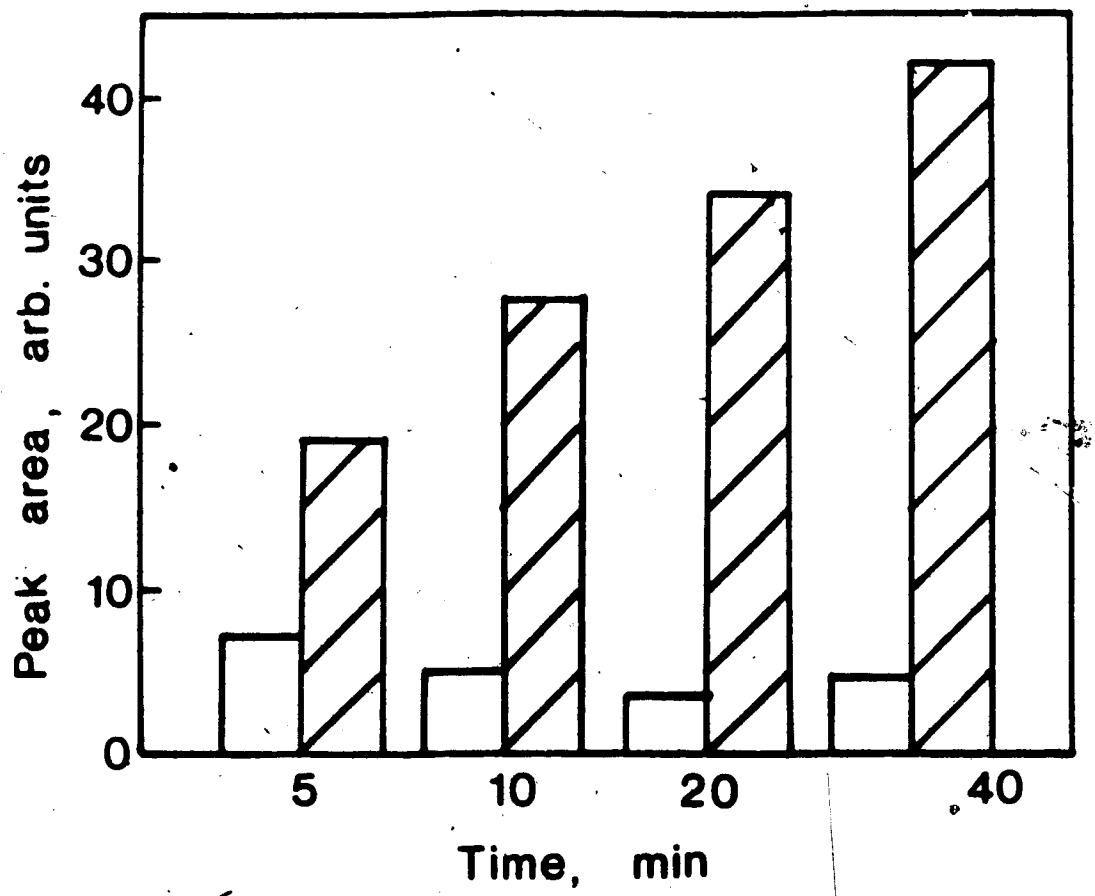

Peak area, arb. unit
80
60
40
20
a
BADS
LDS
BADS
LDS
No Addi.
Addi.

Figure III.3-B

Experimental details are as given under Fig. III.3-A with the exception that initial binding was performed for up to 40 min prior to temperature shift at 37 °C for 10 min. One ml of fresh Band 3 extract was added and incubated for 15 min at 4 °C. Open bars, 1 mM BADS-5P8 eluate; hatched bars, subsequent 1% LDS-5P8 eluate.


Peak area, arb. units
0
10
20
30
40
5
10
20
40
Time, min

following this, if 1 ml of fresh Band 3 extract was added to the resin and shaken at 4 °C for 15 min, a portion of Band 3 was eluted with 1 mM BADS (Fig. III.3a). Only the Band 3 that bound from the fresh batch was eluted by 1 mM BADS since Band 3 bound earlier was rendered BADS unelutable by exposure to 37 °C. The majority of Band 3 in the subsequent LDS eluate was the Band 3 bound during the initial incubation and rendered BADS-unelutable by incubation at 37 °C. This shows that even at apparent saturation, not all ligand sites were bound by the protein.

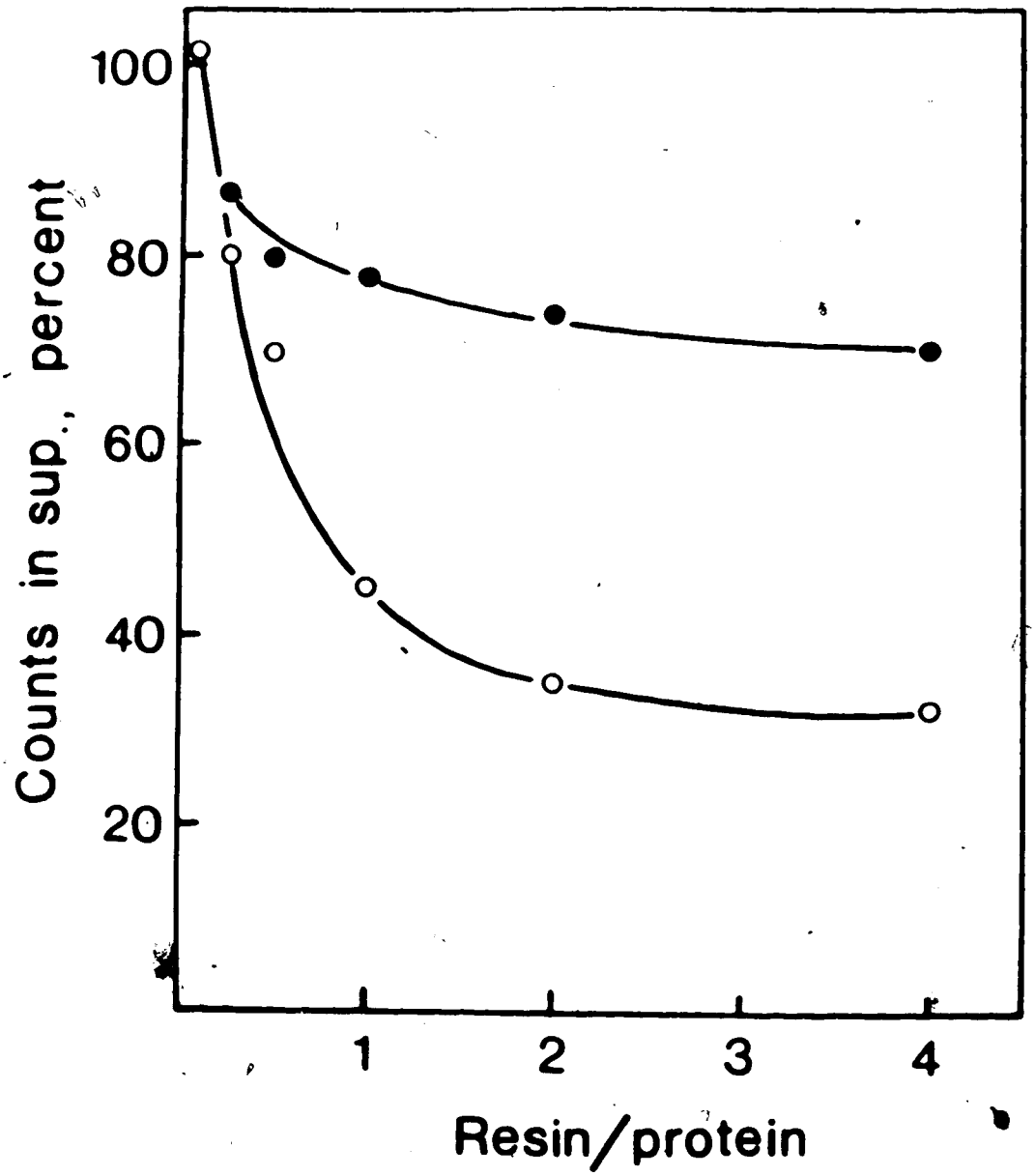
The above observation was further confirmed by performing a time-course binding experiment. Band 3 was incubated with the resin at 4 °C for varying time periods and at different time points aliquots of resin were removed, washed (to remove the unbound protein) and then incubated at 37 °C for 10 min. By doing this, increasing amounts of Band 3 were locked on the resin. Thus, a decreasing number of ligand molecules will remain unoccupied and available for subsequent binding. Fresh Band 3 was then added to these resin preparations and incubated at 4 °C for 15 min. Following this, the bound protein was eluted as usual initially with BADS and later with LDS. Figure III.3b shows that as an increased number of ligand sites were locked, the amount of Band 3 bound during the subsequent incubation decreased and then remained constant. This reflects the decreasing number of ligand molecules available for the protein binding. Upon reaching apparent saturation (30 min and beyond) only a constant number of sites will be available for the freshly added Band 3 for binding. Therefore, these data show that binding of Band 3 to the immobilized inhibitor ligand follows an equilibrium relationship of adsorption and desorption as suggested by Graves and Wu (1974).

3. Interaction of DIDS Labelled Band 3 with Immobilized SITS

Band 3 exists mostly as a dimer or a tetramer in the membrane and in solutions of non-ionic detergents such as $C_{12}E_8$ (Jennings, 1984). Thus Band 3 can bind to the immobilized SITS via one or both monomers. We studied this phenomenon by partially

Figure III.4

Effect of increasing amount of the affinity resin on removal of Band 3 from the supernatant. [^{3}H]-H_2DIDS labelled Band 3 was incubated with SITS-Affi-Gel 102 resin for 15 min at 4 °C. After letting the resin settle for 5 min, radioactivity in the supernatant was counted. Control with equal volumes of Sepharose 4B resin was also run. o———o - Band 3 labelled with 3 μM [^{3}H]-H_2DIDS; ●———● - Band 3 labelled with 30 μM [^{3}H]-H_2DIDS.


100
80
60
40
20
Counts in sup., percent
1
2
3
4
Resin/protein

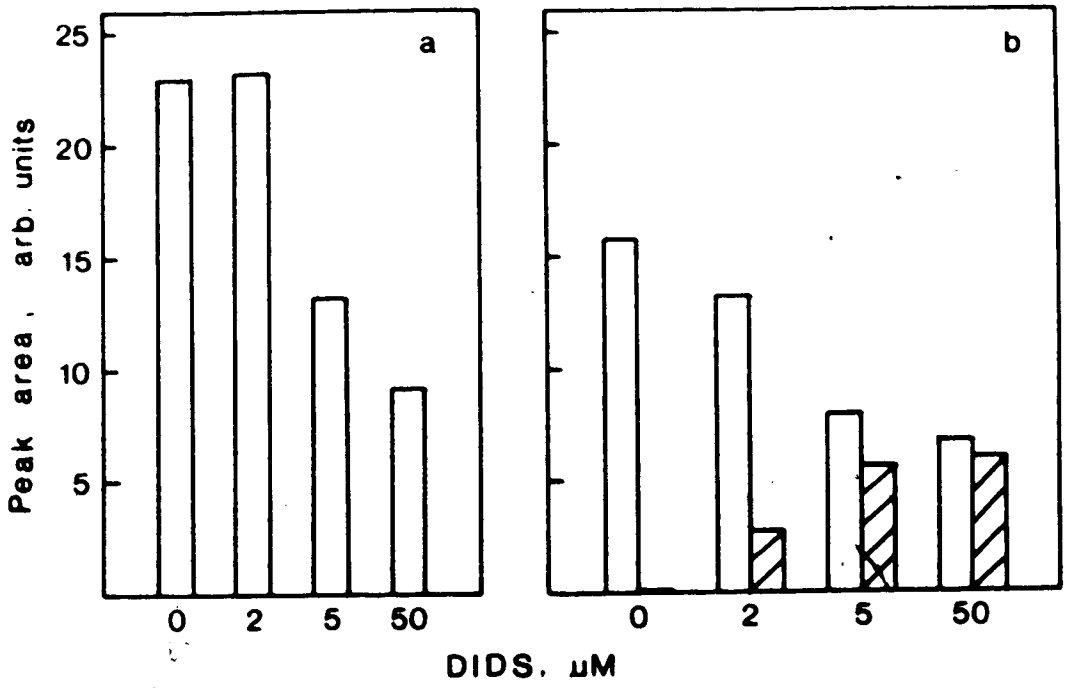
labelling Band 3 with [^{3}H]-H_2DIDS or DIDS. Under these conditions we will have a mixture of Band 3 populations with both, one or no monomers covalently labelled with DIDS. Ghost membranes prepared from control or DIDS-labelled erythrocytes were stripped with 0.2 mM EDTA, pH 7.5, followed by 1 M KI as described earlier (Pimplikar and Reithmeier, 1986). The KI-extracted pellet was solubilized in citrate buffer with 1% $C_{12}E_8$ (Band 3 extract). The protein binding assay was performed at 4 °C as described earlier (Pimplikar and Reithmeier, 1986) with the few modifications described under figure legends.

In order to determine the proportion of Band 3 dimers that have both inhibitor sites occupied by [^{3}H]-H_2DIDS, protein binding assays were performed with increasing amounts of resin. At the resin to sample volume ratio of 4, all of the unlabelled Band 3 was bound to immobilized SITS (data not shown), suggesting that at this ratio of resin to protein, the amount of ligand available is sufficient to bind all the Band 3 present. Figure III.4 shows that when Band 3 was labelled with 3 μM [^{3}H]-H_2DIDS, only up to 70% of the counts were removed from the supernatant with increasing amounts of resin. Thus, the remaining 30% represents labelled Band 3 with both sites occupied by [^{3}H]-H_2DIDS and unable to bind to the resin. When Band 3 was labelled with 30 μM [^{3}H]-H_2DIDS (conditions to fully label the protein), 80% of Band 3 did not bind to the resin. The 20-25% that bound represents protein with one site free or perhaps non-specifically bound Band 3. The Band 3 that was bound to the resin and thus removed from the supernatant was labelled by [^{3}H]-H_2DIDS only in one monomer and bound to the resin via the unlabelled monomer. The Band 3 that did not bind to the resin and therefore remained in the supernatant must have both monomers labelled by [^{3}H]-H_2DIDS.

In order to see if [^{3}H]-H_2DIDS labelled Band 3 is bound specifically, Band 3 bound was eluted by BADS followed by LDS. Table III.1 shows that Band 3 labelled with 3 μM [^{3}H]-H_2DIDS bound to immobilized SITS and was eluted by 1 mM BADS. A portion of

Figure III.5

Effect of DIDS labelling on Band 3 binding to immobilized SITS. Band 3 isolated from erythrocytes labelled with 0, 2, 5 or 50 μM DIDS was used in a protein-binding assay as described under "Materials and Methods". Panel a, total amount (BADS eluate + LDS eluate) of Band 3 bound to the resin; panel b, open columns, amount of Band 3 eluted by BADS at 4 °C; hatched columns, amount of Band 3 eluted by BADS upon incubation at 37 °C for 10 min prior to elution.


a
b
25
20
15
10
5
Peak area, arb. units
0
2
5
50
0
2
5
50
DIDS, μM

DIDS-labelled Band 3 was also eluted subsequently by 1% LDS. DIDS-labelled Band 3 did not bind to Sepharose 4B resin. This shows that Band 3 dimer labelled with

Table III.1 Binding of partially [^{3}H]-H_2DIDS-labelled band 3 to SITS-Affi-Gel 102 resin

Fraction	Seph. 4B	SITS-Affi-Gel 102
	dpm	*dpm*
Total added	25,171	25,171
Supernatant	26,725	19,716
BADS eluate	---	2,877
LDS eluate	---	1,698

Band 3 was prepared from erythrocytes labelled with 3 μM [^{3}H]-H_2DIDS. 50 μl resin was shaken with 60 μl band 3 extract (1mg protein per ml) for 15 min at 4 °C. Band 3 was eluted with 60 μl 1 mM BADS-5P8 followed by 60 μl 1% LDS.

[^{3}H]-H_2DIDS in one monomer does bind to immobilized SITS and this binding must be mediated via the stilbene disulfonate binding site on Band 3, since the bound Band 3 can be eluted by BADS.

We have earlier shown that Band 3 bound to the SITS-Affi-Gel 102 resin becomes tightly bound upon incubation at 37 °C and cannot be eluted with 1 mM BADS (Fig. III.2). When Band 3 dimers, labelled with 3 μM [^{3}H]-H_2DIDS, were bound to immobilized SITS and temperature shifted to 37 °C prior to elution, up to 40% of the counts were eluted by 1 mM BADS (data not shown, see below) suggesting that not all DIDS-labelled Band 3 was converted to the tightly-bound form. Under these conditions unlabelled Band 3 is totally converted to BADS-unelutable form.

In order to see if there was any correlation between the level of DIDS labelling and the inability of Band 3 to acquire tight-binding (BADS-unelutable) form, a binding and temperature-shift experiment was performed with Band 3 reacted with increasing amounts of DIDS. Figure III.5a shows that with increasing DIDS label, the amount of Band 3 bound to the resin decreased. Figure III.5b shows that whereas unlabelled Band 3 was

completely rendered BADS-unelutable following incubation at 37 °C, a part of DIDS-labelled Band 3 was still present in the BADS-elutable form. Moreover, the proportion of Band 3 that remained in BADS-elutable form increased with increasing DIDS label. When labelled with 2 μM DIDS, only 20% of Band 3 remained in the weakly-bound state. When labelled with 50 μM DIDS, more than 90% of Band 3 that bound to immobilized SITS failed to acquire the tight-binding (BADS-unelutable) form. Thus, labelling of Band 3 with DIDS and the inability of such a molecule to acquire tight binding are strongly correlated. Since the tight binding is achieved through a conformational change in the protein molecule (Verkman *et al.*, 1983), it can be concluded from the present data that binding of stilbene disulfonates in one monomer results in the inability of such a dimer to acquire the tight-binding conformation.

4. Interaction of Citrate-modified Band 3 with Immobilized SITS

Water-soluble carbodiimides which react with carboxyl groups under mild conditions (Carraway and Koshland, 1972) inhibit anion transport (Wieth *et al.*, 1982; Craik and Reithmeier, 1984). A Band 3 extract was made from the erythrocytes that had been reacted with citrate and EAC under conditions that inhibited the anion transport by 50%. EAC seems to activate a carboxyl group of citrate which then covalently reacts with Lysine 'a' which is involved in stilbene disulfonate binding (P.K. Werner, personal communication).

A Band 3 extract was also made from control erythrocytes that were treated identically except that EAC was missing from the reaction mixture. One ml of Band 3 extract was shaken with 25 μl SITS-Affigel-102 resin for 15 min at 4 °C and the bound protein was eluted with 250 μM BADS-5P8 followed by 1% LDS-5P8. Both control Band 3 and citrate-modified Band 3 were eluted by 250 μM BADS (Fig. III.6, lane 1 and lane 2) and a portion of Band 3 was also present in the subsequent 1% LDS eluate (lane 5 and lane 6). When Band 3 was initially bound to the affinity resin at 4 °C for 15 min, free Band 3

Figure III.6

Effect of citrate modification on Band 3 binding to immobilized SITS. Binding assay was performed as described under "Materials and Methods". BADS-5P8 and LDS eluates were electrophoresed on a 10% SDS-polyacrylamide gel prepared according to the procedure of Laemmli. Lanes 1 to 4- 250 μM BADS-5P8 eluates; lanes 5 to 8- subsequent 1% LDS-5P8 eluates. Lanes 1,5- control Band 3 at 4 °C; 2,6- citrate-modified Band 3 at 4 °C; lanes 3,7- control Band 3 temperature-shifted to 37 °C following the initial binding; lanes 4,8- citrate-modified Band 3 temperature-shifted to 37 °C following the initial binding.

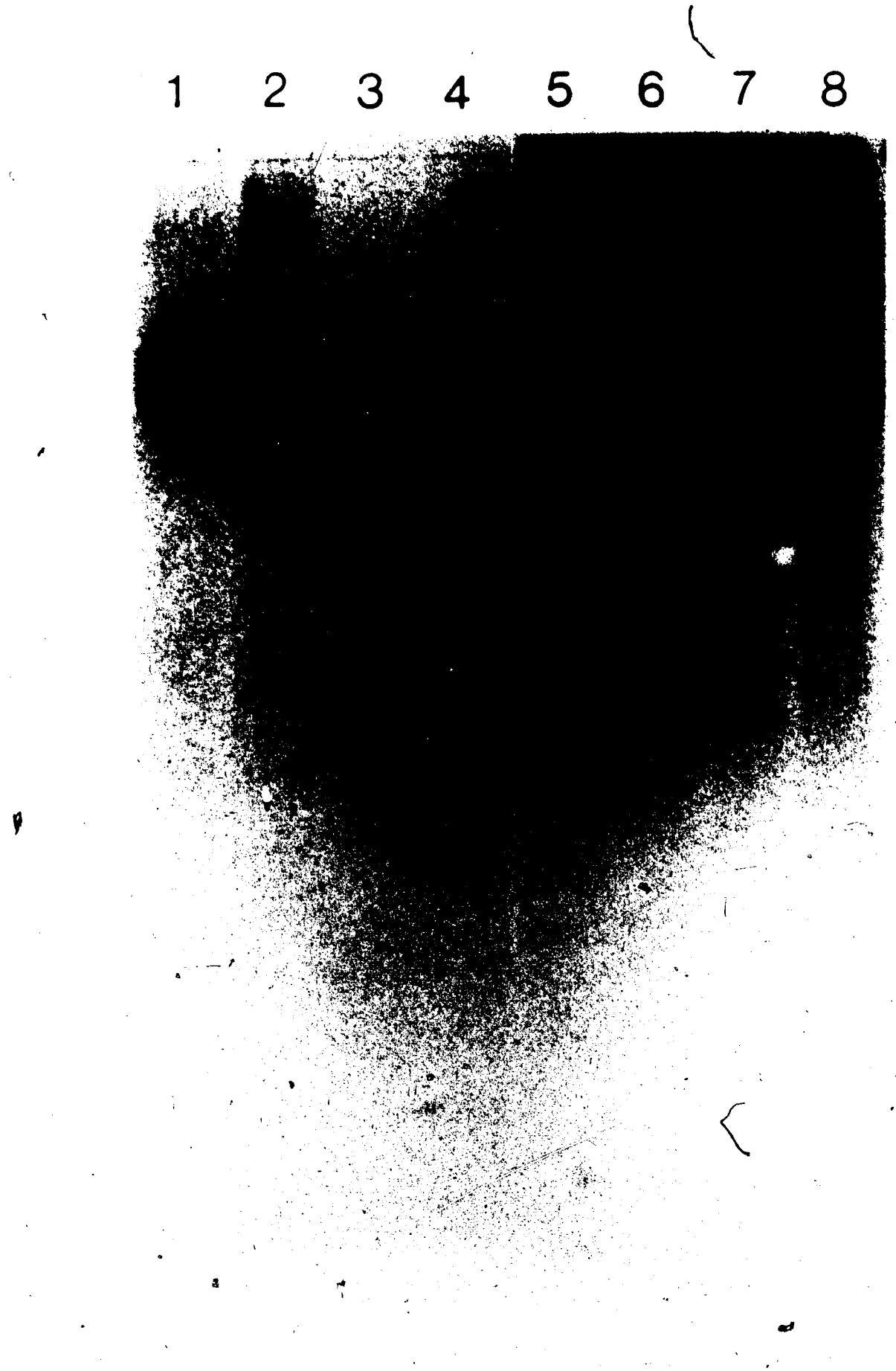
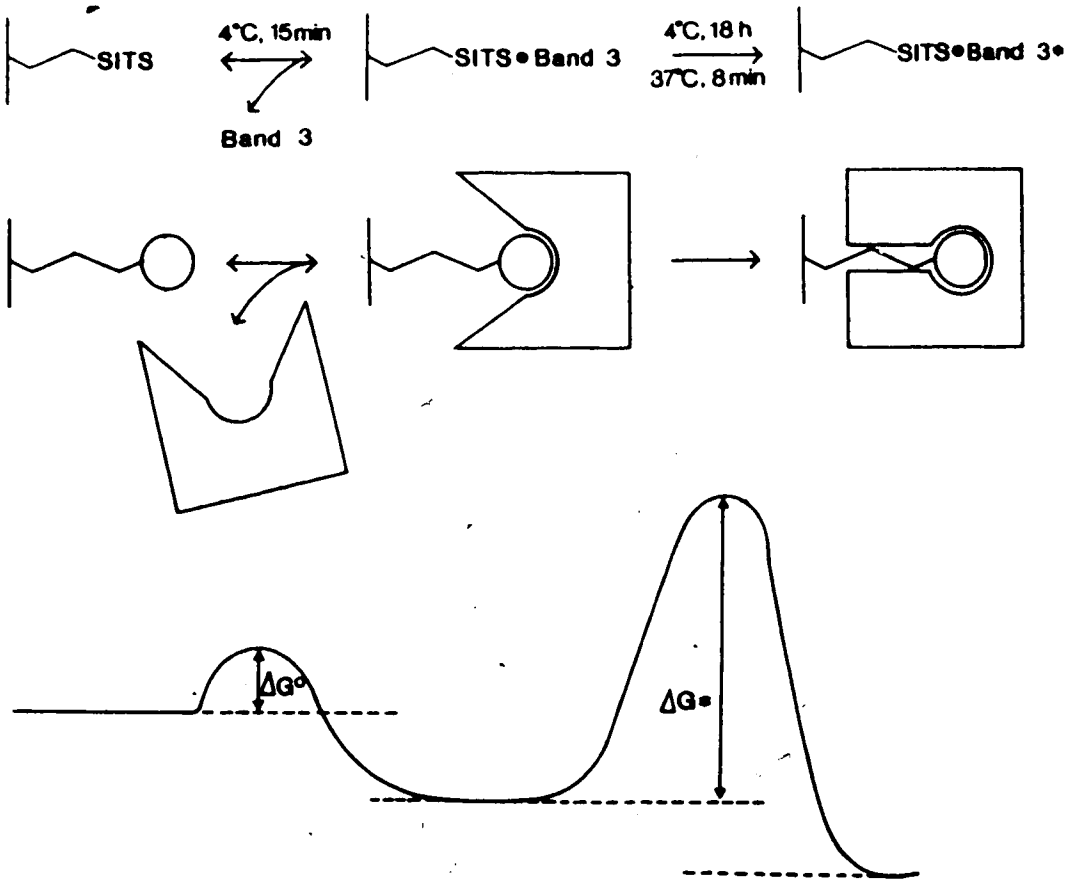

1 2 3 4 5 6 7 8

Figure III.7

Schematic representation of Band 3 binding to SITS-Affi-Gel 102 resin and the subsequent conformational change. For simplicity only one monomer of a Band 3 dimer is shown in this scheme. The lower panel represents the thermodynamic potential (Verkman *et al.*, 1983) for stilbene disulfonate-Band 3 interaction. ΔG° and ΔG^{*} represent transition-state free energies for the initial binding and the conformational change steps, respectively.


SITS
4°C, 15min
Band 3
SITS • Band 3
4°C, 18 h
37°C, 8 min
SITS • Band 3•
ΔG°
ΔG•

was removed and the resin-bound protein was later incubated at 37 °C for 8 min, 250 μM BADS could not elute any Band 3 from both control and citrate-modified Band 3 preparations (lane 3 and lane 4) and was present in the 1% LDS eluate (lane 7 and lane 8).

Figure III.6 also shows that the amount of Band 3 eluted by 250 μM BADS is not the same in control and citrate-modified Band 3 samples (with 1 mM BADS both control and citrate-modified Band 3 were eluted to the same extent). More of the citrate-modified Band 3 is eluted by 250 μM BADS as compared to that of control Band 3 (Fig. III.6, lane 1 vs lane 2; 184 units of citrate-modified Band 3 vs 100 units of control Band 3 by densitometry; data not shown) whereas subsequent elution by 1% LDS shows less citrate-modified Band 3 as compared to control Band 3 (120 units vs 173 units respectively; data not shown). The affinity resin bound the same total amount of control and EAC-treated Band 3 (lanes 7 and 8), only their elution profile was different. In a separate experiment a similar observation was made when the amount of protein was assayed in BADS and LDS elutes by the method of Lowry (data not shown). Since 250 μM BADS eluted more citrate-modified Band 3 than control Band 3, although the same amount of Band 3 was bound to the affinity resin, it indicates that modifying the inhibitor binding site with citrate results in weak binding and as a result citrate-modified Band 3 comes off more easily. It is interesting to note that citrate-modified Band 3, like control Band 3, becomes BADS-unelutable following temperature-shift to 37 °C for 10 min. This means that although the citrate-modified Band 3 binds more weakly to the resin, it is still able to acquire the high-affinity form.

D. DISCUSSION

In order to use SITS-Affi-Gel 102 as a probe for studying stilbene disulfonate interaction with Band 3, it was important to establish that interaction of immobilized SITS follows a pattern similar to that of free ligand with Band 3. It has been suggested that free inhibitor ligand binding to Band 3 occurs in two steps (Verkman *et al.*, 1983; Macara *et al.*,

1983). The initial weak binding is followed by strong binding brought about presumably through a conformational change in the protein (Macara *et al.*, 1983). This may represent a partial translocation of the inhibitor mimicking substrate movement. The interaction of inhibitor (in this case SITS) with Band 3 can be schematically represented as follows:

$$\text{SITS} + \text{Band 3} \rightleftharpoons \text{SITS} \bullet \text{Band 3} \rightarrow \text{SITS} \bullet \text{Band 3}^*$$

Our results show that the interaction of Band 3 protein with SITS immobilized onto a matrix is similar to free ligand-Band 3 interaction. Like free ligand, immobilized SITS molecules bind to Band 3 in two steps. Band 3 initially binds to immobilized SITS to give a SITS ● Band 3 complex which is slowly converted to SITS ● Band 3*, Band 3* being the form of Band 3 that has high affinity for the inhibitor (BADS-unelutable form). Thus, the SITS ● Band 3 complex is present in a quasi-equilibrium state and can follow either of two paths. Band 3 can detach from the resin giving free Band 3 and an unoccupied ligand as indicated by the availability of free ligand sites at equilibrium (apparent saturation with Band 3; Fig. III.3). Alternately, the bound Band 3 can undergo a slow conformational change giving SITS ● Band 3*. When immobilized SITS is incubated with Band 3 for a prolonged period, most of Band 3 is present as SITS ● Band 3* (Fig. III.1). Thus, it appears that SITS-Affi Gel 102 resin provides a simple and elegant system to study protein-stilbene disulfonate interaction.

From stopped-flow and temperature-jump studies of DBDS interaction with Band 3, Verkman *et al.* (1983) have calculated energy profiles for the enthalpies, entropies and free energy changes of each stage of DBDS binding. They conclude that the initial binding step occurs with a slight increase in entropy, a decrease in enthalpy and with a low energy activation barrier. The conformational change step has a very high activation barrier and is accompanied by a decrease in entropy (i.e., an increase in the orderly state of the system) and is mainly enthalpy driven. Our data (Fig. III.2) indicates that the initial

binding step is fast at 4 °C and remains unchanged at 37 °C, suggesting a low activation energy barrier, whereas the conformational change step is very slow at 4 °C but rapid at 37 °C, suggesting a high activation energy barrier. Higher temperature accelerates protein dynamics by increasing the available kinetic energy and thus overcoming the large transition-state free energies. Thus, taking these observations in consideration it can be said that our affinity resin can be used to study protein-stilbene disulfonate interaction and to draw valid conclusions regarding the general nature of such an interaction.

A schematic model based on these and the data disscussed above, together with the thermodynamic profiles for the different steps, is illustrated in Figure III.7. For simplicity, only one monomer is shown. Band 3 initially binds weakly to immobilized SITS to give a SITS●Band 3 complex. This process is reversible and Band 3 can detach from the resin giving free Band 3 and an occupied ligand. Conversion to SITS●Band 3* (the tightly-bound form) is a slow process. A high activation barrier and the stability of SITS●Band 3* complex renders this step essentially irreversible.

A practical point emerges from our work. Inhibitors of transport processes that bind to substrate sites may be used to purify the transport proteins. These inhibitors, however, are often poor substrates. They bind tightly to the translocation site but may be moved through the protein at a slower rate or may be only partially translocated through the proteinacious channel. This partial translocation of inhibitor also occurs in Band 3 with stilbene disulfonates present either in solution (Verkman *et al.*, 1983) or when immobilized on a matrix (Pimplikar and Reithmeier, 1986; Chapter II). The partial translocation buries the transport site in the protein, making it inaccessible to free inhibitor (tightly-bound conformation). The transport protein thereby becomes unelutable by free ligand. Successful purification of Band 3 may be performed only at 4 °C with the binding and the elution done rapidly. Prolonged incubation at 4 °C or the use of higher temperature should be avoided for the successful purification of transport proteins using inhibitor affinity chromatography.

The interaction of DIDS-labelled Band 3 with immobilized SITS provides interesting clues as to the nature of the interaction between the stilbene disulfonate binding sites on adjacent monomers of Band 3 dimers. Our data clearly show that while one monomer is covalently labelled with DIDS, the other monomer still retains its ability to bind SITS. However upon binding to immobilized SITS only through one monomer, Band 3 dimer labelled with DIDS in the other monomer fails to acquire tight binding upon incubation at 37 °C. This indicates that although the inhibitor binding sites on adjacent monomers act independent of each other in achieving initial binding with ligand, they must cooperate to achieve the tight binding that follows initial binding. Occupation of one site on a monomer with DIDS does not prevent the other monomer from binding an inhibitor ligand but seems prevent it from undergoing conformational changes necessary to acquire tight binding.

The possibility that the tight binding is due to both monomers binding to the resin and not due to a conformational change does exist. The distance between two SITS molecules and their orientation has to be very precise for both monomers to bind to the resin. Assuming that immobilized ligands are uniformly distributed in space on the resin and located at each corner of units in a cubic lattice (Lowe and Dean, 1974), the distance between neighboring ligands will be ~65 Å at 12.5 mM ligand concentration (as is the case in SITS-Affi-Gel 102 resin; data not shown). The distance between stilbene binding sites on two adjacent monomers was found to be only 28-52 Å (Macara and Cantley, 1981). Thus, it is possibile that of both monomers bind simultaneously to the resin. We attempted to get a clear answer by reducing the ligand density on the resin and thus making it extremely unlikely that a dimer could simultaneously bind two ligands. Under these conditions, Band 3 binding to the resin was completely lost (most likely due to interference with free charged spacer molecules) and therefore the temperature-shift experiment could not be performed. Thus, at this stage we cannot exclude the possibility that the tight-binding may be due to both monomers binding to the resin.

Verkman *et al.* (1983) have suggested that after rapid binding of one DBDS molecule to one monomer of Band 3 dimer, a slower conformational change takes place which then permits the reaction of a second DBDS molecule to the other monomer. Their studies, however, do not address the question of whether or not the second monomer, upon binding a DBDS molecule also, undergoes the conformational change. Our data with DIDS, a less bulkier inhibitor than DBDS, suggest that the second monomer does not undergo a conformational change upon binding to immobilized SITS. One explanation could be that during the sequential binding only the first monomer that binds inhibitor undergoes the conformational change. Thus, a conformational change in one monomer automatically precludes conformational change in the adjacent monomer. Another possibility is that binding of DIDS in one monomer slows down the conformational change in the other monomer significantly so that within the time frame of the present studies (10 min) it fails to acquire tight-binding. We could not test this possibility since with longer incubations (>60 min) in absence of free Band 3, the bound Band 3 was detected to dissociate from the resin. Alternately, following the binding of inhibitor ligand to one monomer, both monomers undergo the conformational change in concert. Under these circumstances, the unoccupied monomer can only bind an inhibitor ligand but not lock in place. As a result it can now bind to immobilized SITS only weakly and can not become tightly bound upon incubation at 37 °C. Finally, the DIDS labelled monomer may not have undergone the conformational change that must preceed a conformational change in its partner. Whatever the mechanism may be, our data do show that inhibitor binding sites on adjacent monomers interact with each other.

EAC, a water-soluble carbodiimide, reacts with carboxyl groups (Carraway and Koshland, 1972) and inhibits anion transport (Wieth *et al.*, 1982; Craik and Reithmeier, 1984). In presence of citrate, however, it activates citrate carboxyls which then form a covalent bond with a lysine residue involved in inhibitor binding. Data discussed above show that citrate modification decreases the affinity of Band 3 for immobilized SITS since

citrate-modified Band 3 is more easily eluted from the resin as compared to control Band 3. Recent fluorescence enhancement studies from this laboratory have shown that citrate modification of Band 3 in the presence of EAC reduces its affinity for BADS by 2 fold (Werner and Reithmeier, submitted for publication). Under this condition citrate is thought to modify a lysine residue involved in stilbene disulfonate binding and thus reduce the affinity of Band 3 for the inhibitor. Thus SITS-Affi-Gel 102 resin can be used to study changes associated with chemical modification of the inhibitor binding site of Band 3. Our data also indicate that the lysine residue involved in inhibitor binding is not important in tight binding since citrate-modified Band 3 can be rendered BADS-unelutable. Thus our studies show that immobilizing a ligand onto a matrix not only provides a useful system for affinity purification of the desired protein, but also provides a simple and effective tool to study ligand-protein interaction.

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IV. Interaction of Immobilized SITS with Band 3 in Erythrocytes and Vesicles[1]

A. INTRODUCTION

Anion transport inhibitors such as stilbene disulfonates bind specifically to the Band 3 protein of human erythrocytes and inhibit transport of anions across the erythrocyte membrane (Knauf, 1979; Macara and Cantley, 1983). In the plasma membrane, Band 3 is predominantly present as dimers (Jennings, 1984) and one mole of stilbene disulfonate binds per Band 3 monomer (Lepke, *et al.*, 1976; Ship *et al.*, 1977). The stilbene disulfonate binding site on the protein has been the focus of many studies (reviewed in Passow, 1986). The stilbene disulfonate binding site is accessible from the exterior of the red cell and not from the cytoplasmic side (Ship *et al.*, 1977). The binding site is located in a hydrophobic pocket 35-40 Å from sulfhydryl residues on the cytoplasmic domain (Macara *et al.*, 1983). Affinity chromatographic studies using 4-acetamido-4'-isothiocyanostilbene-2,2'disulfonate (SITS) immobilized on Affi-Gel 102 showed that this site was accessible to solubilized Band 3 (see Chapter II; Pimplikar and Reithmeier,1986). Since the length of SITS is approximately 18-20 Å and the length of the spacer molecule is 5-8 Å, it can be concluded that in the solubilized state, this site is 23-28 Å deep from the extracellular surface of Band 3.

We have shown in Chapter II that solubilized Band 3 binds specifically to SITS immobilized on an Affi-Gel 102 matrix (Pimplikar and Reithmeier,1986). This binding is mediated via the stilbene disulfonate binding site on the protein and the binding is abolished in the presence of free ligand or when the binding site is occupied by covalently attached 4,4'-diisothiocyanostilbene-2,2'disulfonate (DIDS). The question addressed in the present studies is whether or not this binding site is accessible to the immobilized ligand when Band 3 is present *in situ* in the intact erythrocyte. The solubilized protein is free to

[1]A version of this chapter has been submitted for publication.

diffuse and thus has an easy access to the immobilized ligand. This is not the case when the protein is membrane bound (either in erythrocytes or in vesicles). Moreover, the erythrocyte surface is covered with a thick coating of oligosaccharides. The most prominent carbohydrate structures on the erythrocyte surface are the poly-N-acetyllactosamine chains which form a sheet of hydration as thick as 160 Å (Fukada *et al.*, 1984). Such structures present on Band 3 protein (Fukada *et al.*, 1984), Band 4.5 protein (Mueller *et al.*, 1979) and glycolipids (Koscielak *et al.*, 1976) may affect the accessibility of the site to the inhibitor. Here we report that intact erythrocytes do bind to SITS immobilized on cellulose as do the rightside-out vesicles. The sealed inside-out vesicles, which normally would not be expected to bind (since they will have their stilbene disulfonate binding site oriented in the opposite direction), exhibit non-specific binding towards the affinity matrix, thereby limiting the potential use of affinity chromatography in separating the rightside-out vesicles from the inside-out vesicles.

B. MATERIALS and METHODS

1. Preparation of SITS-AE-cellulose

α-cellulose was oxidized by a modification of a method described earlier (Kondo *et al.*, 1980). Ethylene diamine was coupled to the oxidized cellulose to produce a 4-atom spacer arm with a free terminal amino group. SITS was coupled to the amino group by nucleophilic substitution. Briefly, 1 g of α-cellulose (8 to 10 ml of hydrated bed volume) was soaked in water for 1 h, washed several times and excess water decanted. The cellulose was incubated with 10 ml of 100 mM sodium periodate in water for 1 h at room temperature. The oxidized cellulose was washed with a large excess of water on a sintered glass funnel, followed by 5 mM sodium phosphate, 150 mM NaCl, pH 8.0 (phosphate-buffered saline), and suspended in 10 ml of phosphate-buffered saline. The above slurry was incubated with 1.35 ml of ethylene diamine (redistilled over KOH) in 5 ml of phosphate-buffered saline and the pH was adjusted to 8 with 6 N HCl. After incubating

for 24 h at 4 °C, the cellulose was washed with a large excess of water and treated with 20 ml of 100 mM $NaBH_4$ in water for 30 min. at 37 °C. The cellulose was washed again with water followed by 500 ml of 0.1 M sodium bicarbonate, pH 8.0, and suspended in 10 ml of 0.1 M sodium bicarbonate. The oxidized cellulose was incubated with SITS (55.2 mg of SITS dissolved in 5 ml of 0.1 M sodium bicarbonate) at 37 °C for 2 h. Following the incubation, the SITS-AE-cellulose was washed with a large excess of 0.1 M sodium bicarbonate followed by water and stored at 4 °C. The amount of SITS ligated to cellulose was calculated by subtracting amount of SITS in the washes from the amount of SITS present during incubation. This procedure gave approximately 50 µmole of ligand/g cellulose.

2. Preparation of erythrocytes

Fresh blood was collected from normal donors in EDTA and was washed at least three times with phosphate-buffered saline to remove leucocytes and platelets. Erythrocytes were then washed with 10 volumes of 28.5 mM sodium citrate, 205 mM sucrose, pH 7.5 (sucrose-citrate buffer), three or four times to remove chloride from the medium. These intact erythrocytes were used in the binding studies.

3. Preparation of ghost membranes and sealed vesicles

Ghost membranes were prepared from outdated blood (kindly provided by the Canadian Red Cross) by hypotonic lysis as described earlier in Chapter II (Pimplikar and Reithmeier,1986). Sealed inside-out and rightside-out vesicles were prepared as described by Steck and Kant (1974). The ghost membranes were suspended in 40 volumes of freshly prepared 0.5 mM sodium phosphate, pH 8.0, at 4 °C for overnight, washed once with the same buffer and centrifuged at 28,000 x g for 30 min. The pellet was then passed through a 27 gauge needle five times. This procedure usually yields a mixture of vesicles containing 30% to 60% of sealed inside-out vesicles.

4. Scanning electron microscopy

The samples (erythrocytes bound to SITS-AE-cellulose) were spread in a flat-bottomed glass dish and fixed in 28.5 mM sodium citrate, pH 7.5, containing 205 mM sucrose and 2% glutaraldehyde for 4 h at 4 °C. The fixative was removed, and the cellulose was washed three times with 28.5 mM sodium citrate, pH 7.5. After washing, the cellulose was dehydrated in a graded series of ethanol, critical point dried, mounted on aluminum stub with silver paint and sputter coated with gold. Samples were examined in a Phillips SEM 505 scanning electron microscope.

5. Analytical Techniques

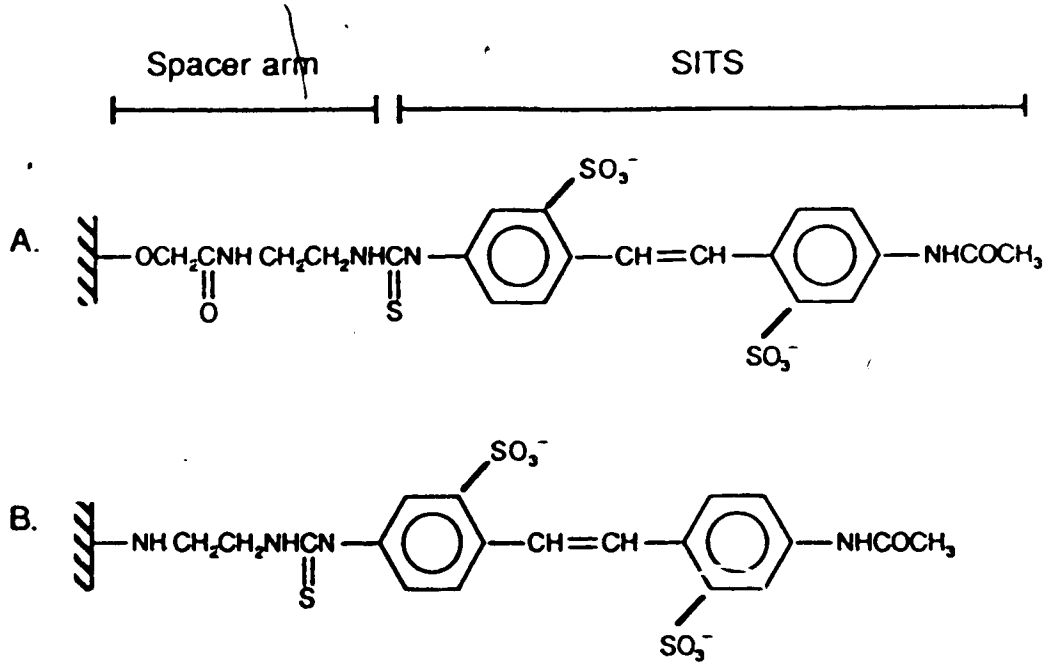
Protein assay was performed according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The proportion of sealed inside-out vesicles was assessed by the inaccessibility of acetylcholinesterase, which is located on the outside of the intact erythrocyte (Steck and Kant, 1974). In the presence of 0.1% Triton X-100, the permeability barrier is broken and the total enzyme activity is exposed. The acetylcholinesterase assay was performed as described earlier (Ellman *et al.*, 1961). The rate of reaction was measured at 412 nm in a Varian DMS 100S spectrophotometer.

C. RESULTS and DISCUSSION

1. Intact erythrocytes bind to SITS-AE-cellulose

In a 24-well microtiter plate, approximately 100 µl of SITS-AE-cellulose per well (for structure, see Fig. IV.1) was placed and washed three times with sucrose-citrate buffer. Excess buffer was removed by a pasteur pipet. Erythrocytes suspended in sucrose-citrate buffer to a 20% hematocrit were added to SITS-AE-cellulose in equal volumes and incubated at room temperature for 20 min. The unbound erythrocytes were removed by adding sucrose-citrate buffer slowly along the side of the well and then gently removing the supernant by a pasteur pipet. The washing procedure was repeated until the

Figure IV. 1. Structures of SITS-Affinity Matrices.



A. SITS-Affi-Gel 102: Affi-Gel 102 was purchased from Bio-Rad and SITS was conjugated to the free amino group of the spacer molecule through the isothiocyano group.

B. SITS-AE-cellulose: α-cellulose was oxidized by periodate, and ethylene diamine was coupled to the oxidized cellulose. SITS was conjugated to the free amino group of ethylene diamine through the isothiocyano group.

Figure IV. 2

Light micrographs showing that the intact erythrocytes bind to SITS-AE-cellulose. In a microtiter plate intact erythrocytes were incubated with cellulose or SITS-AE-cellulose at room temperature for 20 min. The unbound erythrocytes were removed by washing and the wells were examined under an inverted light microscope. Panel A- cellulose incubated with erythrocytes. Panel B- SITS-AE-cellulose incubated with erythrocytes. Panel C- SITS-AE-cellulose incubated with DIDS-labelled erythrocytes. In each panel the photograph on the right shows a 4 X magnified view of the photograph on the left.

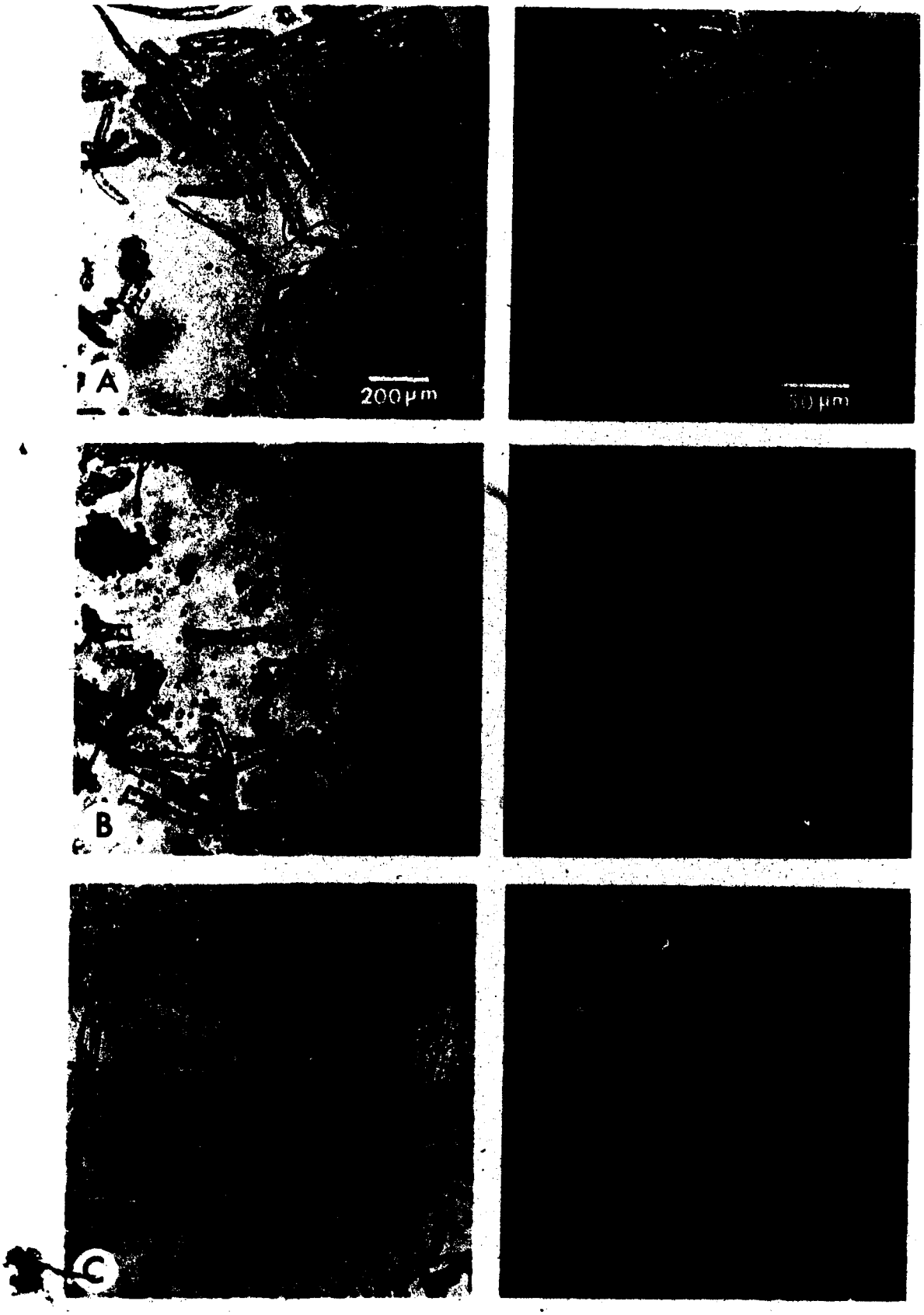
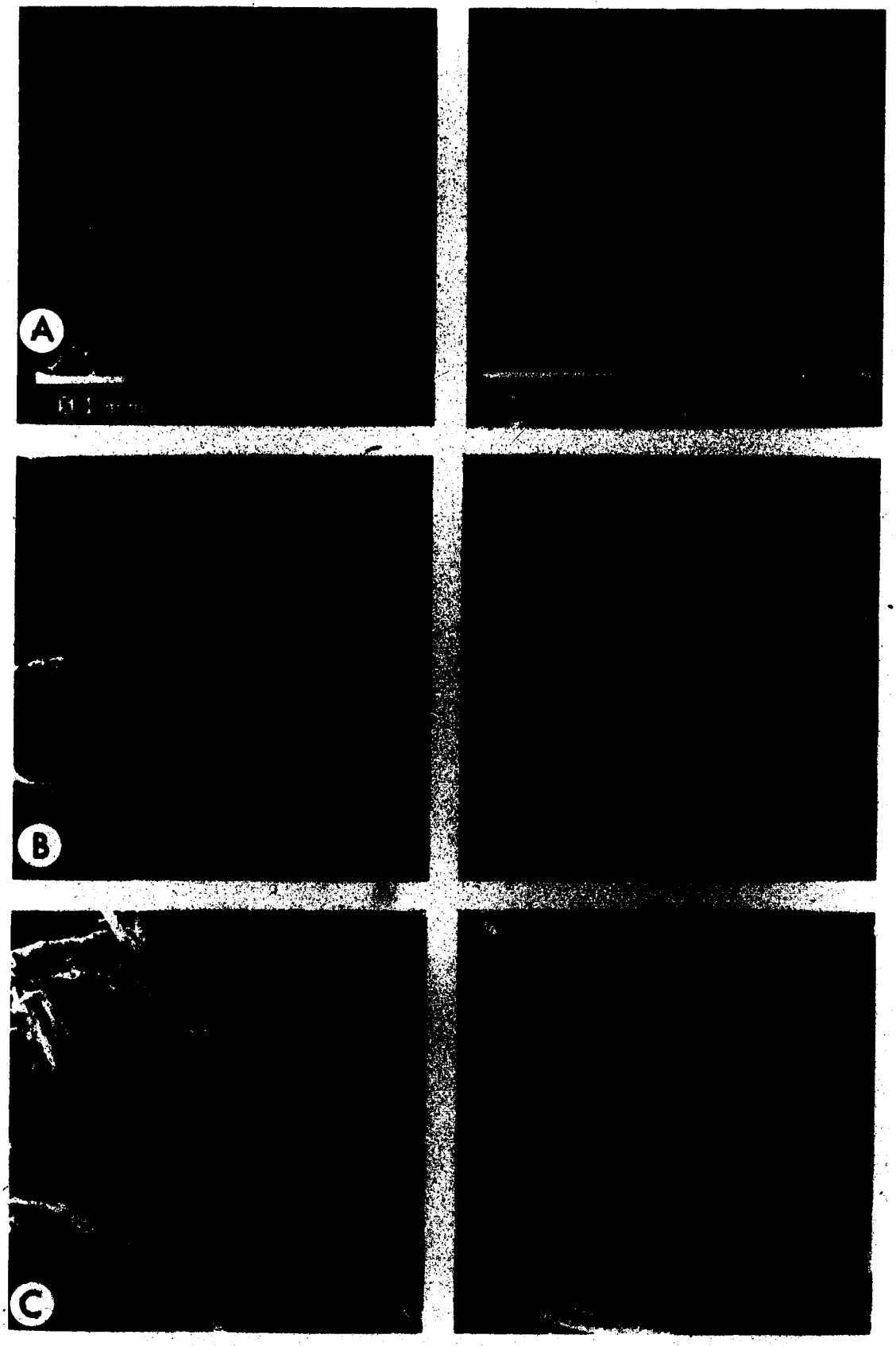

A
200 µm
50 µm
B
C

Figure IV.3

Scanning electron micrographs showing that the intact erythrocytes bind to SITS-AE-cellulose. The details of the binding experiment were as described for Fig. IV. 2. Following the removal of the unbound erythrocytes, cellulose fibers were fixed with glutaraldehyde. The samples were dehydrated in a graded series of ethanol, critical point dried and examined under a scanning electron microscope. Panel A; cellulose incubated with erythrocytes. Panel B; SITS-AE-cellulose incubated with erythrocytes Panel C; SITS-AE-cellulose incubated with DIDS-labelled erythrocytes.


A
B
C

supernatant was free of erythrocytes. At this stage the samples were either processed for scanning electron microscopy or viewed directly under an inverted light microscope.

Fig. IV.2 shows the binding of the erythrocytes to SITS-AE-cellulose fibres by light microscopy. Panel A shows the oxidized-cellulose fibres after incubation with erythrocytes. These fibres were not ligated with SITS and erythrocytes did not bind to these fibres. Intact erythrocytes, however, did bind to SITS-AE-cellulose fibres (Fig. IV. 2, panel B). This binding was mediated via the stilbene disulfonate binding site on Band 3 since erythrocytes covalently labelled with DIDS did not bind to SITS-AE-cellulose (Fig. IV. 2, panel C). Also, when the binding experiment was performed in the presence of excess free 1 mM 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), erythrocytes again failed to bind to the derivatized cellulose (data not shown). Scanning electron micrographs (Fig. IV.3) also show that erythrocytes did not bind to cellulose alone (panel A), but did bind to SITS-AE-cellulose (panel B) and that the binding was abolished when the erythrocytes were labelled with DIDS (panel C).

Preliminary attempts to get binding of erythrocytes to SITS-Affi-Gel 102 (for structure, see Figure IV.1) were not successful. Although solubilized Band 3 bound to SITS-Affi-Gel 102 resin and was successfully eluted later, the intact erythrocytes either failed to bind the resin or came off very easily during the subsequent washing steps (see below). Affi-Gel 102 is a derivatized agarose with a large internal volume. Most of the attached ligand will be present in this internal space. The solubilized proteins can diffuse in and bind to ligand but erythrocytes are excluded due to their size. Thus, the number of ligand sites available to Band 3 in intact cells is limited. On the other hand, cellulose fibres do not have an internal volume (Kondo *et al.*, 1980) and thus all ligand molecules are present on the surface and are available for erythrocytes to bind.

The technique of cell chromatography has been widely applied in the analysis of cell populations of immune system (for reviews see Edelman and Rutishauser, 1974; Bonnafous *et al.*, 1985). The more widely used solid supports in these studies were

plastics, glass and nylon fibers. These supports have no internal volumes and therefore the attached ligands are present on the surface of the matrix. Agarose and polyacrylamide supports have also been used, but not as frequently. Recovery of the bound cells by adding excess ligand is possible in a few cases but the yields are poor (Brunner *et al.*, 1977). Since the cells are bound to the affinity surface via multipoint attachments, addition of excess free ligand does not result in a good recovery of the cells. The recovery can be improved dramatically by mechanical disruption, which has become the method of choice for elution of the bound cells (Bonnafous *et al.*, 1985).

Our data also show that the carbohydrates present on the erythrocyte surface do not affect erythrocytes binding to SITS-AE-cellulose. The precise functions of the carbohydrate chains on erythrocyte membrane, besides their role in blood group specificities and in the antigenic conversion of i-erythrocytes to I-erythrocytes (Watanabe *et al.*, 1979), is not well understood. The role of the carbohydrate chain on Band 3 is even less understood. These data show that the carbohydrates on Band 3 do not interfere with inhibitor-protein interaction.

As in the case of other cells bound to affinity resin (Brunner *et al.*, 1977; Sharma and Mahendroo, 1980), the erythrocytes are also weakly bound to SITS-AE-cellulose. They easily fall off when shaken or when SITS-AE-cellulose is washed vigorously following the binding. This also explains why there were fewer erythrocytes bound to SITS-AE-cellulose when examined under a scanning electron microscope. The bound erythrocytes come off during the fixation and dehydration procedure. Our earlier studies (Chapter II; Pimplikar and Reithmeier,1986) have shown that the presence of a spacer arm, 6-atom long (~6-8 Å in length), between the solid matrix and the ligand is necessary to get optimal binding. Solubilized Band 3, however, did bind to ligand immobilized with a 4-atom spacer molecule (Chapter II; Pimplikar and Reithmeier,1986). This is because the stilbene disulfonate binding site is present in the interior of the protein and the spacer arm helps to overcome the steric hindrance. The spacer arm used in the present studies is 4-

atoms long (4-6 Å in length). With a shorter spacer arm, the ligand probably just reaches the binding site within the protein. Therefore, this could be one of the factors responsible for the weak binding of the erythrocytes to SITS-AE-cellulose.

2. Interaction with the sealed vesicles

The following binding experiments were done on a column rather than in plastic wells to avoid any unnecessary mechanical disturbance generated during the washing procedure. A 5 ml column was packed with 2 ml of SITS-AE-cellulose or SITS-Affi-Gel 102 resin equilibrated with 28.5 mM sodium citrate buffer, pH 8.0. Sealed rightside-out vesicles (1 mg protein/ml) in 28.5 mM sodium citrate buffer, pH 8.0, were loaded on the column (20 ml/h). When the protein sample had completely entered the resin, fresh buffer was added from the top, the flow was stopped, and the protein was allowed to interact with the resin for 20 min at room temperature. Following the incubation, the column was washed with 28.5 mM sodium citrate buffer , pH 8.0, and the absorbance at 280 nm was monitored. Fig. IV.4 shows the A_{280} profile when 400 µl of rightside-out vesicles were loaded either on a SITS-Affi-Gel 102 column or a Sepharose 4B column. The flow-through of the SITS-Affi-Gel 102 column shows a reduced amount of protein, suggesting that the affinity resin had bound and retained the rightside-out vesicles. Thus both the intact erythrocytes and the rightside-out vesicles bind to immobilized SITS. When packed in columns, both SITS-Affi-Gel 102 and SITS-AE-cellulose retained the intact erythrocytes or the rightside-out vesicles to the same extent (data not shown). Since column chromatography was easier to perform with resin rather than cellulose, all the subsequent experiments were carried out with SITS-Affi-Gel 102.

Since the stilbene disulfonates bind to Band 3 only from the outside to inhibit the anion transport, we attempted to quantitatively isolate the inside-out vesicles from the rightside-out vesicles using SITS-Affi-Gel 102 column. The inside-out vesicles will have their stilbene disulfonate binding site on the inside of the vesicle and therefore will not bind

Figure IV.4

Fractionation of sealed rightside-out vesicles on Separose 4B column (Seph.) or SITS-Affi-Gel 102 column. Sealed rightside-out vesicles (1 mg/ml) in volume 400 µl of were loaded on 2 ml of respective resins, incubated for 20 min at room temperature. The peaks show relative amounts of the unbound material. By increasing the amounts of SITS-Affi-Gel 102 resin it was possible to retain all of the rightside-out vesicles on the column.

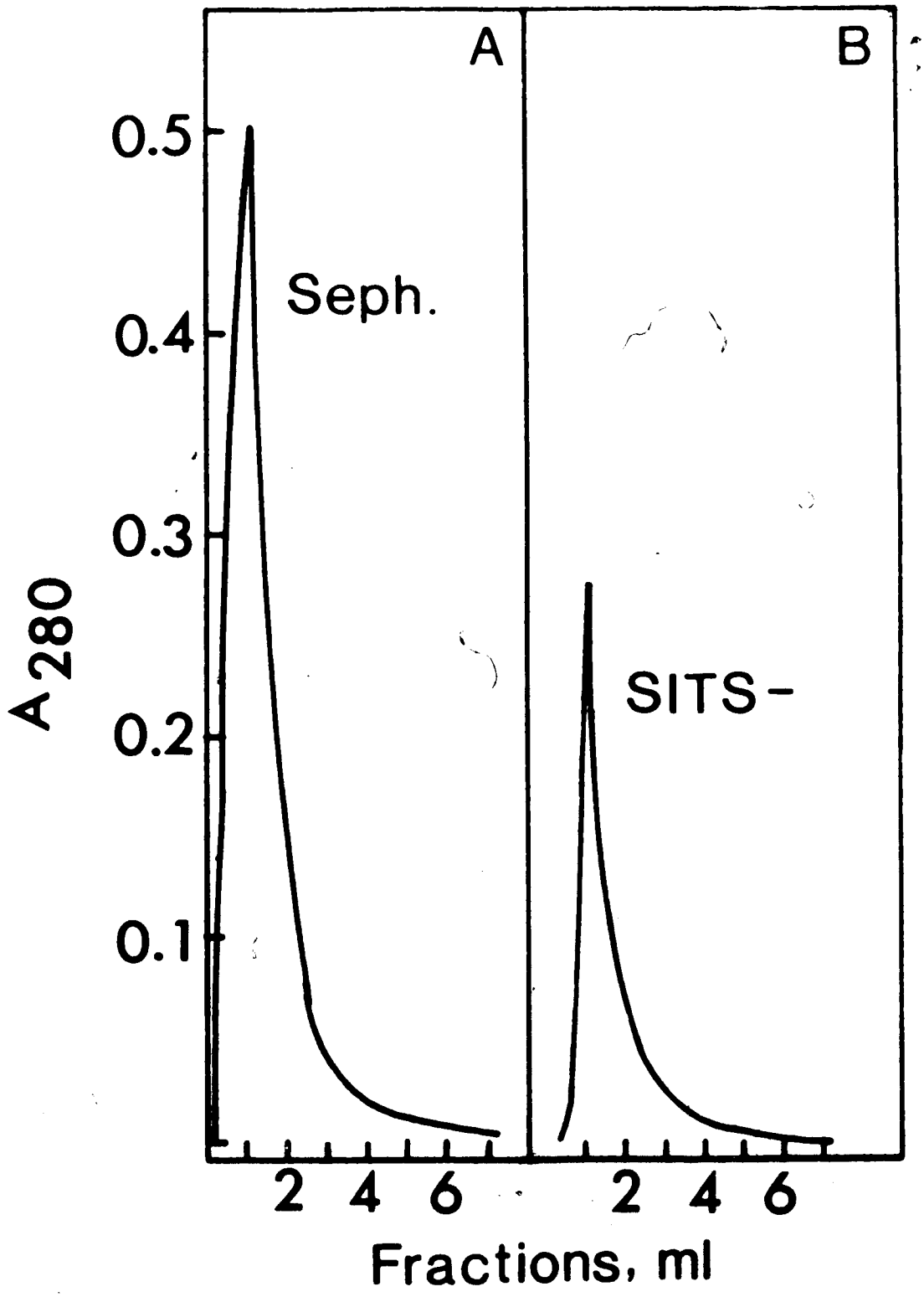

A
B
0.5
0.4
0.3
0.2
0.1
A280
Seph.
SITS-
2 4 6
2 4 6
Fractions, ml

Figure IV.5

Fractionation of crude inside-out vesicles on Separose 4B column (Seph.) or SITS-Affi-Gel 102 column. Crude inside-out vesicles (1 mg/ml) in a volume of 500 µl were loaded on 3 ml of respective resins, incubated for 20 min at room temperature. After elution of the unbound fractions, fresh buffer was added and the resins were stirred gently (shown by the arrows) to elute the bound fraction.

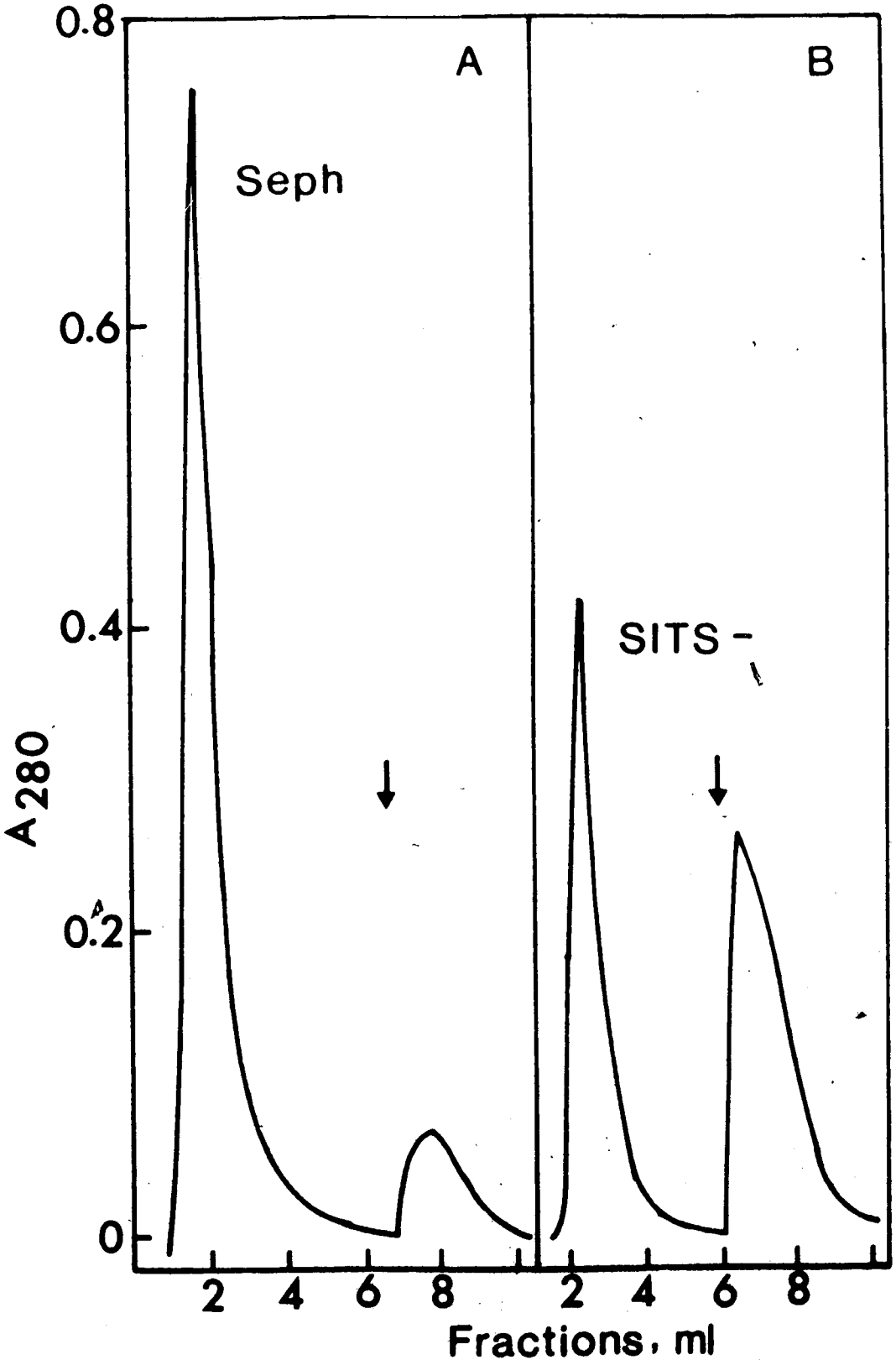

0.8
0.6
0.4
0.2
0
A280
A
B
Seph
SITS -
2
4
6
8
2
4
6
8
Fractions, ml

Table IV.1 Rightside-out vesicles preferentially bind to SITS-Affi-Gel 102 resin

Fraction	Acetylcholinesterase activity[a] -detergent	+detergent[b]	%accessible[c]
Crude inside-out vesicles	1.93	3.19	60
Sepharose 4B			
Unretained	2.14	3.51	61
Retained[d]	-	-	-
SITS-Affi-Gel 102			
Unretained	1.33	3.43	39
Retained	3.08	4.10	75

The crude inside-out vesicles were loaded on a Sepharose 4B or a SITS-Affi-Gel column. The protein was incubated with the column for 20 min and flow-through (unretained fraction) and bound material were collected and assayed for acetylcholinesterase activity. The values for the enzyme actvities represent the means of two separate experiments.

[a]Acetylcholinesterase activity expressed as micromoles of product per mg of protein.

[b]Triton X-100 in a final concentration of 0.1% was added as a detergent.

[c]Accessibility was calculated as (activity without detergent/activity with detergent) x 100

[d]Not determined due to insufficient amount of protein.

to the column. The inside-out vesicles were prepared and loaded on a SITS-Affi-Gel 102 or a Sepharose 4B column as described above. The column was washed with 28.5 mM sodium citrate , pH 8.0, till A_{280} reached zero (Fig. IV.5). The bound material was eluted by gently shaking the resin with the help of a spatula (Brunner *et al.*, 1977). Fig. IV.5 shows that the affinity resin had retained more material as compared to Sepharose 4B and this was subsequently eluted from the column. Table IV.1 shows acetylcholinesterase activities of the various fractions collected. The inside-out vesicle preparation loaded on the columns had 40% sealed inside-out vesicles initially (since 40% of the enzyme activity was not accessible). After passing over a Sepharose 4B column, the proportion of sealed inside-out vesicles remained unchanged. The flow-through of the SITS-Affi-Gel 102 column, however, showed enrichment in the proportion of sealed inside-out vesicles (60% as compared to the initial 40% sealed inside-out vesicles). Correspondingly, the fraction that

Table IV.2 Binding of the rightside-out vesicles to SITS-Affi-Gel 102 resin is blocked by free inhibitor ligand

Fraction	Acetylcholinesterase activity[a] -detergent	+detergent[b]	%accessible[c]
Crude inside-out vesicles			
-DNDS	1.17	2.03	58
+DNDS[d]	1.18	1.91	62
Sepharose 4B[e]			
-DNDS	1.40	2.49	56
+DNDS[d]	1.59	2.58	62
SITS-Affi-Gel 102[e]			
-DNDS	1.04	2.89	36
+DNDS	1.38	2.18	63

300 µl of resin was incubated at room temperature for 2 h with equal volumes of crude inside-out vesicles that were preincubated at 37 °C for 15 min with or without DNDS. Following the incubation, the resin was allowed to settle under gravity and the supernatant (unretained fraction) was collected and assayed for the enzyme activity. The values for the enzyme activities represent the means of two separate experiments.

[a]Acetylcholinesterase activity expressed as micromoles of product per mg of protein.

[b]Triton X-100 in a final concentration of 0.1% was added as a detergent.

[c]Accessibility was calculated as (activity without detergent/activity with detergent) x 100

[d]DNDS in a final concentration of 1 mM was present during the preincubation.

[e]The unretained fraction was assayed for enzyme activity.

bound the resin showed de-enrichment in the proportion of sealed inside-out vesicles (only 25% as compared to the initial 40%). This shows that the rightside-out vesicles bind preferentially to the affinity resin and the sealed inside-out vesicles do not bind as well.

Is the binding of the rightside-out vesicles mediated via the stilbene disulfonate binding site of Band 3 protein ? To answer this question, 300 µl SITS-Affi-Gel 102 resin or Sepharose 4B was incubated with 150 µg of crude inside-out vesicles with or without 1 mM DNDS. After 2 h of intermittent shaking at room temperature, the resin was allowed to settle and the supernatant was assayed for enzyme activity. Table IV.2 shows that DNDS by itself did not change the accessibility of the enzyme. As seen earlier, enrichment of the sealed inside-out vesicles was brought about only by the SITS-Affi-Gel 102 resin and not

by the Sepharose 4B. In the presence of 1 mM DNDS, however, the flow-through of SITS-Affi-Gel 102 column did not show enrichment in the proportion of the sealed inside-out vesicles. Similar results were obtained when the experiment was carried out in presence of free BADS. This shows that the binding of the rightside-out vesicles to the affinity resin was mediated via the stilbene disulfonate binding site of Band 3 protein.

Attempts were made to achieve the purity of sealed inside-out vesicles that was obtained by other methods (Kondo *et al.*, 1980; Steck and Kant, 1974). A density gradient centrifugation procedure resulted in 80% of sealed inside-out vesicles whereas the method described in the present paper yields about 60% of sealed inside-out vesicles. Prolonged incubation during the binding, reapplication of the excluded fractions, binding at various ionic strengths, binding with increased amounts of the resin or incubations at lower or higher temperatures did not result in improvement above 60%. This was most likely due to non-specific adsorption of the inside-out vesicles on the affinity resin. Stilbene disulfonates, such as DIDS, are impermeable to the membrane and when incubated with the intact erythrocytes bind mostly (>95%) to Band 3. When incubated with the leaky ghosts (where the internal surface is now accessible to the inhibitor ligand), DIDS was found to covalently react to other proteins and lipids (Cabantchik and Rothstein, 1974). Since the sealed inside-out vesicles have their internal surface exposed, it seems that binding of the sealed inside-out vesicles to the immobilized SITS was mostly mediated via non-specific interactions. These interactions preclude a one-step separation of the inside-out vesicles from the rightside-out vesicles by affinity chromatography.

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V. Identification, Isolation and Characterization of a Putative Anion Transporter from the Canine Renal Brush Border Membranes[1]

A. INTRODUCTION

Cellular anion transport plays a critical role in many diverse physiological processes such as in the transport of bicarbonate , in the regulation of cell pH and volume and in several electrolyte transport processes (Wieth and Brahm, 1985; Kokko and Jacobson, 1985). Although anion transport systems are known to be present in a variety of tissues, the best-studied system is the one that mediates chloride-bicarbonate exchange across the erythrocyte membrane (Knauf, 1979; Passow, 1986). A 95,000 dalton glycoprotein in the erythrocyte membrane, known as Band 3 protein, mediates the exchange of anions across the erythrocyte membrane. Band 3 is an integral membrane protein and the carboxyl-terminal half (Mr=55,000) spans the bilayer several times (Kopito and Lodish, 1985) and contains the site for ion translocation (Steck *et al.*, 1976; Grinstein *et al.*, 1981).

Anion exchange mediated by Band 3 protein is inhibited by stilbene disulfonate derivatives either reversibly (e.g., by BADS) or irreversibly (Knauf, 1979; Passow, 1986). These inhibitors presumably bind to a single site recessed deep within the interior of the protein (Pimplikar and Reithmeier, 1986; Macara *et al.*, 1983) and compete with the anion substrates. Upon binding to this site on Band 3, inhibitors such as DIDS, under the appropriate conditions, covalently react with one or two lysine residues and cause irreversible inhibition of anion transport. We have recently shown that Band 3 also binds to SITS-Affi-Gel 102 affinity resin and that it can be eluted by BADS (Pimplikar and Reithmeier, 1986; Chapter II).

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In the present work we have employed affinity chromatography to identify an anion transporter from canine kidney membrane fractions (BBM and ALM) using a SITS-Affi-Gel 102 resin. Various anions such as chloride (Warnock and Eveloff, 1982), bicarbonate (Warnock and Eveloff, 1982), phosphate (Schali *et al.*, 1986), sulfate (Pritchard and Renfro, 1983) are transported across the epithelium in different regions of kidney (reviewed in Chapter 1, Anion Renal Exchange, also see Kokko and Jacobson, 1985). A chloride/bicarbonate exchange that is sensitive to SITS or DIDS has been reported in *Necturus* proximal tubule (Spring and Kimura, 1978; Edelman *et al.*, 1981) and in the mouse cortical thick ascending limb of Henle's loop (Andreoli *et al.*, 1981; Friedman and Andreoli, 1981). Band 3-like proteins in kidney have been identified by cross-reaction with anti-Band 3 antibodies (Drenckhahn *et al.*, 1985; Kay *et al.*, 1983; Cox *et al.*, 1985; Schuster *et al.*, 1986). This antigen has been localized to the basolateral membrane of epithelial cells. We report here the isolation and purification of a single polypeptide from the BBM of dog kidney cortex that is labelled with [^{3}H]-H_2DIDS, binds specifically to SITS-Affi-Gel 102 resin but does not cross-react with affinity-purified anti-Band 3 antibodies.

B. MATERIALS AND METHODS

Affi-Gel 102 was purchased from Bio-Rad. SITS was obtained from U.S. Biochemical Corporation, Cleveland, Ohio. BADS was synthesized as described earlier (Rao *et al.*, 1979). $C_{12}E_8$ was from Nikko Chemical Co.,Tokyo. [^{3}H]-H_2DIDS was purchased from Research Development Corporation, Hospital for Sick Children, Toronto, Ontario. All other chemicals were reagent grade or better.

1. BBM Vesicle Preparation

BBM vesicles were isolated by the Mg^{++} precipitation method as described by Silverman and Speight (1986). All steps werec arried out at 0-4 °C and all centrifugation

steps employed an SS-34 rotor and a Sorvall RC-5B refrigerated centrifuge. Briefly, the outer cortex was scraped off and homogenized in 10 volumes of isolation medium (10 mM triethanolamine hydrochloride, 250 mM sucrose, pH 7.6). The homogenate was filtered through a double layer of cheese cloth followed by filtration through a single layer of nylon gauze. It was centrifuged twice at 1250 rpm for 10 min. The supernatant was then centrifuged at 11,500 rpm for 20 min and the pellet (crude membrane fraction) was suspended in isolation medium, frozen and stored at -20 °C. Purified BBM vesicles were prepared within a week. The crude membrane fraction was thawed, suspended in Buffer A (100 mM mannitol, 10 mM HEPES, pH 7.4) and centrifuged at 20,000 rpm for 20 min. The pellet was resuspended in Buffer A containing 10 mM MgCl , incubated on ice for 10 min and centrifuged at 6,000 rpm for 10 min and the resulting supernatant was centrifuged at 20,000 rpm for 20 min. The pellet was suspended in a minimal volume of Buffer A, divided into aliquots and stored in liquid nitrogen until further use. These membranes were enriched over the crude membrane fraction by 15- to 20-fold for alkaline phosphatase (BBM marker), exhibited a 2- to 5-fold reduction of succinic dehydrogenase (mitochondrial marker), and a 2-fold decrease in Na,K-ATPase (ALM marker; Silverman and Speight, 1986). ALM were isolated from homogenized kidney on a percoll density gradient (Pritchard and Renfro, 1983).

2. Purification and Solubilization of BBM

The BBM vesicles were stripped of peripheral membrane proteins by incubation in 10 vol. of 0.2 mM EDTA, 20 µg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.5, at 37 °C for 30 min. The mixture was centrifuged at 19,000 rpm in an SS-34 rotor for 20 min and the pellet was then extracted with 10 volumes 1 M KI, 7.5 mM sodium phosphate, pH 7.5, containing 20 µg/ml phenylmethylsulfonyl fluoride and 1mM dithiothreitol, at 37 °C for 30 min. The mixture was centrifuged at 19,000 rpm for 30 min, the pellet was washed with 5 mM sodium phosphate, pH 8.0 and suspended in a

minimal volume of 28.5 mM or 228 mM sodium citrate, pH 8.0. The KI-extracted membranes were then solubilized at a protein concentration of ~1 mg/ml in 1% $C_{12}E_8$ in 28.5 mM or 228 mM sodium citrate buffer. Following a 20 min incubation on ice, the solubilized membranes were spun at 19,000 rpm for 30 min and the supernatant was stored at 4 °C. This supernatant (KI-E) was used for the binding studies.

3. Affinity Purification

Protein binding to the SITS-Affi-Gel 102 was carried out essentially as described in Chapter II (Pimplikar and Reithmeier, 1986) except that all steps were performed at room temperature unless stated otherwise. The chromatography was carried out in 228 mM sodium citrate, pH 8.0, containing 0.1% $C_{12}E_8$. In a typical assay 50 µl affinity resin in a microfuge tube was shaken with up to 1 ml KI-E sample (~1 mg protein/ml) for 30 min. The resin was allowed to settle under gravity, the supernatant was removed and the resin was washed three times with 500 µl 228 mM sodium citrate, pH 8.0 containing 0.1% $C_{12}E_8$. The bound material was then eluted by shaking the resin for 10 min with 110 µl of 1 mM BADS in 5 mM sodium phosphate buffer containing 0.1%$C_{12}E_8$. Finally, the resin was washed twice with 500 µl 228 mM sodium citrate, pH 8.0, containing 0.1% $C_{12}E_8$ and then extracted for 10 min with 110 µl of 1% lithium dodecyl sulfate in 5 mM sodium phosphate, pH 8.0. Equal volumes of the BADS eluate and lithium dodecyl sulfate eluate were analyzed by SDS gel electrophoresis.

4. Labeling of Proteins with [^{3}H]-H_2DIDS

The solubilized proteins (KI-E) were incubated with up to 40 µM [^{3}H]-H_2DIDS at 37 °C for up to 20 min. Some samples were preincubated with 1 mM BADS at 37 °C for 15 min. At the end of the DIDS incubation the protein samples were boiled with 1 volume of Laemmli sample buffer and the proteins were resolved by SDS gel electrophoresis. The polyacrylamide gel was stained with Coomassie blue, shaken with Enhancer for 30 min at

room temperature, followed by water for 30 min, dried and exposed to Kodak XAR-5 film at -70 °C.

5. Electroblotting Procedure

The proteins were separated by SDS gel electrophoresis according to Laemmli (1970) and were transferred electrophoretically to a nitrocellulose membrane using a Bio Rad Mini Transblot system. Blotting was performed in ice-cold transfer buffer (20%methanol, 192 mM glycine, 25 mM tris, pH 8.3) at 80-100 volts for 2 h. These membranes were then used either for lectin binding or antibody binding.

6. Lectin Blots

The nitrocellulose membrane was blocked with 10% ethanolamine, 0.25% gelatin, 100 mM Tris, pH 9.0, (Olmsted, 1981) for 2 h at room temperature and then washed for 2 h with Buffer 1 (0.25% gelatin, 50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40). Blots were incubated with the biotinylated lectins concanavalin A (Sigma Chemical Co.) and wheat germ agglutinin (Vector Laboratories) for 16-20 h at room temperature, washed with Buffer 1 for 2 h (3 washes) and then incubated with avidin-peroxidase conjugate (diluted 1:800 in Buffer 1) for 2 h. *Ricinus communis* agglutinin (Sigma Chemical Co., Type II) was conjugated directly to peroxidase. Blots were washed with Buffer 1 for 2 h (3 washes) and visualized by reaction with 0.05% diaminobenzedine, 0.1 % H_2O_2, 50 mM Tris-HCl, pH 7.5. Color development was usually allowed to proceed for 5 min at room temperature. Blots were rinsed with distilled water and air-dried.

7. Antibody Production and Affinity Purification

Dog erythrocyte ghost membranes were prepared and the proteins were separated on a 10% Laemmli gel. The Band 3 region was excised and subjected to electroelution (Hunkapiller, *et al.*, 1983). Band 3 thus obtained was dialysed extensively against

150 mM NaCl, 5 mM sodium phosphate, pH 7.4, to remove as much SDS as possible. The protein was emulsified with complete Freund's adjuvant and injected subcutaneously into a white New Zealand rabbit in four locations. Booster injections containing incomplete Freund's adjuvant were administered 21 and 28 days after the initial injection. Blood was collected 7 days after the final booster injection. Affinity purification of antibodies was performed using Band 3 protein transferred to a nitrocellulose membrane (Cox *et al.*, 1985; Olmsted, 1981).

8. Antibody Blots

The nitrocellulose membrane was incubated with 3% Carnation Skim Milk Powder in 137 mM NaCl, 27 mM KCl, 10 mM sodium phosphate, pH 7.4, 0.02% sodium azide (PBS) for 2 h at room temperature and rinsed briefly with antibody buffer (0.05% NP-40, 0,25% bovine serum albumin, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4). The nitrocellulose membrane was then shaken with anti-Band 3 antibodies (1:500 dilution in antibody buffer) at room temperature for 16-20 h. Following a 30 min wash (at least 4 changes) with PBS containing 0.05% Tween 20, the membrane was rinsed with antibody buffer and incubated with alkaline phosphatase conjugated anti-rabbit IgG (1:1000 dilution in antibody buffer) for 1 h. The membrane was then washed 4 times with PBS containing Tween-20, twice with PBS and briefly rinsed with 0.1 M Tris-HCl, pH 9.2. Alkaline phosphatase activity was detected using the substrate 5-bromo-4-chloroindoxyl phosphate and nitro blue tetrazolium using 0.1 M Tris-HCl, pH 9.2. The reaction was continued at room temperature for 30 min or until the desired intensity was obtained. Blots were rinsed with distilled water and air-dried.

9. Endo-β-N-acetylglucosaminidase F Treatment of Solubilized Proteins

The solubilized membrane proteins (50-100 μg) or affinity purified 130-kDa protein (5-20 μg) was incubated with 0.5 U of endo-β-N-acetylglucosaminidase F for 16-

20 h at room temperature. The incubation buffer contained 28.5 mM sodium citrate, 1 mM EDTA, 0.1% 2-mercaptoethanol and the pH was adjusted to ~6.8 before the addition of the enzyme. The reaction was carried out in 50 µl volumes and at the end of the incubation 50 µl of Laemmli sample buffer was added and the samples were boiled for 3 min. The digested proteins were analyzed by sodium dodecyl sulfate gel electrophoresis to determine their apparent molecular weights. Control incubations were carried out with the addition of water instead of the enzyme solution.

10. Endo-β-N-acetylglucosaminidase F Treatment of the BBM vesicles

The orientation of the oligosaccharide chains of the 130-kDa protein in BBM vesicles was determined by studying the accessiblity of the glycosylation site(s) to the endo-β-N-acetylglucosaminidase F. The experimental conditions were the same as described above except that the reaction was carried out in Buffer A (100 mM mannitol, 10 mM HEPES buffer at pH ~6.8).

11. Endo-β-N-acetylglucosaminidase H Treatment of the 130-kDa Protein

The experimental conditions used were identical to those used in endo-β-N-acetylglucosaminidase F treatment except that endo-β-N-acetylglucosaminidase H was used at a final concentration of 50 mU/ml.

12. Protease Treatment of the BBM vesicles

The BBM vesicles were washed at least twice in Buffer A, pH 8.0, and suspended in the same buffer at a protein concentration of ~1 mg/ml. They were treated with either chymotrypsin (1 mg/ml) or proteinase K (1 mg/ml) or trypsin (0.5 mg/ml) at 37 °C for 60 min. The reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride. Identical incubations were carried out in presence of 0.1% $C_{12}E_8$ in order to break the membrane barrier to make the internal sites accessible to the proteases. Control incubations were

carried out with Buffer A without the proteases. Purified 130-kDa glycoprotein in 0.1% $C_{12}E_8$, was treated with trypsin or chymotrypsin under similar conditions. The digested proteins were then analyzed by SDS gel electrophoresis.

13. Amino Acid Analysis

Protein samples were hydrolyzed in 6 N HCl at 110 °C for 24 h under vacuum and the released amino acids were quantitated using a Beckman Amino Acid Analyzer.

14. Analytical techniques

Protein assay was according to Lowry *et al.*(1951). Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (1970). Protein bands were stained with Coomassie Blue and scanned using a Joyce-Loebl Chromoscan 3 densitometer at 530 nm. The molecular weight markers used included β-galactosidase (116 kDa), phosphorylase-B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20 kDa).

C. RESULTS

1. Affinity Chromatography of BBM, ALM and Ghost Membrane Extracts

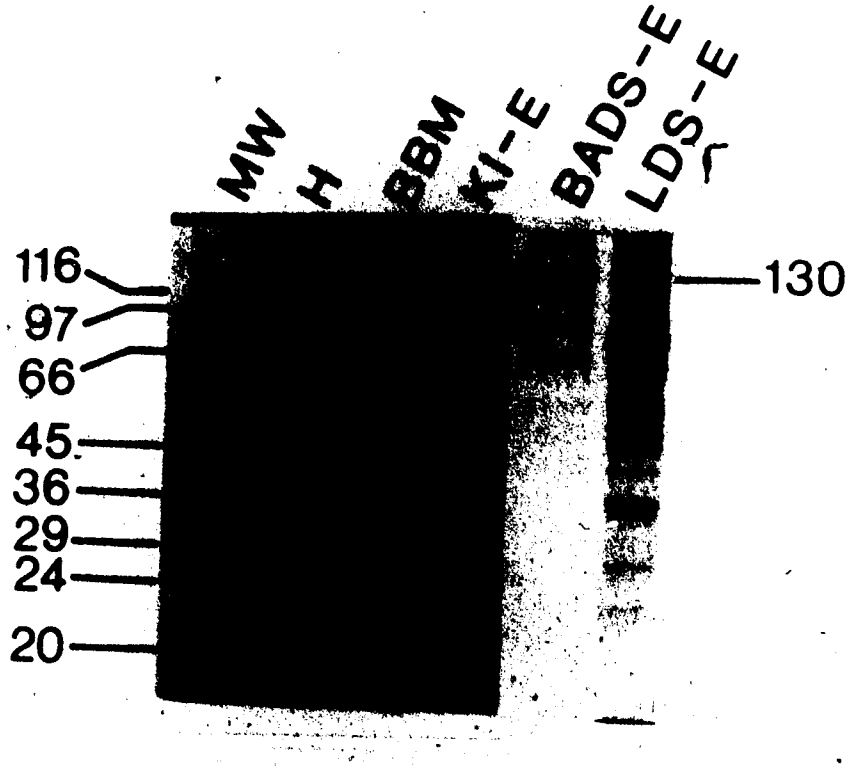
A typical SDS polyacrylamide gel showing the initial fractionation of renal brush border membrane vesicles and the purification of a 130-kDa glycoprotein by affinity chromatography is shown in Fig. V.1a. Lane H shows the protein profile of kidney cortex homogenate. BBM vesicles (Fig. V.1a, lane BBM) have a different protein profile, showing that during the isolation of BBM from the homogenate certain proteins are lost whereas other proteins are enriched. It is apparent that the BBM contains a complex mixture of polypeptides that range in molecular weight from over 100,000 to under

Fig. V.1a

Protein profile of kidney membrane proteins and purification of the 130-kDa protein. SDS gel electrophoresis was performed on a 10% gel prepared according to the procedure of Laemmli. MW, molecular weight markers; H, kidney cortex homogenate; BBM, brush border membranes prepared from the homogenate; KI-E, brush border membranes following EDTA and KI extraction; BADS-E, protein fraction eluted from SITS-Affi-Gel 102 resin by 1 mM BADS; LDS-E, protein fraction subsequently eluted by 1% LDS. The experiment was performed as described under "Materials and Methods".

Fig. V.1b

Interaction of kidney anti-luminal membranes (ALM) and dog ghost membranes (Gh) with SITS-Affi-Gel 102 resin. SDS gel electrophoresis was performed on a 10% gel prepared according to the procedure of Laemmli. Solubilized ALM and KI-extracted dog ghost membranes were incubated with the affinity resin, washed and eluted as described for Fig. V.1a S, samples of solubilized ALM or ghost membranes that were incubated with theresin; B, protein fraction eluted from SITS-Affi-Gel 102 resin by 1 mM BADS; L, protein fraction subsequently eluted by 1% lithium dodecyl sulfate. Arrow indicates the position of erythrocyte Band 3.


MW
H
BBM
KI-E
BADS-E
LDS-E
116
97
66
45
36
29
24
20
130

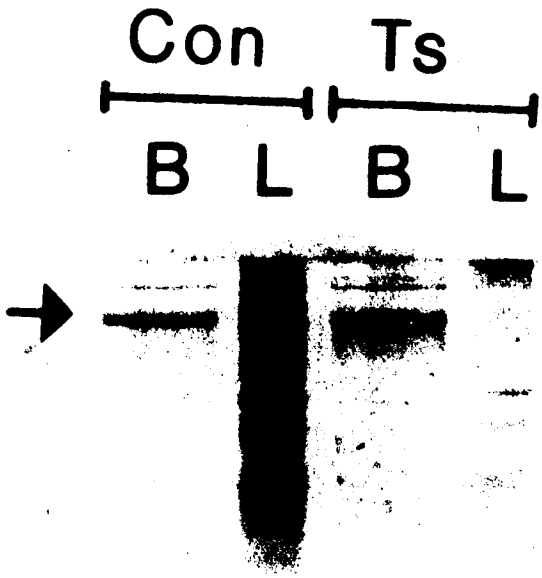

ALM
Gh
S
B
L
S
B
L

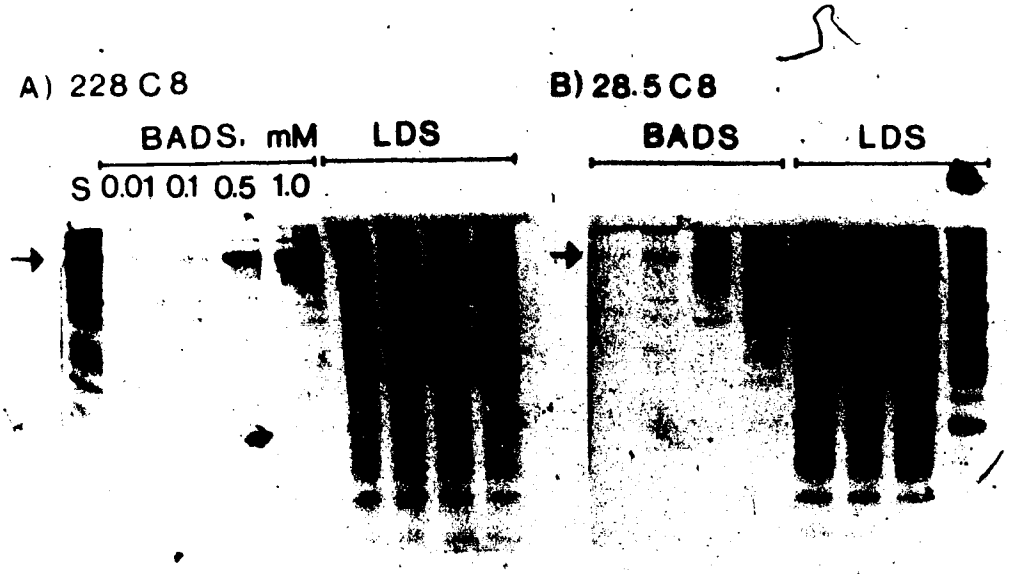
Fig. V.2

Effect of temperature shift on the elution of 130-kDa protein from the SITS-Affi-Gel 102 resin. KI-E sample was incubated with the affinity resin at 4 °C for 30 min. After removing the unbound proteins with at least three washes of 228 mM sodium citrate, pH 8.0 containing 0.1% $C_{12}E_8$, the resin-bound protein was incubated either at 4 °C (Con) or at 37 °C (Ts) for 30 min. At the end of the incubation both samples were left at 4 °C for 2 min and eluted with 1 mM BADS in 0.1 % $C_{12}E_8$, 5 mM sodium phosphate, pH8.0 (B) followed by 1% lithium dodecyl sulfate in 5 mM sodium phosphate, pH 8.0 (L). Except for the 30 min incubation at 37 °C, all other steps were performed at 4 °C. The arrow indicates the position of the 130-kDa protein.

Fig. V.3

Protein fractions eluted from SITS-Affi-Gel 102 resin. A) 228C8-Protein binding was performed at high ionic strength (228 mM sodium citrate, pH 8.0) as described under "Materials and Methods". The bound proteins were eluted with 0.01, 0.1, 0.5 or 1.0 mM BADS in 0.1 % $C_{12}E_8$, 5 mM sodium phosphate, pH 8.0 (4 lanes under BADS). The affinity resin was finally eluted with 1% LDS in 5 mM sodium phosphate, pH 8.0. The four lanes under LDS correspond to the subsequent LDS elution of resin following BADS elution. The arrow shows the 130-kDa protein. S, KI-extract of BBM. B) 28.5C8-Protein binding was performed at moderate ionic strength (28.5 mM sodium citrate, pH 8.0). Details of lanes (BADS and LDS) are as given for A) 228C8.


Con
Ts
B
L
B
L


A) 228 C 8
BADS. mM
LDS
S 0.01 0.1 0.5 1.0
B) 28.5 C 8
BADS
LDS

20,000. By extracting BBM vesicles with 0.2 mM EDTA followed by 1 M KI (Bennett, 1983), weakly associated and extrinsic proteins can be separated from intrinsic membrane proteins (Fig. V.1a, lane KI-E). The protein components of the EDTA and KI-extracted BBM vesicles could be solubilized by 1% $C_{12}E_8$ in 228 mM citrate buffer, pH 8.0. The proteins contained in the detergent extract may be classified as intrinsic membrane proteins since they were not eluted from the membrane by low or high ionic strength extractions but they could be solubilized with the aid of a mild non-ionic detergent ($C_{12}E_8$).

When the solubilized proteins were incubated with SITS-Affi-Gel 102 resin, a 130-kDa protein bound to the resin and it was the predominent (>90% by densitometry) polypeptide eluted by 1 mM BADS (Fig. V.1a, lane BADS-E). The 130-kDa protein binds to the affinity resin only when the ligand SITS is coupled to Affi-Gel 102. This protein did not bind to the Affi-Gel 102 resin without the attached ligand SITS (data not shown).

Some minor polypeptides with molecular weights of 160,000, 110,000, 90,000 and 65,000 were also eluted from the affinity resin by BADS. The amount of these contaminating polypeptides varied, however, in various BADS elutes (for comparison see Fig. V.1a, Fig. V.2 and Fig. V.3). The resin that had been eluted with BADS was subsequently incubated with 1% lithium dodecyl sulfate to elute non-specifically bound proteins (Fig. V.1a, lane LDS-E). The proteins in this fraction represent non-specifically bound proteins or proteins that have a high affinity for the immobilized ligand and are not readily eluted by BADS (see Chapter II). Little of the 130-kDa protein was left associated with the affinity resin after the BADS elution (Fig. V.1a, lane LDS-E). The 130-kDa protein therefore bound to the affinity resin and could be eluted effectively by 1 mM BADS at room temperature.

The possible presence of a stilbene disulfonate-binding protein in kidney ALM was examined by affinity chromatography (Fig. V.1b). No single polypeptide from a $C_{12}E_8$ extract of ALM proteins (Fig. V.1b, lane S, under ALM), when incubated with the affinity

resin, was purified by the BADS elution (Fig.1b, lane B under ALM). A band migrating at 130-kDa was present in variable amounts in the BADS and lithium dodecyl sulfate eluates of the ALM preparations but the presence of this polypeptide was likely due to contamination by BBM. The one or more of the ALM proteins bound to the affinity matrix and eluted by BADS may represent anion transport proteins present in the ALM.

As a control experiment, solubilized dog erythrocyte ghost membranes (Fig 1b, lane S, under Gh) were incubated with the affinity resin under identical conditions. The Band 3 protein was the predominant absorbed protein that was eluted from the resin by 1 mM BADS (Fig. V.1b, lane B under Gh). Substantial Band 3 was eluted by a subsequent incubation of the resin with lithium dodecyl sulfate (lane L under Gh). We have previously noted that human Band 3 binds to the affinity resin in two states. At low temperature (ie. 0-4 °C) Band 3, bound to the affinity resin, can be eluted by BADS (Pimplikar and Reithmeier, 1986). If the matrix-bound protein is incubated at room temperature or 37 °C, Band 3 is converted to a form that is not elutable by BADS but the protein can be removed from the resin by denaturation with dodecyl sulfate. This property of human Band 3 was also exhibited by canine Band 3 in these experiments. The tight binding of canine Band 3 to the affinity resin was expected since the binding and elution were carried out at room temperature.

The effect of temperature on the binding and subsequent elution of the130-kDa protein was determined. Incubating the KI extract with the affinity resin at 4 °C, 22 °C or 37 °C did not affect binding and the subsequent elution of the 130-kDa polypeptide by BADS (data not shown).The 130-kDa protein was allowed to bind to the resin, free protein was removed and the bound protein was incubated at 4 °C or 37 °C. Erythrocyte Band 3 after the temperature shift to 37 °C is no longer elutable by BADS (Pimplikar and Reithmeier, 1986). Fig. V.2 shows that when the 130-kDa protein bound to the SITS-Affi-Gel 102 resin was incubated at 37 °C for 30 min (Lane B, under Ts), it could still be

eluted by BADS. This shows that unlike erythrocyte Band 3 protein the 130-kDa protein does not acquire the tight binding conformation by incubation at 37 °C.

2. Interaction of the 130-kDa Protein with the Affinity Resin

In order to optimize the binding and elution of the 130-kDa protein, the effect of ionic strength, BADS concentration, time of incubation, and the specificity of the interaction were examined.

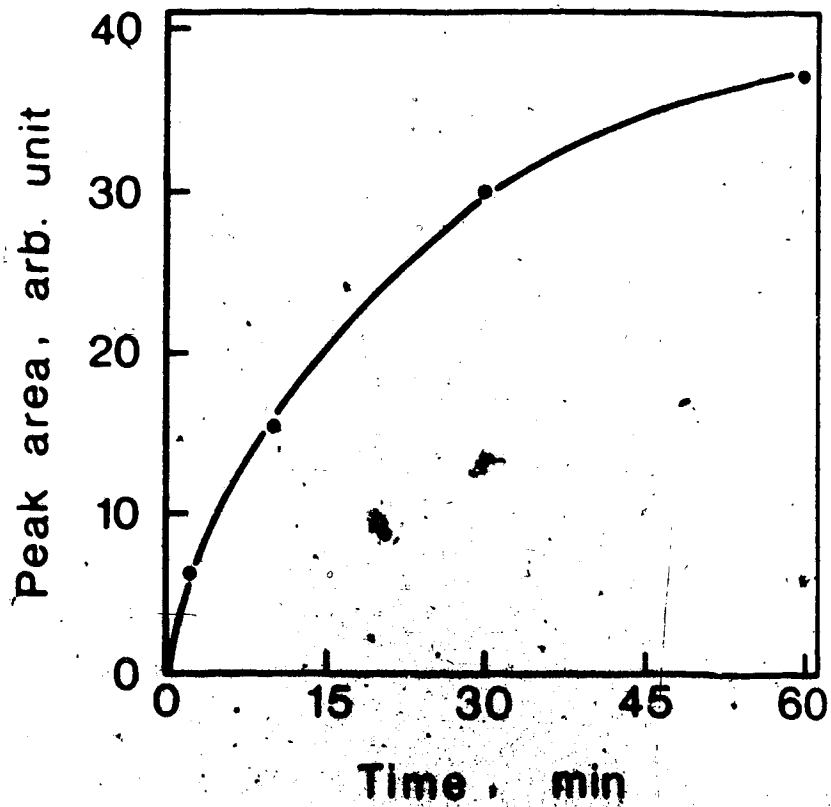
The binding of the protein was performed either at moderate ionic concentrations (1 % $C_{12}E_8$, 28.5 mM sodium citrate, pH 8.0) or at high ionic concentrations (1 % $C_{12}E_8$, 228 mM sodium citrate, pH 8.0). Upon removing unbound protein by washing with respective buffers, the bound protein was eluted with increasing amounts of BADS in 0.1% $C_{12}E_8$, 5 mM sodium phosphate, pH 8.0. The amount of 130-kDa protein eluted increased with higher concentrations of BADS at both moderate and high ionic strength (Fig. V.3). It should be noted that at high ionic strength the amount of contaminating proteins in these fractions remained constant, thus showing that the 130-kDa is eluted specifically by BADS under these conditions (see Fig. V.3 panel A, 228C8, four lanes under BADS). When binding was performed at moderate ionic strength, the subsequent BADS elute contained relatively higher amounts of contaminating proteins and lower amounts of the 130 kDa protein (Fig. V.3 panel B, 28.5C8; four lanes under BADS). The fraction of 130-kDa protein not eluted by low amounts of BADS was present in the subsequent lithium dodecyl sulfate elutes (Fig. V.3, four lanes under LDS). 1 mM BADS eluted the 130-kDa protein effectively since the subsequent dodecyl sulfate eluant contained little of this polypeptide. If the incubation of the solubilized BBM membrane with the resin was carried out in low ionic strength (1% $C_{12}E_8$, 5 mM sodium phosphate, pH 8.0), no binding of the 130-kDa protein was observed (data not shown). The binding of the 130-kDa protein is ionic-strength dependent and the bound protein can be optimally eluted by 1 mM BADS.

Fig. V.4

Time course of protein binding to SITS-Affi-Gel 102 resin. KI-E sample was incubated with the resin as described under "Materials and Methods" for varied lengths of time. Samples eluted with 1 mM BADS in 0.1 % $C_{12}E_8$, 5 mM sodium phosphate, pH 8.0 were electrophoresed on a 10% Laemmli gel, stained with Coomassie Blue and scanned on a Joyce Loebl Chromoscan 3 densitometer at 540 nm. The area under the peak corresponding to the 130-kDa protein is defined as amount of protein bound to the resin.

Fig. V.5

Specificity of 130-kDa protein binding to SITS-Affi-Gel 102 resin. Protein binding assay was performed as deseribed under "Materials and Methods". Top panel shows the effect of presence of free ligand on the protein binding. KI-E, KI extract of BBM used for binding experiments; BADS-E, the three lanes show protein fraction eluted with 1mM BADS when the binding was performed in the presence of 0, 0.1 or 1.0 mM BADS respectively; LDS-E, subsequent respective 1% lithium dodecyl sulfate elutes; MW, molecular weight markers. Bottom panel shows the effect of DIDS prelabelling on the protein binding. BADS-E, the six lanes show protein fractions eluted with 1 mM BADS when the binding was performed with KI-E sample pre-labelled with 0, 2.5, 5, 10, 50 or 100 µM DIDS. DIDS prelabelling was performed at 37 °C for 15 min. Subsequent 1% LDS elute is not shown. KI-E*, KI extract BBM labelled with 100 µM DIDS.


Peak area, arb. unit
40
30
20
10
0
0
15
30
45
60
Time, min

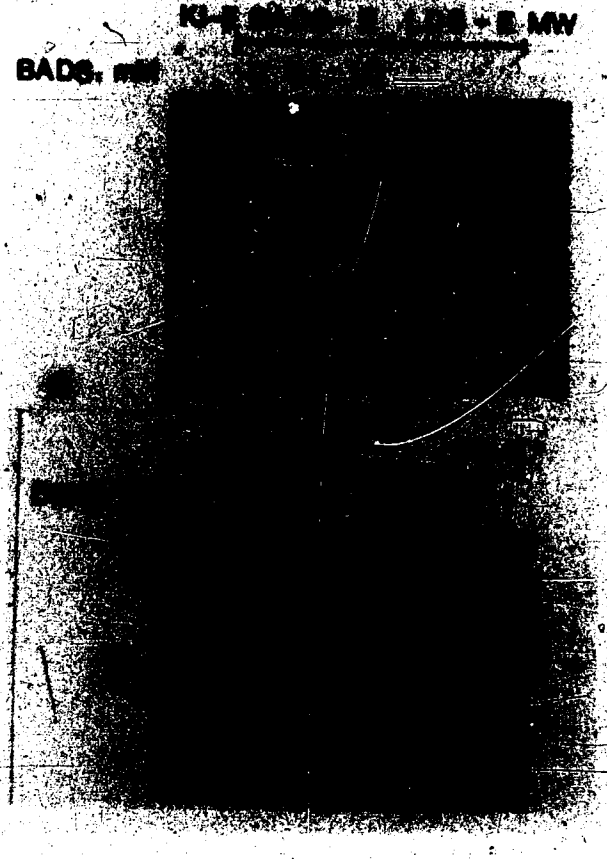


Fig. V.4 shows the time course of the binding of the 130-kDa protein to the affinity resin. The solubilized BBM was incubated with the resin for up to 1 h. The resin was washed with 228 mM citrate buffer, pH 8.0 containing 0.1% $C_{12}E_8$ to remove unbound proteins and the 130-kDa protein was eluted with BADS. The amount of the 130-kDa protein bound to the resin was quantitated by resolving the BADS eluants by SDS gel electrophoresis and scanning the Coomassie Blue-stained gels. The amount of protein bound to the affinity matrix at room temperature increased with time and by 1 h was close to reaching maximum. In the subsequent experiments binding was performed for 30 min.

In the previous sections we have clearly shown that the 130-kDa protein found in renal BBM binds to a stilbene disulfonate affinity column and it can be eluted selectively and quantitatively by BADS. If this protein contains a stilbene disulfonate binding site like erythrocyte Band 3, then the binding of the 130-kDa protein to the affinity matrix should be blocked by free BADS. Fig. V.5 shows that this is the case. In the presence of 0.1 mM BADS, the amount of 130-kDa protein bound to the resin was reduced whereas in the presence of 1 mM BADS the binding was almost completely prevented (Fig. V.5, top panel). The subsequent lithium dodecyl sulfate eluants also lacked the 130-kDa protein but other non-specifically bound proteins were present. This shows that the 130-kDa protein bound the affinity resin specifically via its stilbene disulfonate binding site.

DIDS is a stilbene disulfonate derivative that can irreversibly inhibit anion transport in erythrocytes by binding to the stilbene disulfonate site in Band 3 and then reacting with either one or two lysine residues in the protein (Passow, 1986). When the KI extract was prelabelled with increasing amounts of DIDS, the binding of the 130-kDa protein was totally abolished at a DIDS concentration of 50 μM (Fig. V.5, bottom panel). The other minor contaminating proteins, however, still bound and eluted, showing that the 130-kDa protein interacted with resin specifically through the stilbene disulfonate binding site. Again, the subsequent lithium dodecyl sulfate elutes did not contain the 130-kDa protein (data not shown).

Fig. V.6

Labelling of KI-E with [^{3}H]-H_2DIDS. The experiment was performed as described under "Materials and Methods" with 40 μM [^{3}H]-H_2DIDS in 228 mM sodium citrate, pH 8.0, containing 1% $C_{12}E_8$ for 2, 10 or 20 min at 37 °C either in absence (-) or presence (+) of 1 mM BADS. At the end of incubation the samples were boiled in sample buffer for 3 min and electrophoresed on a 10% Laemmli gel. A) CB, Coomassie Blue stained gel; B) Fluoro, fluorogram of the same gel. The arrows show the position of the 130-kDa protein. M, molecular weight markers.

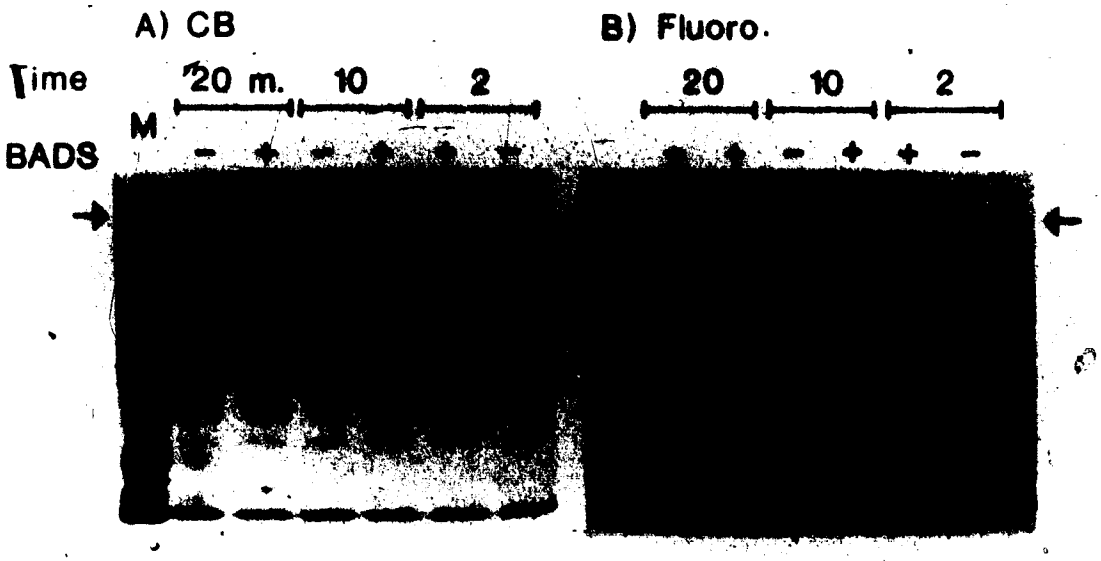

A) CB
B) Fluoro.
Time
20 m.
10
2
20
10
2
M
BADS
- + - + + +
+ + - + + -

Fig. V.7

Effect of endo-β-N-acetylglucosaminidase treatments on the 130-kDa protein. The enzyme treatment was carried out as described under "Materials and Methods". BBM, intact brush border membrane vesicles; KI-E, EDTA and KI extracted BBM solubilized in 1% $C_{12}E_8$, BADS-E, affinity purified 130-kDa protein isolated by BADS elution from SITS-Affi-Gel 102 column; - and + denotes absence or presence of endo-β-N-acetylglucosaminidase F during incubation; * denotes sample treated with endo-β-N-acetylglucosaminidase H; MW, molecular weight markers. The arrow shows the position of the 130-kDa glycoprotein.

Fig. V.8

Lectin blots. Affinity purified 130-kDa protein (BADS-E) was electrophoresed on a 10% Laemmli gel and transferred to a nitrocellulose membrane. WGA- as described under "Materials and Methods" the membranes were incubated with biotinylated wheat germ agglutinin (WGA) followed by avidin-peroxidase conjugate. The membranes were visualized with diaminobenzedine. C, control untreated affinity purified 130 kDa glycoprotein; F, endo-β-N-acetylglucosaminidase F treated affinity purified 130 kDa glycoprotein; H, endo-β-N-acetylglucosaminidase H treated affinity purified 130 kDa glycoprotein. Con A- the membranes were incubated with biotinylated Concanavalin A (Con A) and developed as above. RCA- the membranes were incubated with peroxidase conjugated *Ricinus communis* agglutinin (RCA) and binding was visualized with diaminobenzedine.

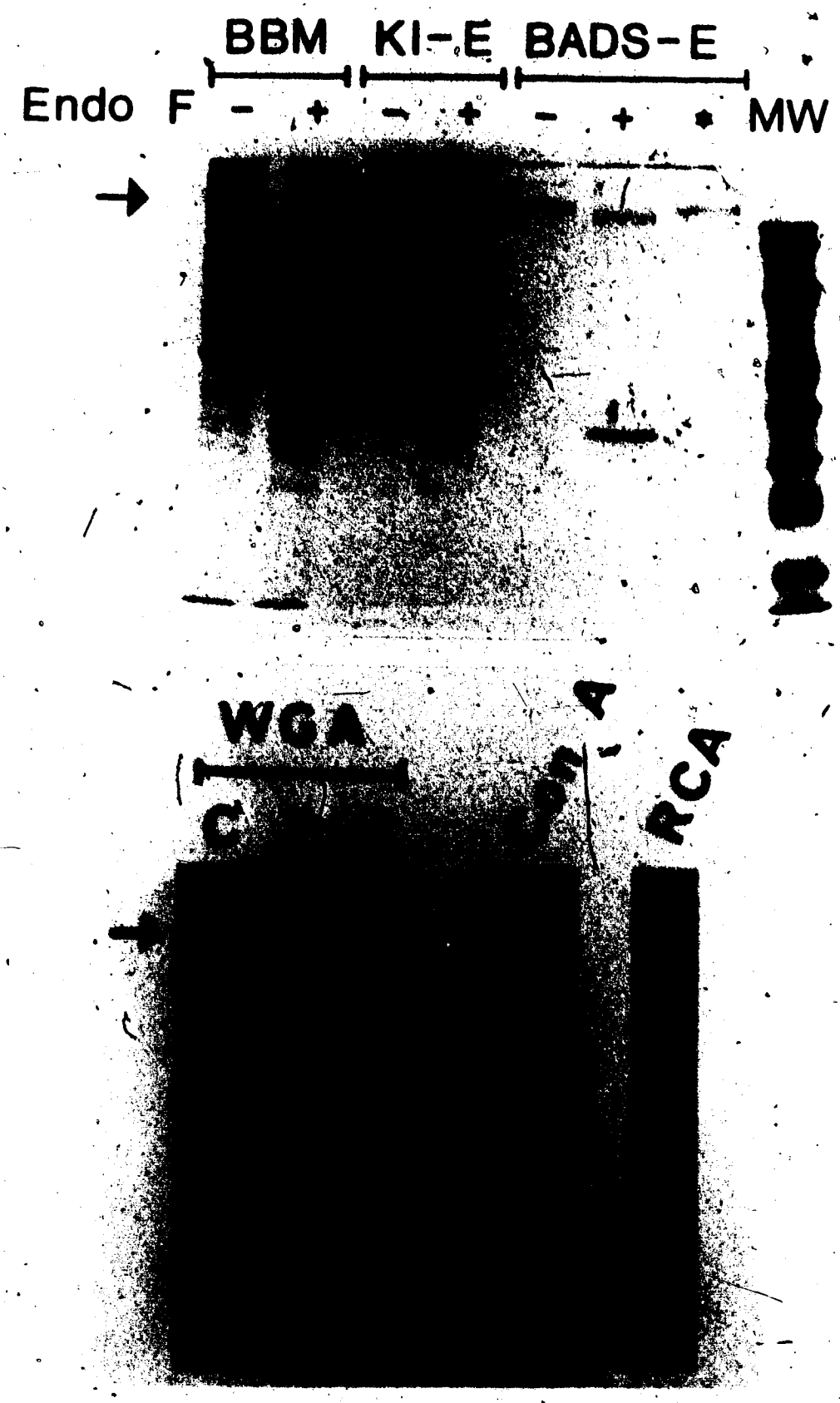
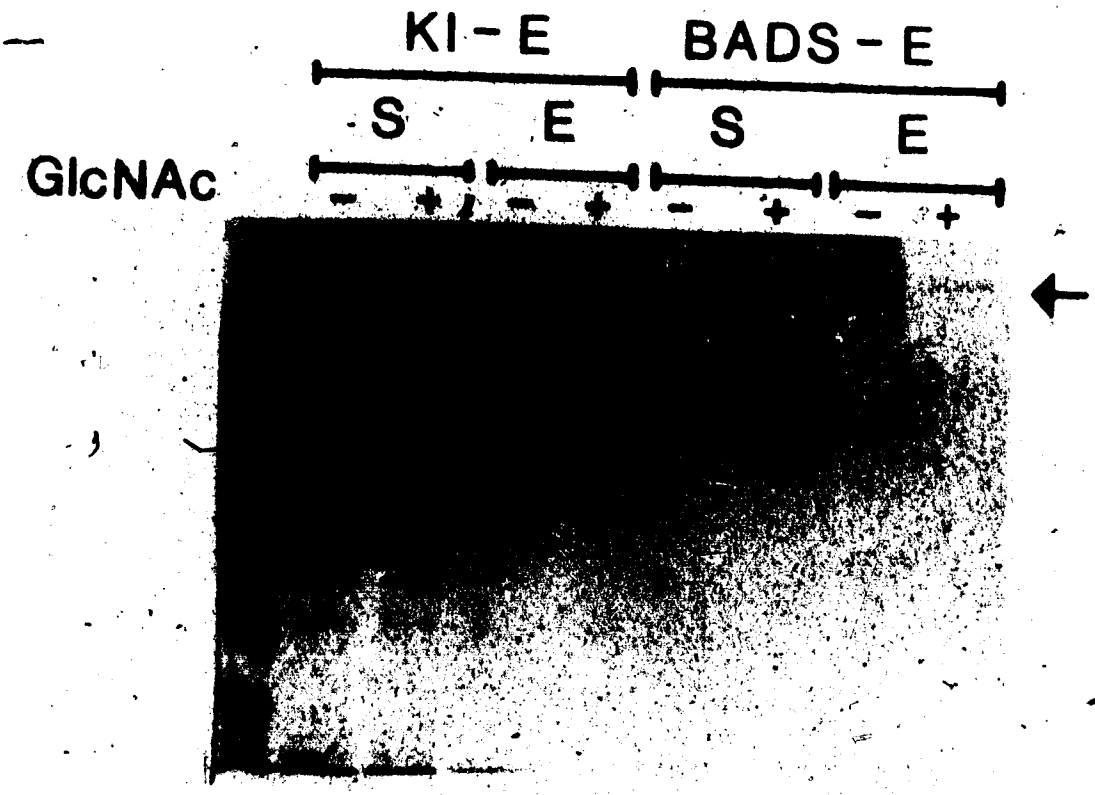

BBM
KI-E
BADS-E
Endo F
- + - + - + *
MW
WGA
RCA

Fig. V.9

Binding of 130-kDa glycoprotein to wheat germ agglutinin-agarose. The binding was performed as described under "Materials and Methods". KI-E, EDTA and KI-extracted BBM solubilized in 1% $C_{12}E_8$, 28.5 mM sodium citrate, pH 8.0 sample was used for the binding studies. The binding was performed either in absence (-) or presence (+) of 0.1 M GlcNAc. S, protein fraction left in supernatant after incubation with WGA-agarose; E, protein fraction eluted with 0.1 M GlcNAc. BADS-E, affinity purified 130-kDa glycoprotein was used for the binding studies. Details of lanes are as given for KI-E.


KI-E
BADS-E
S
E
S
E
GlcNAc
- + - + - + - +

3. [^{3}H]-H_2DIDS labelling

Since DIDS prevents the binding of the 130-kDa protein to the affinity matrix this reagent may be useful for labeling the protein if a reactive amino acid residue is present in the appropriate position in the stilbene disulfonate binding site. The KI extract was labelled with 40 μM [^{3}H]-H_2DIDS at 37 °C for 2, 10 and 20 min in 228 mM citrate buffer pH 8.0, 0.1% $C_{12}E_8$ (Fig. V.6). The fluorogram on the right shows that the 130-kDa protein, together with some other polypeptides, are labeled in a time-dependent manner by this reagent. The Coomassie Blue-stained gel in Fig. V.6 shows that DIDS labelling up to 20 min did not cause any major aggregation of the proteins in the KI extract. The radioactivity at the dye front is likely due to free reagent, labelled phospholipids or small peptides. The specificity of DIDS labeling of the 130-kDa protein was checked by performing the experiment in the presence of BADS. In the presence of 1 mM BADS, [^{3}H]-H_2DIDS labelling of the 130-kDa protein but not of the other polypeptides was prevented. When the binding was performed at pH 6 the 130 kDa protein did not label with [^{3}H]-H_2DIDS (data not shown). This shows that the 130 kDa protein has a specific binding site for stilbene disulfonates and secondly that an amino acid (probably lysine) is present at or near this binding site and can form a covalent bond with DIDS. However, lack of covalent binding at pH 6 suggests that, unlike erythrocyte Band 3 that has a reactive lysine in or near the stilbene disulfonate binding site in the protein, the 130-kDa protein does not have such a reactive residue.

4. Glycosidase Treatment of the 130-kDa Protein

Treatment of intact BBM vesicles, the KI extract and affinity-purified 130-kDa protein with endo-β-N-acetylglucosaminidase F resulted in the removal of carbohydrate as indicated by the change in the electrophoretic mobility of the protein (Fig. V.7). In both the BBM vesicles and the KI extract, other proteins were not affected by the enzyme

treatment. Following endo-β-N-acetylglucosaminidase F treatment, the protein had an apparent molecular weight of 120,000. The 130-kDa glycoprotein was resistant to endo-β-N-acetylglucosaminidase H treatment (Fig. V.7). These experiments show that the 130 kDa protein is a glycoprotein and suggest that sugars are not high-mannose type but rather complex-type. The fact that the carbohydrate component of the 130-kDa protein is accessible to endo-β-N-acetylglucosaminidase F in intact BBM vesicles indicates that the carbohydrate is on the outside of the vesicles.

5. Lectin Blots

In order to confirm the glycoprotein nature of the 130-kDa protein, the ability of the protein to bind various lectins was determined. Affinity-purified 130-kDa glycoprotein was transferred to nitrocellulose paper and incubated with various biotinylated lectins. This glycoprotein bound wheat germ agglutinin, concanavalin A as well as *Ricinus communis* agglutinin (Fig. V.8). The 130-kDa glycoprotein still bound wheat germ agglutinin after endo-β-N-acetylglucosaminidase F or H treatments (Fig. V.8). This may be due to incomplete cleavage of the carbohydrate since not all of the carbohydrate chains are N-linked but some could be attached to the protein through O-linkages. Treatment of the purified 130-kDa glycoprotein with neuraminidase also resulted in slightly faster migration of the protein on SDS polyacrylamide gels . The asialoglycoprotein also retained its ability to bind wheat germ agglutinin, concanavalin A and *Ricinus communis* lectins .

6. Interaction of the 130-kDa Glycoprotein with wheat germ agglutinin-agarose

In order to study the functional role of the 130-kDa protein and to see if it could be purified without using BADS, the interaction of the 130-kDa protein with wheat germ agglutinin-agarose was studied. The KI extract or the affinity-purified 130-kDa protein were incubated with wheat germ agglutinin-agarose at room temperature for 20 min. After.

washing away the unbound protein, the bound protein was eluted with 0.1 M GlcNAc. Fig. V.9 shows when the KI-extract was incubated with wheat germ agglutinin-agarose resin and subsequently eluted with GlcNAc, the 130-kDa glycoprotein was the predominent eluted protein (lane E- under KI-E). When the binding was done in the presence of 0.1 M GlcNAc, much less of the 130-kDa glycoprotein bound and was subsequently eluted from the resin (lane E+ under KI-E). When a limiting amount of affinity-purified 130-kDa glycoprotein was incubated with the resin, 0.1 M GlcNAc completly prevented the protein binding to the resin (lanes S- vs S+ under BADS-E). In the absence of excess free ligand, however, the 130-kDa glycoprotein bound to the wheat germ agglutinin-agarose resin and was eluted with 0.1 M GlcNAc (lane E- under BADS-E).

7. Protease Treatments

In order to determine the accessibility of the protein domains within the 130-kDa glycoprotein in the BBM, vesicles were treated with chymotrypsin, trypsin or proteinase K. Treatment of the intact BBM vesicles with chymotrypsin resulted in a total breakdown of proteins other than the 130-kDa polypeptide (Fig. V.10). The 130-kDa protein, however, now migrated with an apparent molecular weight of ~122,000, indicating that chymotrypsin cleaved ~8,000 daltons of peptide sequence from the protein. This chymotrypsin cleavage site must be on the exterior of the BBM, showing that a portion of the 130-kDa protein is exposed on the luminal side of the BBM membrane. This protein was trypsin resistant under the conditions used but was completely degraded by the non-specific protease proteinase K. Surprisingly, after permeabilizing the membrane with 0.1% $C_{12}E_8$ (Fig. V.10, +det), the proteolytic cleavage pattern did not change. This suggests that the resistance of the 130-kDa protein to trypsin and the limited cleavage by chymotrypsin was not due to the permeability barrier but must be a property of the solubilized protein itself. This conclusion was confirmed by treating purified 130-kDa

protein (Fig. V.10, BADS-E) with trypsin or chymotrypsin for 5, 15 and 60 min. Trypsin at 0.5 mg/ml at 37 °C (Fig. V.10, Trypsin) failed to digest the purified protein although removal of a small peptide can not be excluded. Chymoptrypsin treatment (1 mg/ml) of the 130-kDa protein again produced a 122-kDa fragment after 60 min. of digestion at 37 °C (Fig. V.10, Chymo).

8. Cross-Reaction With Anti-Band 3 Antibodies

BBM, ALM and dog ghost proteins were separated on a SDS polyacrylamide gel and then transferred to a nitrocellulose membrane. This membrane was probed either with antibodies raised against dog erythrocyte Band 3 protein, with affinity-purified anti-dog Band 3 antibodies, or with non-immune serum (Fig. V.11). The 130-kDa protein from the BBM vesicles did not cross react with the unpurified antibody whereas a 95,000 dalton protein from the ALM preparations cross reacted strongly (Fig. V.11). With the affinity-purified monospecific antibodies, the 130-kDa protein of the BBM again failed to cross-react whereas the 95-kDa ALM protein still cross reacted. Other minor bands from both BBM and ALM preparations that cross reacted with unpurified antibodies, did not cross react with the affinity purified antibodies. Dog erythrocyte Band 3 reacted strongly with both antibody preparations. Western blots using more dilute antibody or less ghost membrane protein showed that only erythrocyte Band 3 reacted with the antibody. The blot illustrated in Fig. V.11 was overdeveloped with respect to the erythrocyte Band 3 in order to detect any cross reacting protein in the BBM preparations. When probed with antibodies raised against human erythrocyte Band 3, the 95,000 dalton ALM protein but not the 130-kDa BBM protein cross reacted (data not shown).

9. Amino Acid Analysis

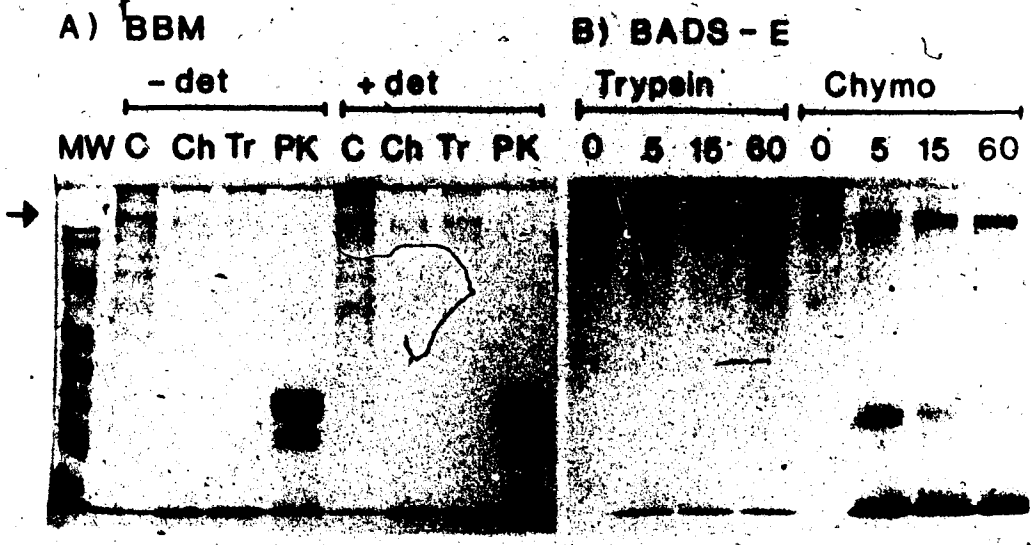
The 130-kDa glycoprotein was purified by affinity chromatography and electroelution of the band from SDS polyacrylamide gels. The amino acid composition of

Fig. V.10

Effect of protease treatment on the 130-kDa glycoprotein in sealed BBM vesicles, (A) BBM; or after purification by affinity chromatography, (B) BADS-E. BBM vesicles without detergent (-det) or with 0.1% $C_{12}E_8$ (+det) were incubated without any protease (C),or with chymotrypsin (Ch, 1 mg/ml), trypsin (Tr, 0.5 mg/ml) or proteinase K (PK, 1 mg/ml) for 1 h at 37 °C. Affinity-purified 130-kDa glycoprotein was treated with trypsin or chymotrypsin under similar conditions for 0, 5, 15, and 60 min. Digestions were stopped by addition of 0.2 mM phenylmethylsulfonyl fluoride and the proteins were resolved by electrophoresis on a 10% acrylamide gel. The arrow shows the position of the 130-kDa glycoprotein. MW, molecular weight markers as defined in Fig. V.1.

Fig. V.11

Antibody blots. B, BBM; A, ALM and G, dog ghost membrane proteins were electrophoresed on a 7.5 % Laemmli gel and transferred to a nitrocellulose membrane. The cross-reacting proteins were detected as described under "Materials and Methods". M, molecular weight markers.1) CB, Coomassie Blue stained gel; 2) Ab, blot incubated with anti-dog Band 3 antibodies; 3) MS-Ab, blot incubated with affinity purified monospecific anti-dog Band 3 antibodies; 4) NIS, blot incubated with nonimmune serum. The arrow shows the position of the 130-kDa glycoprotein from BBM.


A) BBM
B) BADS - E
- det
+ det
Trypsin
Chymo
MW C Ch Tr PK C Ch Tr PK
0 5 15 60 0 5 15 60

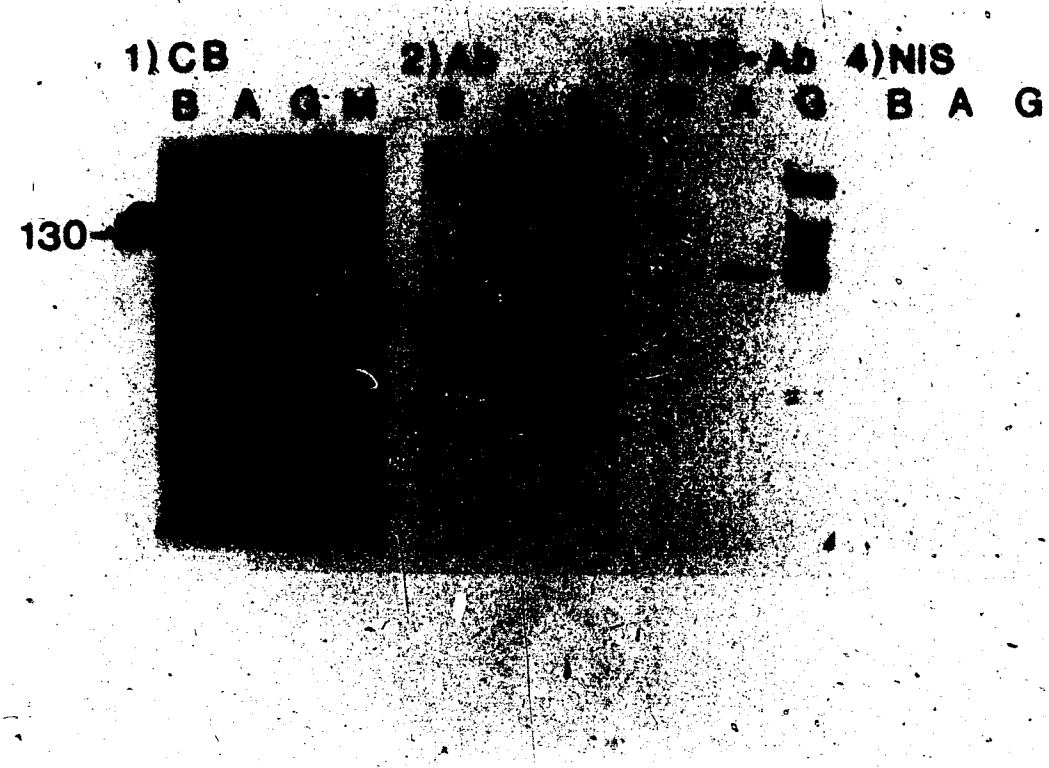

1) CB
4) NIS
B A G
B A G
130

Table V.1 Amino Acid Compositon of the 130-kDa Glycoprotein and Erythrocyte Band 3

Amino acid	130-kDa Glycoprotein	Band 3[a]
	Mol %	
Asp	12.7	6.5
Thr	6.8	5.0
Ser	9.0	6.8
Glu	11.2	12.0
Pro	4.4	5.7
Gly	8.2	7.7
Ala	8.1	7.4
Val	6.0	7.6
Met	0.7	2.2
Ile	4.0	4.7
Leu	9.4	13.4
Tyr	3.9	3.1
Phe	4.4	5.5
His	2.3	2.2
Lys	5.0	3.3
Arg	4.0	5.2
Cys	N.D[b].	0.7
Trp	N.D.	1.2

[a]Taken from Steck, T.L. et al. Biochem. 17, 1216-1222 (1978)
[b]N.D., not determined.

the 130-kDa protein is listed in Table I. This protein contains a moderate content (28 %) of hydrophobic amino acids (Val, Met, Ile, Leu, Tyr, Phe). Of particular note however is the high content (24 %) of acidic amino acid residues. The overall amino acid composition is similar to the composition of erythrocyte Band 3.

D. DISCUSSION

The renal transport systems that mediate excretion and reabsorption of various anions seem complex. Unlike the erythrocyte Band 3 protein which can utilize a number of anions as substrate, the renal transport systems seem specific for different anions. The transporters are less abundant, localized in various parts of a nephron and are functionally intertwined with each other. Due to these difficulties, the proteins mediating various transport functions have not been identified, isolated and biochemically characterized. Most of the information on these systems has been derived from studies involving a micropuncture technique, microperfusion of isolated nephrons or from transport studies using BBM and ALM vesicles and kidney epithelial cells grown in culture (for review see Kokko and Jacobson, 1985; Warnock and Eveloff, 1982).

These transport systems have been well characterized by physiological techniques, however, regarding their localization, substrate specificity, transport kinetics and various inhibitors that impair a given transport process. A number of studies have shown that stilbene disulfonates such as SITS and DIDS inhibit chloride/base exchanger systems (Spring and Kimura, 1978, Andreoli *et al.*, 1981). Studies have reported the presence of chloride/bicarbonate (Warnock and Eveloff, 1982), chloride/hydroxide (Warnock and Yee, 1981), and chloride/formate (Alpern, 1987) exchangers on the luminal side of tubules. Such findings are, however, not without contradictory reports (Seifter *et al.*, 1984; Schwartz, 1983). Recent studies have suggested the presence of DIDS-inhibitable chloride/bicarbonate exchangers in cell lines derived from kidney such as the MDCK cell line (Kurtz and Golchini, 1987) and Vero cell line (Madshus and Olsnes, 1987). The

presence of such exchange systems, inhibited by SITS or DIDS, in the ALM has also been implied (Pritchard and Renfro, 1983, Edelman *et al.*, 1981). Despite these conflicting reports, an overwhelming body of evidence (see Chapter 1, Renal Anion Exchange) supports the presence of a chloride/base exchanger in the brush border membranes of kidney tubules (Warnock and Eveloff, 1982).

The anion transport protein of erythrocytes contain a stilbene disulfonate binding site and this protein can be purified by affinity chromatography using SITS-Affi-Gel 102 resin (see Chapter 2). It is clear that there are a number of anion transport processes in kidney and that they are inhibited by stilbene disulfonates. These anion exchange proteins in luminal membranes contain a stilbene disulfonate binding site (Bastlein and Burckhardt, 1986) and they should bind specifically to the affinity resin. The present studies unambiguously show that a 130-kDa glycoprotein from the brush border membranes specifically binds stilbene disulfonates and that it can be purified using inhibitor affinity chromatography. We feel that this protein is an excellent candidate for the Cl^-/anion transporter present in renal luminal membranes.

Recent evidence has shown that specific DIDS binding sites are present in membranes isolated from the luminal (Kd = 19.3 μM, 31.7 nmol/mg protein) and the basolateral side (Kd = 5.5 μM, 10.9 nmol/mg protein) of epithelial cells from renal proximal tubules (Talor *et al.*, 1987). In the same study DIDS was found to inhibit electroneutral sulfate uptake into luminal vesicles (Ki = 20 μM). Pritchard (1987) also found that BBM vesicles take up sulfate (Km = 0.4 mM) in exchange for bicarbonate or chloride in the absence of Na^+ and that this process was inhibited by DIDS. Our studies have shown that 50 μM DIDS completely prevents binding of the 130-kDa protein to the affinity resin while 10 μM DIDS had no effect (Fig. V.5). This is consistent with the presence of a DIDS binding site in BBM with an affinity of 19.3 μM (Talor *et al.*, 1987). The number of sites (31.7 nmol/mg protein) in BBM vesicles as determined by Talor *et al.* is, however, too high to represent binding to a single site in a transport protein. DIDS is

apparently binding to multiple sites in these membranes even at 4 °C for short time periods. Our radiolabelling experiments (Fig. V.6) show that at 37 °C, DIDS labels many proteins in the BBM and only the 130-kDa protein is protected from labelling by free stilbene disulfonate.

The 130-kDa glycoprotein from the brush border membranes of canine kidney has some similarities with the human erythrocyte chloride/bicarbonate exchanger. Both proteins are major intrinsic glycoproteins of relatively high molecular weight. They both bind to SITS-Affi-Gel 102 affinity resin and can be labelled specifically with [^{3}H]-H_2DIDS. In addition, the amino acid composition of the 130-kDa protein is similar to the composition of erythrocyte Band 3.

The kidney 130-kDa glycoprotein and erythrocyte Band 3 are not similar in other ways. The kidney glycoprotein does not acquire a tight binding conformation to the affinity resin at higher temperatures and these two proteins are immunologically distinct. Since antibodies raised against Band 3 protein are generally directed towards the immunodominant amino-terminal domain which is not involved in the transport function, lack of antibody reactivity is not very surprising. Earlier studies using anti-Band 3 antibodies (Drenckhahn *et al.*, 1985, Cox *et al.*, 1985) indicated the presence of a cross reacting protein in ALM but not in BBM. A monoclonal antibody that recognizes the membrane domain of human erythrocyte Band 3 bound to the basolateral membrane in a subpopulation of cells in the cortical and outer medullary collecting tubules (Schuster *et al.*, 1986). No immunoreactivity was found with the antibody in proximal tubules. Antibodies raised against intact Band 3 or the cytoplasmic domain bind to the basal region of cells of cortical collecting ducts (Hazen-Martin *et al.*, 1987). Our present data also supports this view (Fig. V.11).

The 130-kDa glycoprotein is clearly distinguishable from these Band 3-like proteins. It does not cross react with antibodies raised against human or dog Band 3. These antibodies do react with a 95,000 protein present in the ALM. We can not, at

present, rule out the possibility that the cross-reacting ALM protein is contaminating erythrocyte Band 3 protein although that seems unlikely due to the extensive washes and the density gradient centrifugation involved in preparing ALM. The 95,000 dalton antigen is not present in the BBM. Conversely, the 130-kDa protein is present in the BBM but not the ALM.

The 130-kDa glycoprotein in BBM vesicles is resistant to trypsin digestion and chymotrypsin removes only a small portion of the protein. It seems to be heavily glycosylated with a complex carbohydrate structure, since it binds to all three lectins used in these studies and is sensitive to endo-β-N-acetylglucosaminidase F but not to endo-β-N-acetylglucosaminidase H. The carbohydrate is exposed on the exterior of the BBM vesicles. Since the lectins still bound to endo-β-N-acetylglucosaminidase F-treated protein, some of the oligosaccharide chains may be O-linked. We are currently using wheat germ agglutinin-agarose resin to purify this protein so that it can be reconstituted into membrane vesicles and then its transport function studied. We believe the 130-kDa glycoprotein that we have isolated from dog kidney BBM represents a likely candidate for a SITS- or DIDS-inhibitable anion exchanger.

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VI. SUMMARY AND DISCUSSION

The studies reported in this thesis were directed towards designing and synthesizing an affinity resin for studying Band 3, the anion exchange protein from human erythrocyte membrane. A stilbene disulfonate derivative which specifically binds to Band 3 in intact erythrocytes and inhibits the anion transport was to be used as a ligand. A spacer molecule of appropriate length and nature was to be used to overcome possible steric hindrance. This affinity matrix was then to be used for three main purposes; 1) for purification of erythrocyte Band 3, 2) for studying Band 3-stilbene disulfonate interactions and 3) for purifying Band 3-like proteins from cells other than erythrocytes.

A. Purification of Erythrocyte Band 3 Protein

Using the SITS-Affi-Gel 102 resin, Band 3 from human erythrocytes could be purified to homogeneity (>97% purity by SDS-PAGE). Band 3 is extensively associated with the cytoskeletal network, and it was necessary to remove these peripherally associated proteins by a low-salt and a high-salt extraction. If not removed prior to solubilization, these proteins remained associated with Band 3 and were co-eluted with Band 3 from the affinity resin. After the low- and the high-salt extraction, Band 3 constitutes to about 60-70% of the total membrane protein. Thus, the purity of the Band 3 protein achieved after the affinity chromatography may not seem spectacular but is nevertheless significant (Fig. II.1).

The SITS-Affi-Gel 102 resin can also be used to purify the Band 3 protein from nonhuman erythrocytes. To our knowledge this is probably the first report of a successful purification of a membrane transport protein using inhibitor-affinity chromatography. In the present studies a number of affinity matrices were synthesized and tested for their usefulness in purifying Band 3. Since SITS-Affi-Gel 102 gave the best results in the preliminary experiment, this resin was used in the subsequent experiments. An extensive

characterization of the binding and the elution conditions was done in order to optimize purification of the Band 3 protein. We also characterized a number of chemical compounds to study their properties and to determine their utility as spacer molecules.

B. Ligand-Protein Interaction

These studies have revealed some interesting aspects of the stilbene disulfonates-Band 3 interaction that were not known until now and provide experimental evidence for some phenomenon that were inferred to occur from earlier work. These studies have provided evidence that Band 3 binds to stilbene disulfonates in two states. By exploiting the differential elution sensitivities of these two forms by BADS, it was shown that one form is weakly-bound while the other is strongly-bound to immobilized SITS. The weakly-bound form can be rapidly converted to the strongly-bound form at elevated temperatures. Earlier work by Solomon and coworkers (Verkman *et al.*, 1983) and by Macara *et al.*, (1983) had suggested that such a phenomenon occurs presumably through a conformational change in the protein following inhibitor binding. The present work provides evidence that the stilbene disulfonates inhibitors do become tightly-bound to Band 3 following the initial weak binding. This process is slow at 4 °C but rapid at 37 °C and therefore this observation strongly supports but does not prove that such a change is brought about by a conformational change in the protein.

Band 3 exists primarily as a dimer in membranes and in nonionic detergent. Earlier work from this and other laboratories had indicated that the stilbene disulfonates binding site is present in between two monomers of a dimer and that the two monomers interact with each other. Experiment described in Chapter III show that when one monomer is covalently labelled with DIDS, the other monomer still binds to the immobilized ligand. Such a dimer, however, displays a reduced capacity to acquire the tightly bound form. This shows that when a stilbene disulfonates binds to one monomer, the behavior of the second monomer is influenced by such binding although each monomer has a separate

stilbene disulfonates binding site. When Band 3 is covalently labelled with citrate, the protein binds to the resin with a lower affinity but the protein still retains its capacity to acquire the tight binding. This shows that the residue modified by citrate is involved in the initial binding but does not affect the process of conformational change.

Since the stilbene disulfonates binding site on the protein is accessible only from the outside, intact erythrocytes and rightside-out vesicles but not inside-out vesicles should bind to the resin. Data presented in Chapter IV shows that both the intact erythrocytes and the rightside-out vesicles do bind to the resin via the stilbene disulfonates binding site of the Band 3 protein. An important observation was made that for cell chromatography, a matrix with internal space (such as agarose) is not suitable. With cellulose, all the immobilized ligand is present on the surface and is accessible for binding. Significant proportion of inside-out vesicles also bound to the affinity resin. This binding was mediated via nonspecific hydrophobic and electrostatic interactions. It has been observed that in leaky ghosts, stilbene disulfonates bind nonspecifically to lipids and proteins from the cytoplasmic side of cells. This nonspecific interaction precluded use of the affinity resin as a novel one step procedure for the separation of rightside-out vesicles from inside-out vesicles.

C. Identification of Analogous Proteins

Band 3 mediates electroneutral exchange of anions across the erythrocyte membrane. This exchange is inhibited by stilbene disulfonates. The kidney membranes also exhibit a similar electroneutral exchange of anions that is inhibited by stilbene disulfonates. Thus, in principle it is possible that the protein(s) that mediate anion-exchange in the kidneys would bind specifically to the affinity resin and thus can be identified and isolated. Results summarized in Chapter V show that a single polypeptide of molecular weight 130,000 binds specifically to the resin. This protein was also specifically labelled with [^{3}H]-H_2DIDS. It is a glycoprotein and is present on the luminal side of

kidney epithelial cells. The stilbene disulfonate-inhibitable anion exchange also takes place on the luminal side.

The purification of the 130-kDa glycoprotein achieved by affinity chromatography looks very impressive (Fig V.1a). Prior to chromatography, the 130-kDa glycoprotein was only about 10% of the total brush border membrane proteins and by one step purification with the affinity resin, purity of the protein was improved to >90%. This observation proves the potential of affinity chromatography in purifying a protein. It seems likely that the 130-kDa glycoprotein is not extensively associated with other kidney membrane proteins. This is also suggested by the observation that anti-Band 3 antibodies do not cross react with the 130-kDa glycoprotein. The N-terminal cytoplasmic domain of Band 3 which is involved in binding with other proteins also happens to be the immunodominant area of the Band 3 protein. A majority of polyclonal antibodies raised against Band 3 are usually directed against this region of the protein. The lack of antibody cross-reactivity suggests that the 130-kDa glycoprotein does not have a domain similar to to the Band 3 cytoplasmic domain involved in binding with cytoskeletal elements. This makes purification of the 130-kDa glycoprotein much easier than that of Band 3. Thus it can be said that if Band 3 protein was not associated so extensively with other proteins, one step purification of the Band 3 protein by affinity chromatography would also be possible.

An interesting observation was made with both the erythrocyte membrane proteins and the kidney brush border membrane proteins. Besides Band 3 and the 130-kDa glycoprotein, SITS-Affi-Gel 102 resin retained many other proteins as well. These proteins, however, could not be eluted by adding excess free BADS suggesting that these proteins were bound nonspecifically to the immobilized ligand (or as in a few cases, they may be bound very tightly). This nonspecific interaction was probably due to the predominant hydrophobic nature of the ligand. The increased nonspecific adsorption by the affinity resin was also observed when BADS, which is more hydrophobic than SITS, was used as an immobilized ligand. These studies using SITS as the immobilized ligand

suggest that even if the ligand retains some contaminating proteins, the eluting conditions can be made to selectively desorb the desired protein.

Although, this glycoprotein binds to stilbene disulfonates, it does not cross react with anti-Band 3 antibodies. Reasons for the lack of cross-reactivity have already been discussed. A number of studies have indicated presence of anti-Band 3 antibodies cross-reacting protein(s) on the basolateral side but not on the luminal side of the kidney epithelial cells (Drenckhahn *et al.*, 1985, Cox *et al.*, 1985). Recent data shows that this protein is not present in proximal tubules but is present in medullary collecting tubules (Schuster *et al.*, 1986). Moreover, only the basolateral membrane showed immunoreactivity. Data presented in Fig. V. also shows the presence of an anti-Band 3 antibodies cross-reacting protein from the basolateral membranes but not from the brush border membranes. The cross-reacting protein from the basolateral side is shown to be colocalized with cytoskeletal-like proteins (Drenckhahn *et al.*, 1985). Thus it may be possible that the two functions of the erythrocyte Band 3 protein (the anion-transport and the cytoskeletal attachment site) are performed by two different proteins in kidneys.

Although a likely candidate for anion transporter of the kidney membranes, the function of the 130-kDa glycoprotein remains unknown. A direct evidence that the 130-kDa glycoprotein mediates anion-exchange has yet to be presented. Secondly, the kidney membranes seem to house a number of distinct anion transporters each with a different substrate specificity, different sensitivity to stilbene disulfonates and some exhibiting cation dependence (Madshus and Olsnes, 1987; Alpern and Chambers, 1987). The situation is further complicated by the fact that these transport systems are functionally linked with each other since they share some of the substrates. Moreover some other transport systems which do not use inorganic anions as substrates are also shown to be inhibited by stilbene disulfonates. Thus, assigning a precise transport function to the 130-kDa glycoprotein must await extensive functional characterization.

Identification of the 130-kDa glycoprotein as a stilbene disulfonates binding protein from kidneys is probably the most exciting outcome of the present work. The next logical step would be to functionally characterize this polypeptide. This should primarily involve reconstitution of the purified protein into vesicles and studying its transport characteristics. These studies can be supported by determining its tissue distribution using ultrastructural immunological approaches. Since various parts of a nephron have been identified with particular transport functions, such studies could be used to support in vivo function of the 130-kDa glycoprotein. Finally the amino acid sequence can be determined either by a biochemical approach or preferably deduced by cloning cDNA that codes for the 130-kDa glycoprotein. The molecular biological approach will be more rewarding since it can be used to mutate the 130-kDa glycoprotein in precise locations along the polypeptide chain. This information should be useful to delineate regions of the protein responsible for its function.

The technique of affinity chromatography is, however, not without limitations in all the three areas discussed above and applied in the present work. The biggest drawback of this technique is that it is still an 'inexact science'. Although this technique has been successfully exploited in the basic research as well as in industries, it has not received a sound theoretical treatment. As a result, as in the area of crystallography, success of affinity chromatography cannot be ensured and almost always depends on the earlier failed attempts and on a trial and error approach. A big obstacle in purification by affinity chromatography is the nonspecific association of the immobilized ligand or the spacer molecule with contaminating proteins. Elution of the bound protein in native non-denatured state can sometimes become a problem (Weber *et al.*, 1985). Studying the ligand-protein interaction generally yields only qualitative information not quantitative. Finally, the nonspecific interactions observed with affinity resins also interfere with identification and isolation of a related protein from a different system. Despite these

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drawbacks, the present work has made significant contributions to the application of affinity chromatography for studying integral membrane proteins.