University of Alberta

Structural and Functional Analysis of the C-terminal Region of Human Plasma Cl^-/HCO_3^- Anion Exchanger, AE1

by

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Abstract

Human plasma Cl^-/HCO_3^- anion exchange protein 1 (AE1), is the major integral membrane protein in the erythrocyte, performs tightly coupled one to one electroneutral exchange of Cl^- for HCO_3^- across the erythrocyte membrane. The C-terminal region of the AE1 membrane domain is implicated as involved in anion translocation and has been the subject of extensive characterization. An understanding of the folding structure of this region and its role in the substrate translocation will directly relate to the understanding of AE1 transport mechanism.

This thesis focuses on the determination of topology and the identification of pore-lining residues in the C-terminal region of human AE1, from residue Phe806 to Cys885. Cysteine scanning mutagenesis coupled with treatment with sulfhydryl reagents indicated that the C-terminal region of AE1 has an unusual topology, with an extracellular medium-accessible transmembrane loop, two short transmembrane segments (TM) connected by a very small loop accessible to the extracellular medium, composed of four amino acids, and an intracellular-localized C-terminus.

Measurements of Cl^-/HCO_3^- exchange activity before and after treatment with small sulfhydryl reagents revealed that, among the eighty introduced cysteine mutants, only those in the region of Val849-Leu863 are involved in forming the anion transport site. Ser852-Ala858 in the extracellular portion of the last two TMs were identified to form the substrate charge filter.

On the basis of these results, we propose a topology model for the C-terminal region of the membrane domain. We also identified the residues in the region that

directly interact with the substrate, and the anion permeability barrier in the human AE1 protein.

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Table of Contents

List of Tables

Chapter 1:

Chapter 3:

List of Figures

Chapter 1:

Chapter 3:

Chapter 4:

Abbreviations

AE, anion exchange

AE1, Cl^-/HCO_3^- anion exchange protein 1

AE1C$^-$, cysteineless AE1

AE2, Cl^-/HCO_3^- anion exchange protein 2

AE3, Cl^-/HCO_3^- anion exchange protein 3

BCECF-AM, 2',7'–bis(2-carboxyethyl)–5(6)-carboxyfluorescein-acetoxymethyl ester

BIDS, 4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate

Biotin maleimide, 3-(N-maleimidylpropionyl)biocytin

BSSS, bis(sulfosuccinimidyl)suberate

CA, carbonic anhydrase

C-terminus, carboxy-terminus

CTAB, hexadecyltrimethylammonium bromide

$C_{12}E_8$, octaethylene glycol monododecyl ether

$C_{12}E_9$, nonaethylene glycol monododecyl ether

DEPC, diethyl pyrocarbonate

DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

DMEM, Dulbecco's modified Eagle's medium

DNA, deoxyribonucleic acid

DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate

dRTA, familial distal renal tubular acidosis

eAE1, erythrocyte AE1

ECL, enhanced chemiluminescence

EDTA, ethylenediaminetetraacetic acid

EMA, eosin 5-maleimide

ER, endoplasmic reticulum

FBS, fetal bovine serum

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GPA, glycophorin A

GPB, glycophorin B

GST, glutathione-S-transferase

H_2DIDS, 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate

HEK, human embryonic kidney

HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HPLC, high performance liquid chromatography

HRP, horseradish peroxidase

HS, hereditary spherocytosis

kAE1, kidney AE1

IgG, immunoglobulin G

LYIA, lucifer yellow iodoacetamide

mRNA, messenger RNA

MTSEA, (2-aminoethyl)methanethiosulfonate

MTSES, (2-sulfonatoethyl) methanethiosulfonate

MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate

NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate

NIP-taurine, N-(4-isothiocyano-2-nitrophenyl)-2-aminoethylsulfonate

N_{exo}/C_{cyt}, N-terminus extracellular and C-terminus intracellular orientation

NMR, nuclear magnetic resonance

N-terminus, amino-terminus

PBS, phosphate buffered saline

pCMBS, parachloromercuibenzenesulfonate

PCR, polymerase chain reaction

PG, phenylglyoxal

PLP, pyridoxal 5'-phosphate

PMSF, phenylmethylsulfonyl fluoride

qBBr, bromotrimethylammoniumbimane bromide

SAO, Southeast Asian ovalocytosis

SCAM, substituted-cysteine accessibility method

SDS, sodium dodecyl sulfate

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

SLC4A1, solute carrier family 4, anion exchanger 1

TLCK, N-p-tosyl-L-lysine chloromethyl ketone

TM, transmembrane segment

TPCK, N-tosyl-L-phenylalanine chloromethyl ketone

Tris, Tris(hydroxymethyl)aminomethane

WRK, Woodward's reagent K

Chapter 1

Introduction

Introduction

Human plasma Cl^-/HCO_3^- anion exchange protein 1 (AE1), also called band 3 or SLC4A1, belongs to a widely distributed membrane transport protein family (Alper, 1991; Alper et al., 2002; Reithmeier, 1993; Sterling and Casey, 2002). AE1 facilitates tightly coupled one to one electroneutral exchange of Cl^- for HCO_3^- across the erythrocyte plasma membrane (Frohlich and Gunn, 1986; Jay and Cantley, 1986; Jennings, 1989b; Passow, 1986). AE1 is the most abundant integral membrane protein in human erythrocytes (Fairbanks et al., 1971), and has been the best-characterized mammalian transport system. Thus, AE1 has served as a model for our understanding of membrane transport mechanism for decades. Yet, key questions remain regarding the three-dimensional structure, the anion translocation site and the transport mechanism of AE1.

Many aspects of human AE1 have been studied, including structural organization, functional regulation and gene expression (Alper, 1991; Alper et al., 2002; Jennings, 1989b; Reithmeier, 1993; Sterling and Casey, 2002). This introduction focuses on the structural studies of human AE1, especially, the oligomeric structure of AE1 in the cell membrane, the topology of the AE1 membrane domain, and the structure of the AE1 substrate translocation site. The body of the thesis will focus on the structure and functional role of the C-terminal portion of AE1, studied using a combination of introduced cysteine mutants and sulfhydryl specific protein chemistry.

1.1 Physiological function of AE1

1.1.1 CO_2 metabolism

AE1 plays a critical role in the CO_2 transport system (Wieth et al., 1982a). CO_2 is the end product of tissue metabolism and must be removed from the body. CO_2 is transported mainly as HCO_3^- ion in the blood (about 70%), since CO_2 dissolves poorly in the plasma. In the systemic capillaries, CO_2 diffuses into the erythrocyte due to high local CO_2 partial pressure, and is converted to HCO_3^-, catalyzed by intracellular carbonic anhydrase II (CAII) (Sterling and Casey, 2002). HCO_3^- is then transported out of the erythrocyte by AE1 in exchange for Cl^-, driven by the increased intracellular HCO_3^- concentration. This process prevents the alkalization of the erythrocyte. Conversion of CO_2 to HCO_3^- benefits the body in two ways: enhancement of blood capacity to carry CO_2 and pH buffering to stabilize the body's pH. When the blood reaches the lung, the whole process is reversed. The lowered concentration of CO_2 inside the cell drives CAII to catalyze HCO_3^- conversion to CO_2. This conversion decreases the intracellular HCO_3^- level and subsequently activates AE1 to bring in more substrate in exchange for Cl^-. Through this whole process, CO_2 is removed from the body rapidly (Figure 1.1). AE1 deficient mice and cattle showed acidosis, chronic hemolytic anemia and high rates of neonatal death (Inaba et al., 1996; Peters et al., 1996).

1.1.2 Erythrocyte biconcave shape

Human erythrocytes have a unique biconcave shape, which makes it flexible as it passes through the capillaries during gas exchange and resilient to shear stress in the circulation (Jay, 1996). This unique shape is formed and maintained by the interaction of integral membrane proteins, cytoskeletal proteins and some cytoplasmic peripheral

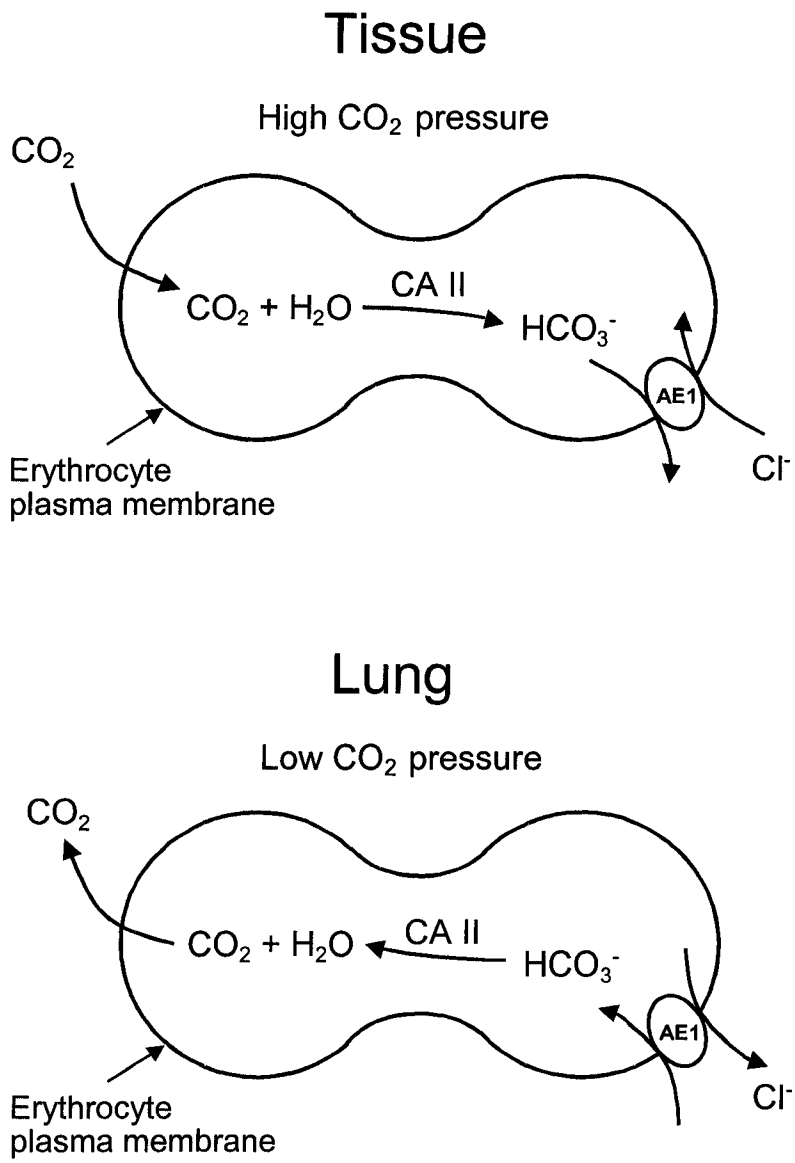


Figure 1.1 CO_2 clearance from the human body
The upper panel shows an erythrocyte in the systemic capillaries and the lower panel shows an erythrocyte in the pulmonary capillaries. (Modified from Lodish et al., 1999)

proteins. AE1 is needed to organize and maintain this unique shape of erythrocyte (Inaba et al., 1996; Peters et al., 1996). The cytoplasmic domain of AE1 interacts with the peripheral proteins ankyrin, protein 4.1 and protein 4.2 to anchor the lipid bilayer to the spectrin-actin cytoskeleton (Zhang et al., 2000). AE1 also interacts with the Rh membrane protein complex forming a macrocomplex in the erythrocyte membrane, to stabilize the structure of the cell membrane (Bruce et al., 2003a). When AE1 is deficient, erythrocytes change their shape from biconcave to spherical, and spontaneously shed membrane vesicles and tubules (Inaba et al., 1996; Peters et al., 1996).

1.1.3 Senescent cell antigen

The average life span of human erythrocytes in the circulation is 120 days (Schlüter and Drenckhahn, 1986). Aged erythrocytes are bound with autoantibodies and subsequently removed from the circulation by phagocytic cells of the reticuloendothelial system (Bartosz et al., 1982; Kay, 1978; Khansari and Fudenberg, 1983; Shaw et al., 1980; Todd et al., 1984). Extensive biochemical analysis revealed that the membrane protein that bound to the IgG autoantibodies is AE1 protein (Kay, 1978; Kay et al., 1983; Lutz, 1981; Lutz and Stringaro-Wipf, 1983). Two mechanisms have been proposed that lead to the formation of the senescence antigen by AE1; one is AE1 molecule aggregation (Low et al., 1985; Kannan et al., 1991; Schlüter and Drenckhahn, 1986) and the other is structural alteration (Kay, 1984). Clustering of AE1 molecules in the aged erythrocyte membrane has been observed by immunofluorescence microscopy (Low et al., 1985) and size exclusion HPLC studies (Casey and Reithmeier, 1991). The isolated aggregated AE1 indeed bound with IgG autoantidodies (Kannan et al., 1991). In contrast, a synthetic peptide corresponding to a loop region of AE1 protein blocked the binding of IgG

autoantibodies to the aged erythrocytes. Thus, it was proposed that the senescent cell antigen is created by exposure of a normally hidden region in the AE1 protein (Kay et al., 1990). Recently, mild oxidative modification of the carbohydrate of AE1 was reported to be responsible for the binding of the IgG autoantibodies to the AE1 protein (Ando et al., 1997). The exact mechanism of erythrocyte senescent antigen formation needs to be further explored.

1.1.4 Acid-base balance

One of the major functions of the kidney is to maintain the body's acid-base balance. To achieve this balance, the kidney must excrete an amount of acid equal to the nonvolatile acid production. In the meantime, the kidney must prevent the loss of HCO_3^- in the urine (Koeppen and Stanton, 1996). Both the reabsorption of the filtered load of HCO_3^- and the excretion of acid are accomplished through the process of H^+ secretion. The acid secretion in kidney takes place in the epithelial cells of the proximal tubule, distal renal tubule and in the type A intercalated cells of the collecting ducts (Alper, 2002). A kidney AE1 spliceform (kAE1) exists on the basolateral membrane of the type A intercalated cell (Sabolic et al., 1997; Wagner et al., 1987). In these cells, CO_2 is hydrated by cytoplasmic carbonic anhydrase II to produce carbonic acid, which then dissociates to H^+ and HCO_3^- ions. The H^+ ions are pumped out of the cell *via* the H^+-ATPase in the apical membrane, while the HCO_3^- ions are exchanged for Cl^- *via* kAE1 in the basolateral membrane (Figure 1.2). Thus, kAE1 co-operates with H^+-ATPase in the type A intercalated cells to acidify the urine and reabsorb HCO_3^- (Tanner, 1996). Mutations of kAE1 cause human distal renal tubule acidosis, in which the kidneys are

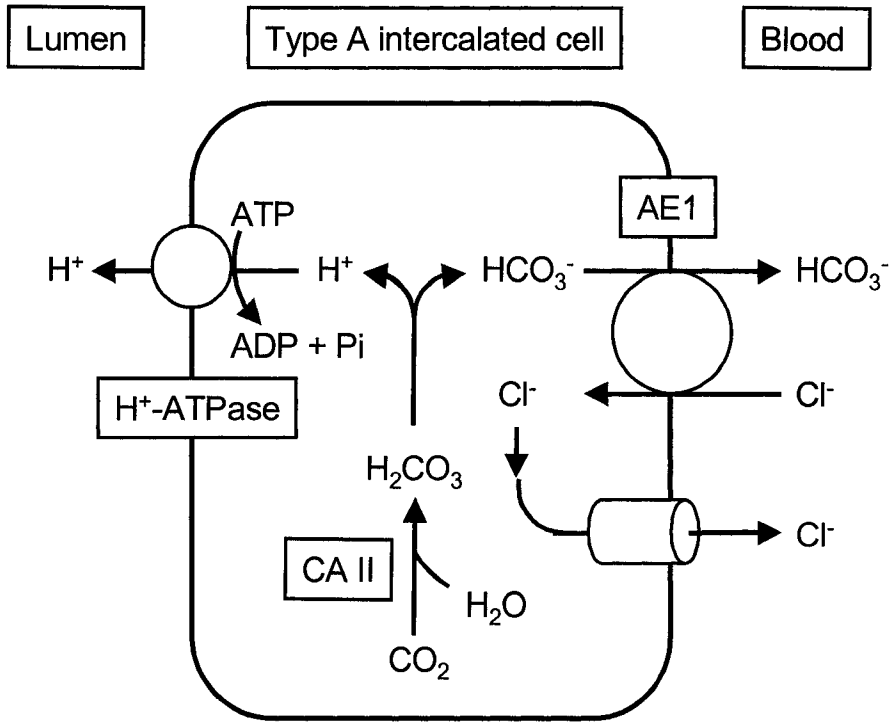


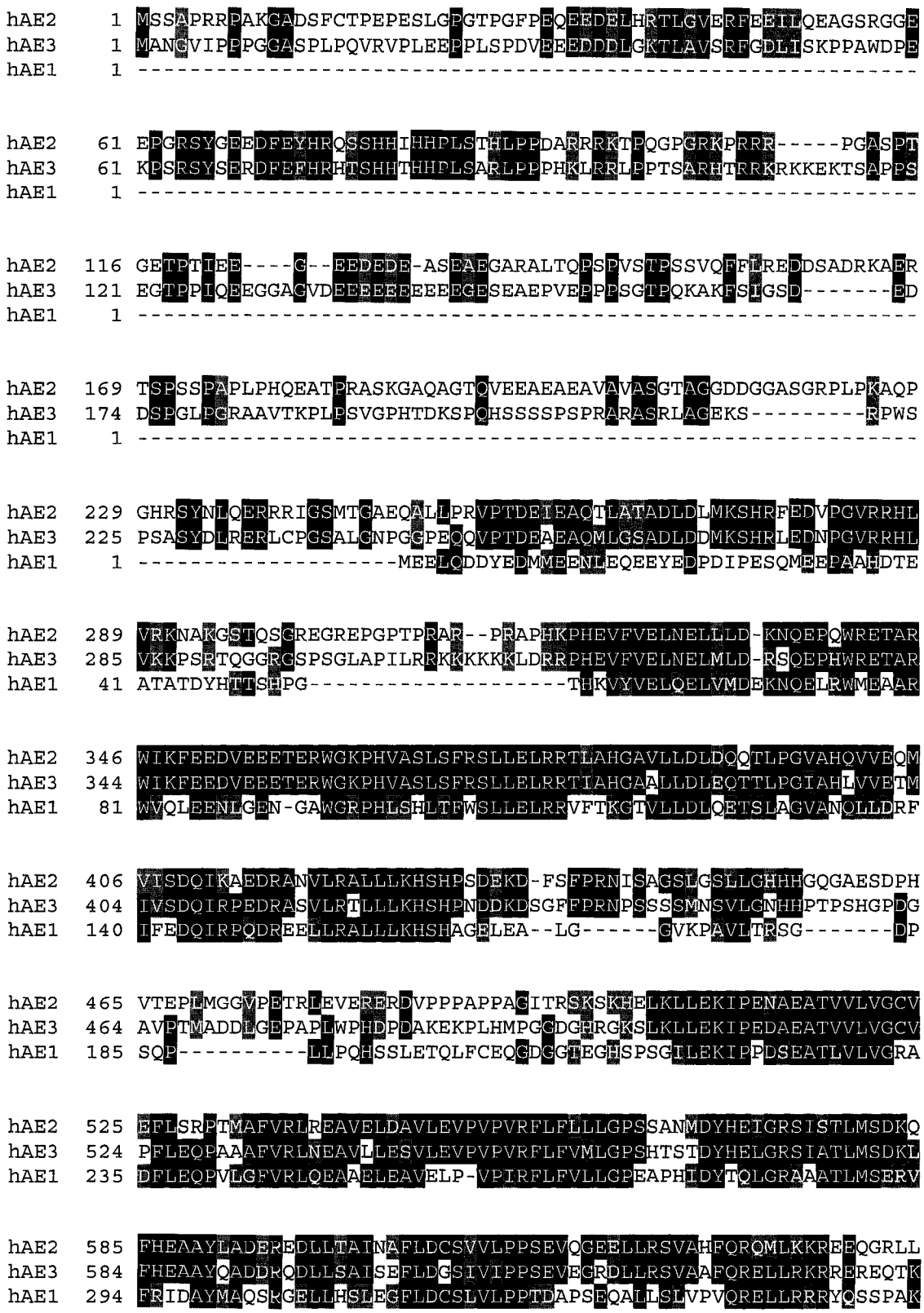
Figure 1.2 Co-operation of H^+-ATPase and AE1 in acid secretion in type A intercalated cells

Type A intercalated cells secrete acid into the lumen of the kidney collecting duct. CO_2 is hydrated by CA II to produce H^+ and HCO_3^-. H^+ is pumped out of the cell by H^+-ATPase in the apical membrane, and HCO_3^- is exchanged for Cl^- *via* AE1 in the basolateral membrane. Cl^- leaves the cell *via* Cl^- channel in the basolateral membrane. (Adapted form Bruce and Tanner, 1999)

unable to excrete a sufficient amount of net acid to balance nonvolatile acid production (Alper, 2002).

1.2 Human anion exchanger family

Human AE1 belongs to a multi-gene family (SLC4A), which consists of three members: AE1, AE2 and AE3 (Alper, 1991; Alper et al., 2002). They all mediate HCO_3^- transport across the plasma membrane and share sensitivity (to varying degrees) to the disulfonic stilbene class of antagonist, electroneutrality of transport, and ion substrate specificities. Human AE1 is composed 911 amino acids (Lux et al., 1989; Tanner et al., 1988); human AE2 is composed of 1240 amino acids (Gehrig et al., 1992), and human AE3 is composed of 1232 amino acids (Yannoukakos et al., 1994a). AE family members have two-domain structure: a highly conserved membrane domain and a more divergent cytoplasmic domain (Alper, 1991; Reithmeier, 1993). Amino acid sequence alignment of all cloned AE members revealed that the membrane domains share 64-69% identity overall and 80% identity in the transmembrane segments (Alper, 1991) (Figure 1.3). The region of greatest difference in the membrane domain is the loop between the putative transmembrane segment (TM) 5 and 6 (Alper et al., 1988; Kopito and Lodish, 1985) (bold line in Figure 1.3). This loop differs not only in the protein sequence and length, but also the glycosylation state. In AE2 and AE3, it serves as a probable N-glycosylation site, whereas the glycosylation site in AE1 is in the loop between TM7 and 8 (Alper, 1991; Reithmeier, 1993). AE1 has the smallest cytoplasmic domain, compared to AE2 and AE3. The regional amino acid sequence similarity of the cytoplasmic domain between AE1 and AE2, AE3 is only about 30%, whereas between AE2 and AE3, it is about 60% (Alper, 1991). The membrane domains of all the family members are



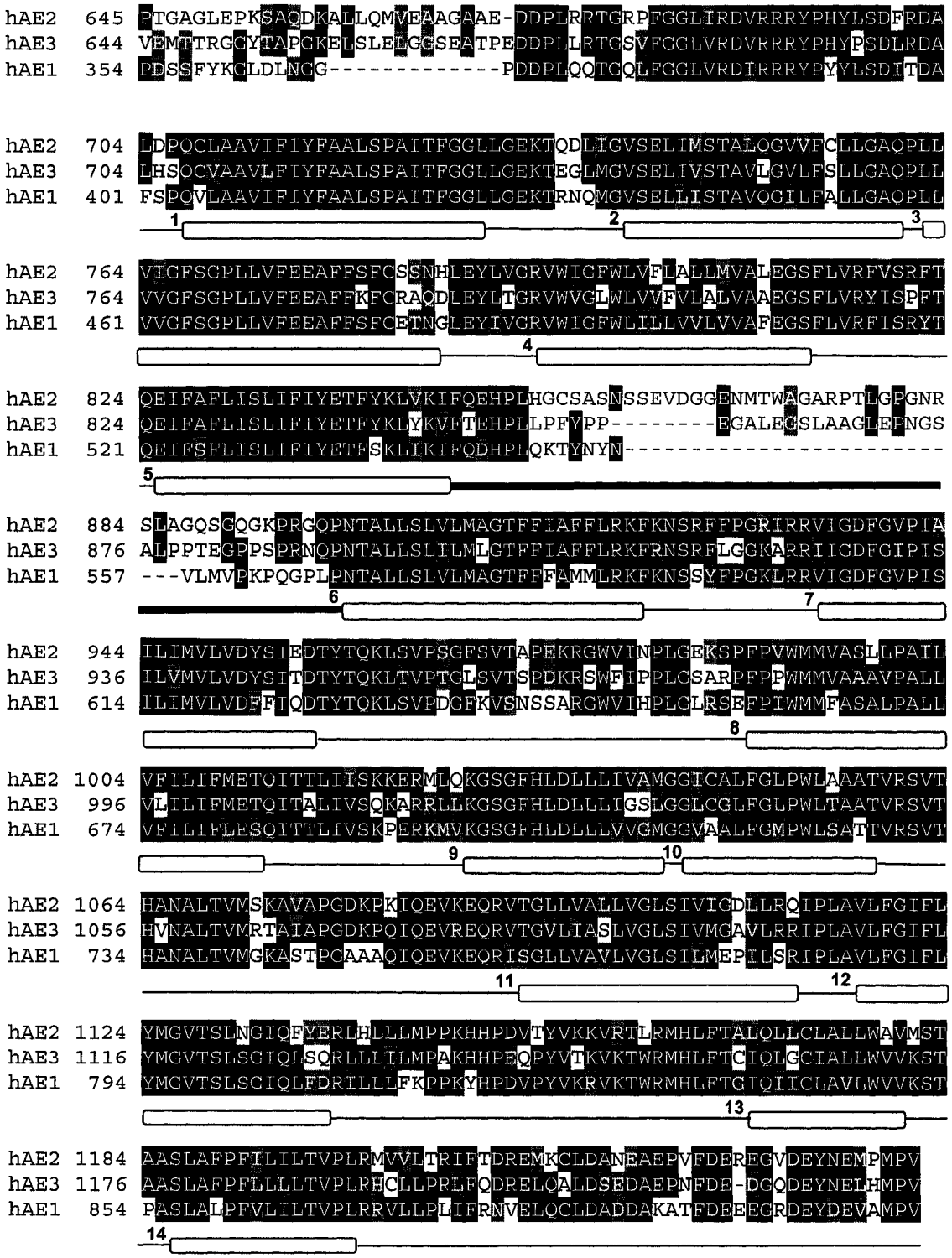


Figure 1.3 Amino acid sequence alignment of human AE1, AE2 and AE3
The first letter of each sequence name refers to the species: h (human). AE1, (Tanner et al., 1988); AE2, (Gehrig et al., 1992); AE3, (Yannoukakos et al., 1994a). The overall sequence identity of the three AE isoforms is 65%. ▭, TMs; —, loops.

responsible for the anion transport function, whereas the cytoplasmic domains seem to have different functions based on the protein sequences.

1.2.1 AE1

1.2.1.1 Molecular cloning of AE1 protein

Molecular cloning of AE1 was difficult because of the inability to obtain significant amounts of messenger RNA (mRNA) encoding the protein, until Braell and Lodish solved the problem by utilizing anemic mouse spleen as the source of AE1 mRNA (Braell and Lodish, 1981; Braell and Lodish, 1982). Judged by the proteolysis pattern, murine AE1 demonstrated structural similarity to the human AE1 protein. On the basis of the obtained mRNA, Kopito and Lodish successfully cloned murine AE1 (Kopito and Lodish, 1985). This work laid the foundation for the future structural and functional studies of the AE1 protein, as well as the molecular cloning of various AE1 isoforms from different species. In 1988 and 1989, Tanner and Lux independently cloned human AE1 by screening a human fetal liver cDNA library, using different complementary oligonucleotides (Lux et al., 1989; Tanner et al., 1988). The size of cloned AE1 cDNA is about 3.5 kb, encoding 911 amino acids. Human AE1 gene has been localized to chromosome 17q21-q22 (Lux et al., 1989; Showe et al., 1987).

1.2.1.2 Alternative forms of human AE1

Human AE1 is expressed as two splicing-forms resulting from different transcriptional initiation: erythroid AE1 (eAE1) and kidney AE1 (kAE1) (Alper et al., 2002). eAE1 is a full length AE1 that consists of 911 amino acids. It is the most abundant integral membrane protein in the human erythrocyte (50% of integral membrane protein, 1.2 x 10^6 copies per cell) (Fairbanks et al., 1971), where it performs

tightly coupled one to one electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane (Jay and Cantley, 1986; Passow, 1986). eAE1 is a 110 kDa glycoprotein with a single glycosylated site at residue Asn642, which is responsible for the diffuse appearance of the protein on SDS-PAGE (Lux et al., 1989; Markowitz and Marchesi, 1981; Tanner et al., 1988). The 40 kDa N-terminal cytoplasmic domain of eAE1 interacts with ankyrin to anchor the lipid bilayer to the cytoskeleton, whereas the 55 kDa C-terminal membrane domain performs the anion exchange function (Jay and Cantley, 1986; Jay, 1996), even after the cytoplasmic domain is proteolytically removed (Grinstein et al., 1978; Lepke and Passow, 1976).

kAE1 is an N-terminal truncated form of full length AE1 consists of 856 amino acids, which lacks the first N-terminal 65 amino acids (Kollert-Jons et al., 1993). It is produced by transcriptional initiation of the mRNA from a promoter in intron 3 of the same gene that codes for eAE1 (Sahr et al., 1994). kAE1 is located on the basolateral surface of the type A intercalated cells in the renal collecting duct (Sabolic et al., 1997; Wagner et al., 1987). This location is critical for the normal function of the kidney. Intracellular retention or mis-targeting of kAE1 to the apical surface of the type A intercalated cell causes distal renal tubular acidosis (Devonald et al., 2003; Toye et al., 2002). Recent studies revealed that the C-terminus of AE1 contains a putative tyrosine-based sorting signal, which implicates it in basolateral targeting in polarized epithelia (Devonald et al., 2003).

1.2.2 AE2

Shortly after molecular cloning of AE1, a non-erythroid form of anion exchanger protein, AE2, was isolated and cloned from the human erythroleukemic cell line K562

(Demuth et al., 1986) and subsequently from several other species (Alper et al., 1988; Kudrycki et al., 1990; Lindsey et al., 1990). Human AE2 protein is 1240 amino acids in length (Gehrig et al., 1992). The AE2 gene produces alternative mRNA transcripts, resulting from transcription initiation from three promoters, which produces five AE2 variants, AE2a, AE2b1, AE2b2, AE2c1, AE2c2. These variants are N-terminally truncated at different places within the first 200 amino acids (Medina et al., 2000; Wang et al., 1996). Northern blots have shown that AE2 is expressed very widely, with highest expression in gastric parietal cells (Stuart-Tilley et al., 1994), choroid plexus epithelial cells (Alper et al., 1994), colonic surface enterocytes (Alper et al., 1999), renal thick ascending limb cells (Martinez-Anso et al., 1994), and osteoclasts (Teti et al., 1989). AE2 has been proposed to be involved in the acidification of gastric mucosa, secretion of cerebrospinal fluid and bone remodeling (Alper, 1991). The human AE2 gene has been localized to chromosome 7q35-q36 (Palumbo et al., 1986).

1.2.3 AE3

The AE3 gene has been cloned from human (Yannoukakos et al., 1994b), mouse (Kopito et al., 1989) and rat (Kudrycki et al., 1990). AE3 is mainly expressed in the excitable tissues, heart (Linn et al., 1992), brain (Kopito et al., 1989) and retina (Kobayashi et al., 1994). The human AE3 gene employs two promoters to generate two N-terminal variants: a full length isoform AE3 that consists of 1232 amino acids, and a cardiac isoform AE3 consisting of 1034 amino acids (Yannoukakos et al., 1994b). The C-terminal amino acid sequence of both polypeptides are identical, but the cardiac isoform contains a unique N-terminal sequence of 73 amino acids, which replaces the first 270 amino acids of the brain isoform (Yannoukakos et al., 1994b). Heart and retina

co-express both AE3 isoforms (Kobayashi et al., 1994; Linn et al., 1995; Linn et al., 1992). Cardiac isoform AE3 is the dominant AE protein expressed in the heart (Linn et al., 1995), whereas both AE3 isoforms are expressed at similar level in the retina (Kobayashi et al., 1994). *In situ* hybridization studies demonstrated that transcription of AE3 brain isoform takes place in virtually all types of neurons of the brain (Kopito et al., 1989). Cardiac isoform AE3 has been proposed to contribute to the pH recovery after cellular-acid loading of the cells (Sterling and Casey, 1999). The gene of human AE3 has been localized to chromosome 2q36 (Yannoukakos et al., 1994b).

1.3 Post-translational modification of AE1

1.3.1 Glycosylation

AE1 protein contains a single glycosylated site at residue Asn642 on the extracellular loop 4 of the membrane domain (Lux et al., 1989; Tanner et al., 1988). The diffuse appearance of AE1 on SDS-PAGE has been attributed to heterogeneity in the carbohydrate (Markowitz and Marchesi, 1981). Glycosylation of AE1 was proposed to help AE1 processing to the plasma membrane. However, mutation of Asn642 to Gly, Ser and Thr did not affect the migration of the newly expressed AE1 to the Xenopus oocyte surface membrane, except a slightly reduced anion transport was observed (Groves and Tanner, 1994b). In addition, removal of the carbohydrate by endoglycosidase did not affect the protein function, the secondary structure or oligomeric structure of the protein (Casey et al., 1992). This suggests that glycosylation is not functionally important, or required for the expression of AE1 protein to the plasma membrane. Analysis on the heterogeneity of the oligosaccharide chain of AE1 revealed that, about half of the protein contains a large polyactosaminyl oligosaccharide structure and the other half contains a

smaller oligosaccharide (Landolt-Marticorena et al., 1998). The functional role of the carbohydrate on the surface of AE1 may be to contribute to the formation of the senescent antigen on the aged erythrocytes (Beppu et al., 1992).

1.3.2 Fatty acylation

The sulfhydryl group of Cys843 in the C-terminal region of the membrane domain of AE1 was serendipitously found to be fatty acylated in the erythrocyte, because of the inability of determining this residue by a gas-phase sequencer (Kawano et al., 1988). The attached fatty acyl chain was further identified to be palmitic or stearic acid (Okubo et al., 1991). Fatty acylation has been observed in a variety of membrane-attached proteins, especially the G protein family. Fatty acylation of G proteins has been proposed to be dynamic and play an important role in regulating the activation and inactivation of the G protein (Wedegaertner et al., 1995). However, mutation of the palmitoylated Cys843 to Ser or Met had no effect on the anion transport activity (Kang et al., 1994), and mutation of Cys843 to Ala did affect cell surface expression of the protein (Cheung and Reithmeier, 2003). The latter study also suggested that palmitoylation of Cys843 may only take place in the erythrocytes, as no AE1 palmitoylation could be detected when the protein was expressed in HEK293 cells or COS7 cells (Cheung and Reithmeier, 2003). The functional role of this modification is still unknown.

1.3.3 Phosphorylation

AE1 is the major tyrosine kinase phosphorylated protein in erythrocytes (Harrison et al., 1994; Minetti et al., 1998; Tuy et al., 1983). It is phosphorylated primarily on the N-terminal cytoplasmic domain. The major phosphorylation site has been identified as

Tyr8, which is located in a highly acidic amino acid stretch in the cytoplasmic domain (Dekowski et al., 1983; Low et al., 1987; Yannoukakos et al., 1991b). Also, Tyr21, Tyr359 and Tyr904 were reported to be phosphorylated *in vitro* (Yannoukakos et al., 1991a). Recent studies have shown that AE1 is phosphorylated sequentially, first by tyrosine kinase Syk on residues Try8 and Tyr21, and then by tyrosine kinase Lyn on residues Tyr359 and Tyr904 (Brunati et al., 2000). Phosphorylation of AE1 has been proposed to be involved in regulating the association of AE1 with the cytoplasmic proteins (Brunati et al., 2000).

1.4 AE1 associated variants and human diseases

1.4.1 Southeast Asian Ovalocytosis

Southeast Asian Ovalocytosis (SAO) occurs mainly in the Southeast Asia where malaria is endemic (Amato and Booth, 1977). SAO erythrocytes are oval and rigid (Mohandas et al., 1984; Saul et al., 1984). The molecular basis of this cell morphology is the deletion of amino acids 400-408 from AE1 (Jarolim et al., 1991; Mohandas et al., 1992; Schofield et al., 1992b). Despite the deletion of nine residues, SAO AE1 is not degraded inside the cell, but inserted into the erythrocyte membrane. It has been suggested that the deletion removes a flexible region of the protein that causes a non-specific interaction of the cytoplasmic domain of SAO AE1 with the cytoskeleton (Liu et al., 1995b; Mohandas et al., 1992). The SAO erythrocytes have been shown to protect children against fatal cerebral malaria (Genton et al., 1995). Although SAO occurs with high frequency in parts of Southeast Asia, no individuals with homozygosity of the mutant protein have been found, suggesting that the homozygosity of the mutation is lethal (Liu et al., 1994).

The structure of the membrane domain of SAO AE1 is extensively altered compared to normal AE1 (Bruce and Tanner, 1999; Kuma et al., 2002a). The SAO AE1 is misfolded in the membrane and does not transport anions or bind anion transport inhibitors (Moriyama et al., 1992; Sarabia et al., 1993; Schofield et al., 1992a). The structure of the loop carrying the N-glycan chain is also changed; SAO AE1 does not have a polylactosaminyl oligosacchride (Sarabia et al., 1993). However, SAO AE1 retains the ability to form heterodimers with normal AE1 (Jennings and Gosselink, 1995; Salhany and Schopfer, 1996).

1.4.2 Hereditary spherocytosis

Hereditary spherocytosis (HS) is a common congenital hemolytic anemia, characterized by spherocytic erythrocytes with increased osmotic and mechanical fragility. The spherocytic shape of these cells results from loss of membrane surface area relative to intracellular volume. HS can derive from defects in any of the erythrocyte skeletal components that are involved in stabilizing the lipid bilayer, such as spectrin, ankyrin, protein 4.2 and AE1 (Hassoun and Palek, 1996; Tanner, 1997). A large number of mutations in AE1 protein have been associated with HS (Bruce and Tanner, 1996; Bruce and Tanner, 1999; Hassoun and Palek, 1996). Mutations in the cytoplasmic domain of AE1 have been reported to disrupt the interaction of AE1 with ankyrin or protein 4.2. Thus, the lipid bilayer cannot be anchored to the cytoskeleton (Jarolim et al., 1996; Jarolim et al., 1992). In addition, mutations in the membrane domain also result in HS (Bruce and Tanner, 1999; Hassoun and Palek, 1996). These mutations are characterized into two groups: in one, the mutation produces unstable mRNA; in the other, the mRNA is present, but the mutant protein is either degraded or not inserted into

the plasma membrane. In both cases, the amount of AE1 present in the erythrocyte membrane is greatly reduced, and thus destabilizes the lipid bilayer.

1.4.3 Human blood group antigens

Many serologically defined blood group antigens have been linked to AE1 mutations (Bruce and Tanner, 1996; Bruce and Tanner, 1999; Jarolim et al., 1998a; Tanner, 1997). The most studied antigens are Diego blood groups. The Diego blood group is comprised of two allelic antigens: Di^a and Di^b (Jarolim et al., 1998a). The Di^a antigen is common in the indigenous peoples of America and Southeast Asia (Bruce and Tanner, 1999). It is known clinically that anti-Di^a antibody causes severe hemolytic disease of the newborn (Kusnierz-Alejska and Bochenek, 1992; Levine et al., 1956), and immediate hemolytic transfusion reaction (Hinckley and Huestis, 1979). The molecular basis of Di^a results from a single amino acid substitution of Pro854 to Leu in the last extracellular loop of AE1 (Bruce et al., 1994), whereas Di^b is associated with the presence of Pro854. The mutation does not affect the anion transport function mediated by AE1, but causes an increased binding susceptibility to H_2DIDS (an anion exchanger inhibitor) (Bruce et al., 1994). Two possibilities have been proposed to explain structural alterations caused by Pro854 to Leu mutation: an altered local conformation of the extracellular loop, and a widespread conformational changes on the surface of the protein (Bruce et al., 1994).

1.4.4 Familial Distal renal tubular acidosis

Familial distal renal tubular acidosis (dRTA) is a hereditary disease diagnosed by inability to acidify urine in a setting of spontaneous systemic metabolic acidosis or after

an imposed acid load (Alper, 2002). The kidney form of AE1 in the type A intercalated cells co-operates with H^+-ATPase to acidify the urine and reabsorb HCO_3^- in the collecting ducts (Tanner, 1996). A number of different AE1 mutations have been associated with the autosomal dominant form of dRTA. AE1 mutations in residues Arg589 (Bruce et al., 1997; Jarolim et al., 1998b), 613 (Bruce et al., 1997) and Ala858 (Bruce et al., 2000) were reported to cause dRTA. A mutation that truncates the C-terminal 11 amino acids was also reported to cause dRTA (Karet et al., 1998). The inability of AE1 mutant protein to be processed to the plasma membrane has been suggested to be the cause of dRTA (Quilty et al., 2002; Toye et al., 2002). However, a recent report also suggested that mistargeting of the mutant protein to the apical surface of the type A intercalated cell also results in dRTA (Devonald et al., 2003).

1.5 Oligomeric state of AE1 in the plasma membrane

The oligomeric structure of AE1 in the cell membrane has been studied extensively. Evidence from many laboratories using a variety of approaches strongly suggests that AE1 exists as a mixture of dimers (about 70%) and tetramers (about 30%) in the erythrocyte membrane (Casey and Reithmeier, 1991; Jennings, 1984; Low, 1986). Understanding the oligomeric structure of AE1 in erythrocyte membrane is prerequisite to the structural and functional studies of AE1 protein.

1.5.1 Biophysical and biochemical analysis of AE1 oligomeric structure

1.5.1.1 Freeze-fracture analysis

Freeze fracture electron microscopy studies revealed the existence of intramembranous particles in the erythrocyte cell membrane, which are believed to be

largely composed of AE1 (Yu and Branton, 1976). Quantification of these particles per cell (4 x 10^5) (Weinstein et al., 1980) found it is far less than the number of AE1 molecules, which is 1.2 x 10^6/cell (Fairbanks et al., 1971), suggesting each particle contains more than one AE1 molecule. Rotary shadow-cast micrographs further showed a distinct heterogeneity in the particle size and fine structure (Margaritis et al., 1977), providing further support that some particles could consist of dimers or tetramers of AE1 molecule.

1.5.1.2 *In situ* oligomeric analysis

The *in situ* oligomeric state of AE1 was examined by three different approaches: transient absorption anisotropy (Nigg and Cherry, 1979a), fluorescence resonance energy transfer (Dissing et al., 1979; Macara and Cantley, 1981), and radiation inactivation (Cuppoletti et al., 1985). Transient absorption anisotropy measurement of erythrocyte membranes containing eosin maleimide (a fluorescent chemical) labeled AE1 revealed that cross-linking of AE1 by Cu^{2+}-orthophenanthroline did not lower the rotational mobility of the bound eosin (Nigg and Cherry, 1979a), which suggests AE1 is associated as dimers or higher oligomers prior to the covalent cross-linking. Fluorescence resonance energy transfer measurements between pairs of fluorescence probes bound on separate AE1 subunits revealed their distance is only 28 to 50 Å, which is less than half of the estimated mean distance (150 Å) between monomers in the erythrocyte bilayer (Dissing et al., 1979; Macara and Cantley, 1981). This also supports the dimeric association and the presence of tetrameric species in the membrane. Radiation inactivation studies of AE1 further showed that dimers are the basic unit *in situ* of anion

transport (Cuppoletti et al., 1985). These data provided strong evidence that AE1 is not monomeric in the erythrocyte membrane.

1.5.1.3 Crystal structure analysis

The membrane domain and cytoplasmic domain of AE1 have been crystallized separately. The low resolution (20 Å) two dimensional crystal structure of the membrane domain clearly showed that the membrane domain exists as a homodimer in the lipid bilayer (Wang et al., 1993). The 2.6 Å crystal structure of the cytoplasmic domain also revealed that the cytoplasmic domain forms a tight symmetric dimer, stabilized by interlocked dimerization arms contributed by both monomers (Zhang et al., 2000). Thus, the crystal structures of both domains of AE1 support the conclusion that the major form of AE1 in the cell membrane is a dimer.

1.5.1.4 Cross-linking analysis

Early studies by Steck showed that AE1 could be cross-linked into a covalent (-S-S-) dimer in isolated erythrocyte membranes by mild oxidation of Cu^{2+}-orthophenanthroline (Steck, 1972). Trimers and tetramers were also observed in the study. Formation of dimers and tetramers is experimental condition dependent. Cross-linking performed at 0°C on spectrin-depleted membranes yielded only dimers (Reithmeier and Rao, 1979), whereas at room temperature, tetramers were the major product (Wang and Richards, 1974). Further study revealed that the disulfide bond is formed between cysteine 201 of one subunit and cysteine 317 of the paired subunit in the cytoplamic domain (Thevenin et al., 1989). Cross-link of the two residues by disulfide bond formation can only take place in solutions of pH 7.5 and higher (Salhany et al., 1998).

Cross-linking of AE1 was also studied by using membrane-permeant homobifunctional reagents. Treatment of intact erythrocytes with dimethyl-3.3'-dithiobispropionimidate (Wang and Richards, 1975) or a spin labeled *bis*-(N-hydroxysuccinimide ester) (Willingham and Gaffney, 1983) only produced dimers; with maleimidomethyl-3-maleimido propionate (Sato and Nakano, 1981) both dimers and small amount of trimers were produced; photoactivated cross-linker 4,4'-dithiobiphenylazide (Mikkelsen and Wallach, 1976) also were dimers and tetramers. To explore if AE1 could be cross-linked from the extracellular surface, a hydrophilic membrane impermeant reagent, bis(sulfosuccinimidyl)suberate, was applied to intact cells. Results showed only dimers were produced (Jennings and Nicknish, 1985; Staros, 1982; Staros and Kakkad, 1983; Staros et al., 1981).

To argue that AE1 dimerizaion is not formed by random collision, a photoactivatable cross-linker, ethyl 4-azidobenzimidate, with a half-life in the millisecond timescale was applied to the cross-linking study, and subsequently showed the main cross-linking product was dimer (Kiehm and Ji, 1977). Two other photoactivated reagents di-N-(2-nitro-4-azidophenyl)cystamine-S-S-dioxide (Huang and Richards, 1977) and p-azidophenylisothiocyanate (Sigrist et al., 1982), were also found to cross-link AE1 completely to dimers. The fact that covalent AE1 dimers are formed within milliseconds is strong evidence that the dimer is not formed by random collision (Ji and Middaugh, 1980).

1.5.1.5 Isolated AE1 analysis

AE1 exists as noncovalent dimers in Triton X-100, a mild non-ionic detergent (Clarke, 1975; Yu and Steck, 1975). Electrophoresis of purified AE1 in nonionic

detergent ($C_{12}E_9$ and Triton X-100) showed that AE1 migrates as distinct dimers and tetramers (Nakashima et al., 1981). Upon mixing of two populations of AE1 (one labeled with BIDS and the other with eosin maleimide) in Triton X-100 for 24 hr at room temperature, no exchange of partners was detected (Macara and Cantley, 1983). All these suggest that AE1 dimer is very stable in the nonionic detergent. In the mean time, conflicting results were also reported, that freshly prepared purified AE1 could equilibrate reversibly as monomer/dimer/tetramer in nonionic detergent (Pappert and Schubert, 1983; Schubert et al., 1983). To examine if the AE1 dimer is an artifact resulting from time-dependent, detergent-mediated oxidation, and if AE1 is in a monomer/dimer/tetramer equilibration in the detergent solution (Pappert and Schubert, 1983; Schubert et al., 1983), size exclusion HPLC analysis was performed on freshly prepared AE1 (Casey and Reithmeier, 1991). AE1 protein was dissolved from erythrocyte membrane by a mild non-ionic detergent $C_{12}E_8$ and eluted from size exclusion HPLC column. Results confirmed that AE1 dimer is not an artifact, and 70% of AE1 population exists as a dimer and 30% exists as a tetramer and high oligomeric forms in the detergent solution. The study also demonstrated that the AE1 oligomeric forms do not equilibrate in $C_{12}E_8$, and the monomers can only be produced by protein denaturation.

1.5.2 AE1 oligomerization and function

The major form of AE1 in the erythrocyte membrane is dimer, but each monomer performs the anion exchange function independently. Irreversible inhibition of one monomer by DIDS or H_2DIDS does not affect the capability of the paired monomer to carry out the transport function (Lepke et al., 1976; Macara and Cantley, 1981; Ship et

al., 1977; Wieth, 1979). However, DIDS only binds to dimeric, not monomeric AE1, suggesting the functional structure of each monomer depends on the dimerization (Boodhoo and Reithmeier, 1984). A fluorescence resonance energy transfer study revealed the distance between the transport sites of each monomer is very close, suggesting they may share a common cavity between the subunits for the access of substrates to the transport sites (Macara and Cantley, 1981). Thus, the dimer has been proposed to be the basic functional unit of AE1 in the cell membrane. Interestingly, a reconstitution study using purified AE1 monomer claimed that the basic functional unit of AE1 is a monomer in the membrane, but it has not been confirmed (Lindenthal and Schubert, 1991).

The oligomerization state of erythrocyte membrane AE1 is influenced by its attachment to the cytoskeleton. Most studies conclude that ankyrin preferentially binds to tetrameric AE1, with little or no affinity for the dimer (Casey and Reithmeier, 1991; Che et al., 1997; Chen and Barkley, 1998; Thevenin and Low, 1990; Yi et al., 1997). Addition of ankyrin to the erythrocyte membrane could force AE1 to form tetramers (Che et al., 1997; Salhany et al., 1998; Yi et al., 1997). Thus, AE1 tetramers are proposed to be critical in the formation and maintenance of the biconcave shape of erythrocytes. The higher order oligomerization of AE1 was observed to be associated with the Heinz bodies in the erythrocytes, and with the circulating antibodies in blood circulation (Low et al., 1985; Kannan et al., 1991; Schlüter and Drenckhahn, 1986).

1.6 Structure of AE1

1.6.1 Structure of cytoplasmic domain

1.6.1.1 Functional role of cytoplasmic domain

The cytoplasmic domain of human AE1 functions as a major organizing center of the erythrocyte cell membrane (Low, 1986; Zhang et al., 2000). It offers anchoring sites for the membrane-associated proteins in the erythrocytes, including ankyrin (Bennett and Stenbuck, 1980), protein 4.2 (Cohen et al., 1993; Rybicki et al., 1996), protein 4.1 (An et al., 1996; Pasternack et al., 1985), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rogalski et al., 1989), phosphofructokinase (Jenkins et al., 1984), aldolase (Murthy et al., 1981), hemoglobin (Salhany and Cassoly, 1989; Walder et al., 1984), hemichromes (Waugh and Low, 1985), and the protein tyrosine kinase ($p72^{syk}$) (Harrison et al., 1994). Interaction of AE1 with these proteins has important structural and functional consequences for the cell, including cell flexibility and shape control (Low et al., 1991), glucose metabolism regulation (Low et al., 1993), ion transport (Malik et al., 1993), as well as cell lifespan (Kannan et al., 1991). Mutations in the cytolasmic domain alter the interaction of AE1 with ankyrin that lead to erythrocyte spherocytosis (Jarolim et al., 1996; Jarolim et al., 1992).

1.6.1.2 Interaction of cytoplasmic domain with peripheral proteins

Ankyrin is the bridging protein between AE1 and the spectrin-actin cytoskeleton. Interaction of ankyrin with AE1 tethers the AE1 dimers to form tetramers (Che et al., 1997; Salhany et al., 1998; Yi et al., 1997). The ankyrin binding site on the cytoplasmic domain has been mapped to the flexible extreme N-terminus (Davis et al., 1989; Ding et al., 1994; Ding et al., 1996; Wang et al., 1995; Willardson et al., 1989) and the residues

174-186 (Davis et al., 1989), which correspond to the hairpin structure in the crystal structure (Zhang et al., 2000). Recent studies suggested that residues 174-186 constitute the major ankyrin binding site, while the extreme N-terminus is not required (Chang and Low, 2003). Protein 4.1 is another peripheral protein that associates with AE1 and spectrin-actin. The interaction sites have been assigned to the extreme N-terminus (Lombardo et al., 1992) and the LRRRY sequence (residues 434-436) (Jons and Drenckhahn, 1992) in the cytoplasmic domain. The LRRRY sequence is believed to interact with an oppositely charged LEEDY peptide in protein 4.1 (Jons and Drenckhahn, 1992). Association of protein 4.2 with AE1 has been proposed to involve residues Glu40, Gly130 and Pro327, as their mutations lead to a protein 4.2 deficiency and spherocytosis (Inoue et al., 1998; Jarolim et al., 1992; Rybicki et al., 1993). The glycolytic enzymes, deoxyhemoglobin, and hemichromes all bind to the extreme N-terminus of the cytoplasmic domain (Tsai et al., 1982; Walder et al., 1984; Waugh and Low, 1985). As erythrocytes contain sufficient molecules of AE1 to bind all copies of ankyrin, protein 4.1 and other protein simultaneously (Low, 1986), it seems unlikely that competition for binding would regulate the protein complex assembly.

1.6.1.3 Crystal structure of AE1 cytoplasmic domain

The cytoplasmic domain of AE1 has been crystallized to 2.6 Å resolution at pH 4.8 (Zhang et al., 2000). The crystallized cytoplasmic domain is a homodimer. The monomeric unit has a compact structure with a dimension of approximately 43 x 45 x 53 Å. It is composed of 11 β-strands and 10 α-helices, which belongs to the α + β-fold class. Eight of the β-strands assemble into a central β-sheet of mixed parallel and antiparallel strands. Two remaining strands form a β-hairpin structure (residues 176-

185), together with the first 6 helices assemble into a globular domain spanning residues 55-290. The dimerization arm is composed mainly of helices and a single β-strand, composed of residues 304-357. The β-strand forms an interlocked structure with another β-strand from the paired monomer, which plays a critical role in the formation of dimer. The dimerization arm is connected to the large globular domain by a short helix and loop segment, consisting of residues 291-303. Residues 1-54, 202-211, and 357-379 were poorly defined in the crystal structure. The inability to resolve these residues was assumed to be due to regional flexibility, as the cytoplasmic domain was shown to be a highly flexible molecule (Low et al., 1984; Thevenin et al., 1994; Zhang et al., 2000).

1.6.2 Structure of AE1 membrane domain

1.6.2.1 Functional role of membrane domain

The membrane domain of AE1 functions as an anion exchanger in the erythrocyte, where it performs one to one electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane (Grinstein et al., 1978; Jay and Cantley, 1986; Jennings, 1989b; Lepke and Passow, 1976; Passow, 1986). It also forms protein complexes by associating with several integral and peripheral proteins to regulate its transport activity (Bruce et al., 2003b; Sterling et al., 2002; Sterling et al., 2001). Increasing evidence support the view that AE1 has an important role in organizing the integral membrane proteins in the erythrocyte plasma membrane (Bruce et al., 2003a).

1.6.2.2 Interaction of membrane domain with peripheral and integral proteins

1.6.2.2.1 Carbonic anhydrase II

The major two isoforms of carbonic anhydrase (CA) in human erythrocytes are CAI (about 85%) and CAII (about 15%) (Sly and Hu, 1995). CAII is the most catalytically active form of CA in erythrocytes (Sly and Hu, 1995). Several lines of evidence suggested that CAII interacts with the C-terminus of AE1. First, lectin-induced clustering of AE1 also induced clustering of CAII in the erythrocyte membranes; second, CAII could be co-immunoprecipitated with AE1, but only by the antibody that recognizes the N-terminal epitopes; third, the interaction could be blocked by an antibody against the C-terminus of AE1; fourth, the glutathione-S-transferase (GST) AE1 C-terminus fusion protein bound CAII at a stoichiometry of approximately 1 to 1; finally, affinity blotting assays confirmed that CAII but not CAI can bind the C-terminus of AE1 (Vince and Reithmeier, 1998). Subsequent studies using truncation and point mutations of the C-terminus of AE1 identified the CAII binding site in AE1 as the acidic motif L886DADD890 (Vince and Reithmeier, 2000). Recent work demonstrated that interaction of AE1 with CAII activates the AE1 HCO_3^- transport rate (Sterling et al., 2001).

1.6.2.2.2 Carbonic anhydrase IV

CAIV is a glycosylphosphatidylinositol-anchored extracellular plasma membrane protein, which reversibly hydrates CO_2 on the extracellular space of the human erythrocytes (Waheed et al., 1992). CAIV has recently been shown to interact with AE1 to form a physical complex (Sterling et al., 2002). CAIV localizes in lipid rafts of the cell membrane which are rich in sphingolipids and cholesterol (Brown and Rose, 1992). Sucrose gradient sedimentation studies revealed that when CAIV is co-expressed with AE1, it shifts from its original fraction to the AE1 fraction, which suggests that AE1

interacts with CAIV physically (Sterling et al., 2002). Gel overlay assays also showed interaction of CAIV with AE1 expressed in HEK293 cells. Further analysis using GST fusion proteins of AE1 extracellular loop 3 and 4 fusion protein found CAIV could bind specifically to the fourth extracellular loop of AE1, and the interaction site was proposed to be the region of Arg656 – Ile661 (Sterling et al., 2002). Interaction between CAIV and AE1 activated the AE1 HCO_3^- transport rate.

1.6.2.2.3 Glycophorin A

Glycophorin A (GPA) is an integral erythrocyte membrane sialoglycoprotein responsible for the negative charge of the erythrocyte membrane (Poole, 2000). GPA is a single transmembrane segment protein that is present at similar copy numbers as AE1 in the erythrocyte (Anstee, 1990; Cartron and Rahuel, 1992; Poole, 2000). Increasingly evidence supports the notion that GPA interacts with AE1 in the erythrocyte membrane. Binding of anti-GPA antibody to the extracellular domain of GPA decreased the rotational and lateral mobility of AE1 in the erythrocyte membrane (Knowles et al., 1994; Nigg et al., 1980). The expression of blood group antigen Wr^b requires the presence of both GPA and AE1 (Ring et al., 1994; Telen and Chasis, 1990), and anti-Wr^b antibody could co-immunoprecipitate both GPA and AE1 from the erythrocyte membrane (Telen and Chasis, 1990). In addition, movement of nascent AE1 to the cell surface was facilitated by GPA in the Xenopus oocytes (Groves and Tanner, 1992; Groves and Tanner, 1994a). AE1 knockout mice do not express either AE1 or GPA, although the erythroid precursors contain GPA mRNA, indicating that AE1 is also involved in the movement of GPA to the cell surface (Hassoun et al., 1998). The interaction between GPA and AE1 in the membrane has been proposed to occur *via* Glu658 of AE1 (Bruce et

al., 1995; Huang et al., 1996). The GPA associated AE1 has recently been reported to have higher transport activity than AE1 expressed alone (Bruce et al., 2003b).

1.6.2.2.4 Rh complex

A recent study showed that AE1 interacts with the Rh complex in the erythrocyte membrane to form a macro functional protein complex (Bruce et al., 2003a). Rh complex, one of the major blood group antigens in erythrocyte membranes, is composed of Rh-associated glycoprotein (RhAG), Rh polypeptides, glycophorin B (GPB), CD47 and LW (Cartron, 1999; Cartron and Colin, 2001). Immunoprecipitation of AE1 by anti-AE1 antibody also co-immunoprecipitated the Rh complex from normal erythrocyte membranes, suggesting that AE1 is associated with Rh complex to form a single macrocomplex. This interaction is also supported by some previous reports. In the Southeast Asian Ovalocytosis erythrocytes, where AE1 hetero-dimers are formed of normal AE1 and mutant AE1 with a 9-amino acid deletion, a decreased Rh blood group antigen expression was observed (Booth et al., 1977). Co-expression of AE1 and Rh polypeptide in K562 erythroleukemia cells enhanced both expression and cell surface reactivity of the Rh antigens (Beckmann et al., 1998; Beckmann et al., 2001). More recently, Rh complex was shown to link to the cytoskeleton *via* the interaction with AE1 in the erythrocyte membrane (Bruce et al., 2002). These studies suggest that the membrane domain of AE1 also plays an important role in organizing the integral membrane proteins in the erythrocyte.

1.6.2.3 Topology of AE1 membrane domain

The topology of the membrane domain of AE1 has been examined by a variety of approaches. Determination of the topology of the membrane domain will help to locate

the anion transport site, understand the transport mechanism and the human diseases that are derived from mutations or deletion of the AE1 gene.

1.6.2.3.1 Hydropathy plot analysis

Hydropathy plot analysis is a proven approach to predict the transmembrane helices of the integral membrane proteins. A transmembrane helix is an energetically favorable structure for a sequence of hydrophobic amino acid residues. An alpha helix of about 20 residues is sufficiently long to span a lipid bilayer if it is oriented perpendicular to the lipid bilayer. Based on the theoretical tendency for a hydrophobic sequence to form an alpha helix in a lipid bilayer, Kyte and Doolittle published a method for representing the average hydrophobicity of each sequence composed of N residues in a large sequence (Kyte and Doolittle, 1982). The sequences with high hydropathy are more likely to exist as a membrane spanning helix in the method. The deduced amino acid sequence of cloned human AE1 reveal that the protein consists of 911 amino acids (Lux et al., 1989; Tanner et al., 1988). Hydropathy analysis on the protein sequence showed there are 10 strong hydrophobic peaks in the C-terminal portion, which suggests that the membrane domain contains at least 10 transmembrane helices (Lux et al., 1989; Tanner et al., 1988). Hydrophobic peaks 2, 8, 9 and 10 are quite broad, suggesting they may be composed of two tightly associated helices separately (Figure 1.4). Thus, the membrane domain was proposed contain up to 14 TMs. Although detailed information, such as orientation of the TMs, size or location of the connecting loops, cannot be provided by this method, it offers a framework for interpreting the past and future studies on the topology of the protein.

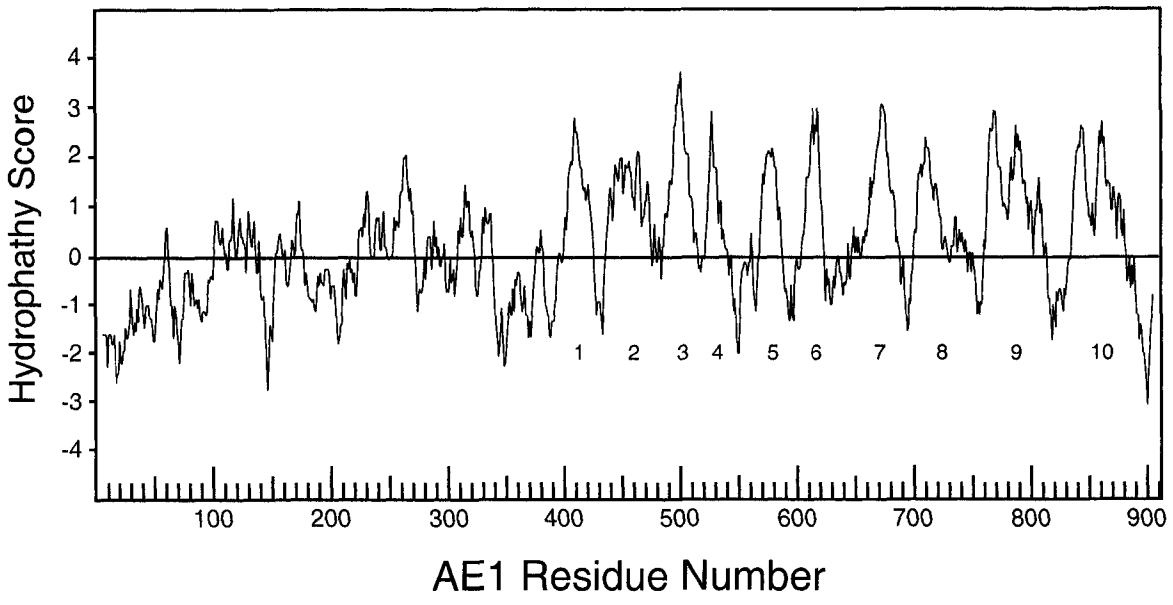


Figure 1.4 Kyte-Doolittle hydropathy analysis of the deduced amino acid sequence of human AE1

Numbers in the plot indicate the ten strong hydrophobic peaks of the membrane domain of AE1. The plot is generated with DNA Strider 1.2 software; running average window is 10 amino acids.

1.6.2.3.2 Proteolytic analysis

AE1 protein is extremely susceptible to trypsin cleavage at an intracellular site Lys360 in the physiological strength medium, yielding a soluble 41 kDa fragment and a membrane bound 55 kDa fragment (Mawby and Findlay, 1982; Steck et al., 1976), which are respectively the N-terminal cytoplasmic domain and the C-terminal membrane associated domain (Drickamer, 1978; Jenkins and Tanner, 1977). The membrane domain gives a diffuse band on gel electrophoresis because of an oligosaccharide at Asn642 (Jay, 1986).

1.6.2.3.2.1 Proteolysis of AE1 in intact erythrocytes

Human AE1 is resistant to trypsin digestion in intact erythrocytes or resealed erythrocyte ghosts under physiological strength medium, but can be cleaved at Lys562 on the extracellular surface in an isosmotic medium of low ionic strength (Brock et al., 1983; Jenkins and Tanner, 1977). Application of chymotrypsin to intact erythrocytes cleaves AE1 at Tyr553, which is quite close to the extracellular trypsin site (Drickamer, 1976; Jennings and Nicknish, 1985). Pronase, thermolysin and subtilisin also cleave close to this site under similar conditions (Cabantchik and Rothstein, 1974; Jenkins and Tanner, 1975). These studies suggest that the region around Tyr553 is highly susceptible to protease action from the outside surface of the cell, and that it makes up an extracellular loop in the protein. Papain produces a somewhat different cleavage pattern of AE1 in the intact erythrocyte, cutting at three extracellular sites, Lys551, Gln564 and Gln630 (Jennings and Adams, 1981; Jennings and Nicknish, 1985). The cleavages release a small peptide from Lys551 to Gln564, a membrane bound 7 kDa fragment and a glycosylated 28 kDa fragment. Digestion of sealed erythrocyte ghosts by pepsin yields

several membrane bound fragments (Tanner et al., 1979), including a sequenced fragment P5, which contains 72 amino acids from Met559 to Gln630 (Brock and Tanner, 1986). This fragment was proposed to form two transmembrane helices on the basis of the hydrophobicity of the amino acid sequence. Asn642 is the only glycosylated site of AE1 (Lux et al., 1989; Tanner et al., 1988). Thus, the Gln630 to Asn642 region is on the extracellular surface of the protein.

1.6.2.3.2.2 Proteolysis of AE1 in unsealed leaky erythrocyte membranes

In unsealed leaky erythrocyte membranes, a new trypsin cleave site was obtained at Lys743 of AE1 in low ionic strength solution (Williams et al., 1979). Further study showed this cleavage could be generated by trypsin trapped inside resealed erythrocyte ghosts; thus, Lys743 was suggested to be intracellular (Jennings et al., 1986). Digestion of ghost membranes by pepsin produced a cleavage at Ile397, suggesting it is an intracellular site (Brock and Tanner, 1986). Application of moderate concentrations of chymotrypsin to unsealed red cell membrane not only cleaves at Tyr553, but also cleaves at a cytoplasmic site Tyr359 (Grinstein et al., 1979; Mawby and Findlay, 1982). The N- and C-terminal membrane domain fragments produced by this digestion are usually described as the 17 and 35 kDa fragments on the basis of their apparent molecular weight on SDS-PAGE. Thermolysin also cleaves after Gly361 on the cytoplasmic side of the membrane (Brock and Tanner, 1986). Thus, both chymotrypsin and thermolysin cytoplasmic cleavage sites are close to the trypsin site at Lys360, which suggests the region around Lys360 is probably not highly-structured, forming a hinge area between the membrane and cytoplasmic domain of AE1 protein. Figure 1.5 summarized all the sites of *in situ* proteolysis.

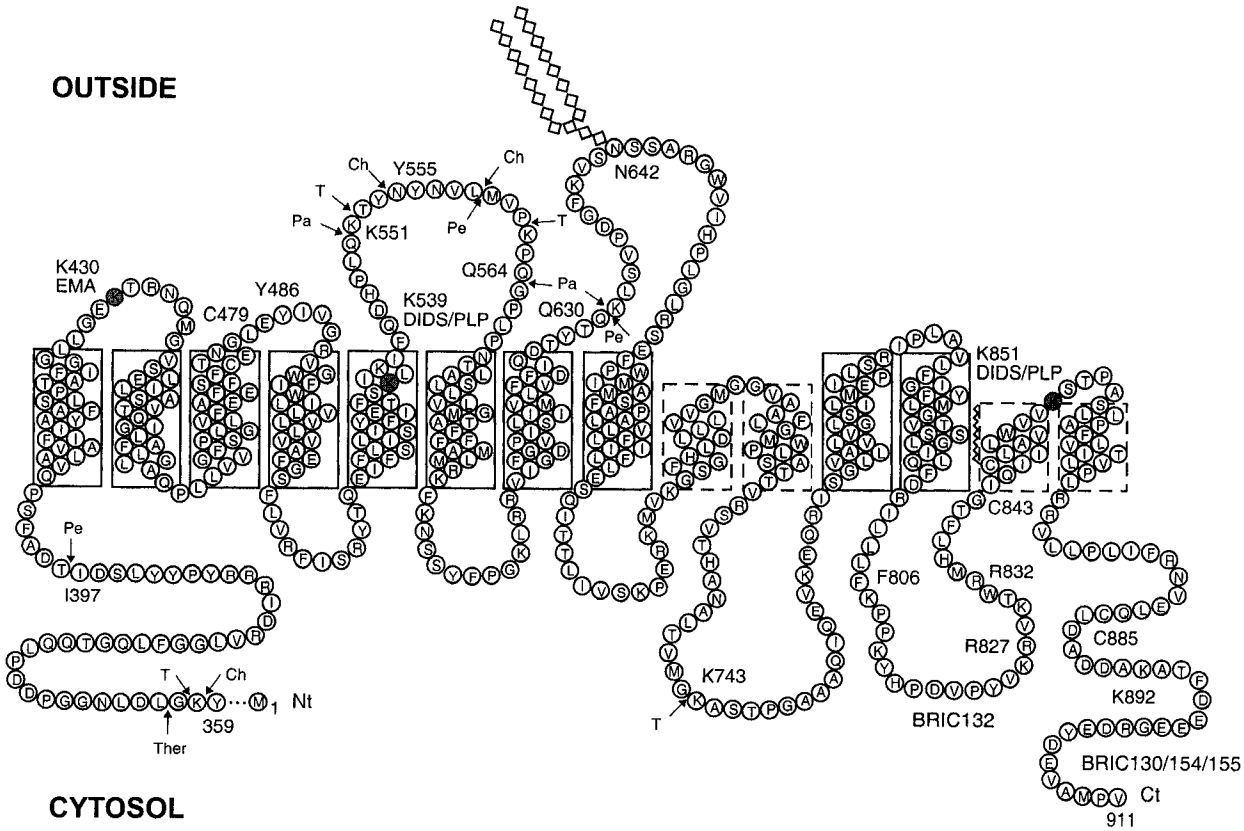


Figure 1.5 Topology model of the membrane domain of AE1 based on hydropathy analysis, chemical and antibody labeling and *in situ* proteolysis

The model contains 14 TMs. The first eight TMs are well established, while the region after TM8 is uncertain. TMs shown in dashed outline may not be exposed to the lipid bilayer. The single site of glycosylation is indicated by diamond and C843 is palmitoylated. Nt, amino terminus; Ct, carboxyl terminus. T, trypsin; Ch, chymotrypsin; Pa, papain; Pe, pepsin; Ther, thermolysin. BRIC, epitopes of AE1 C-terminal monoclonal antibodies. The shadowed residues represent either EMA or DIDS labeled sites. (Modified from Wang, 1994)

1.6.2.3.2.3 Proteolysis of AE1 in alkaline-stripped erythrocyte ghosts

Erythrocyte membranes contain integral membrane proteins and peripheral proteins. AE1 becomes susceptible to extensive cleavage by proteases when peripheral proteins are stripped by high concentrations of NaOH. Alkaline treatment of erythrocyte membranes results in denatured AE1 protein. Digestion of stripped ghost membrane with chymotrypsin produces a 15 kDa and a 8 kDa membrane bound fragment (Ramjeesingh et al., 1983; Ramjeesingh et al., 1980). The 8 kDa fragment contains two cysteine residues, similar to the C-terminal tail of AE1, and the cleavage site was proposed at either of Tyr818 or Tyr824 (Tanner, 1989). Extensive digestion of stripped ghost membranes with high concentrations of papain or pepsin only leaves several membrane-spanning portions of the protein of about 4 kDa (Falke et al., 1985b; Ramjeesingh et al., 1984). The remaining portions represent the transmembrane helices of AE1.

1.6.2.3.3 Chemical labeling

Some useful topology information was obtained from the chemical labeling of certain specific amino acids on the surface of AE1 protein by membrane impermeant reagents. DIDS is a covalent inhibitor of human AE1. Application of DIDS on the extracellular surface inhibits anion exchange activity. The covalent labeling sites for DIDS have been localized to Lys539 and Lys851 (Okubo et al., 1994). Pyridoxal phosphate, another AE1 inhibitor, also labels Lys539 and 851 from the extracellular medium (Kawano et al., 1988; Nanri et al., 1983; Tanner, 1989). Thus, it is clear that regions around Lys539 and 851 are extracellular medium accessible. Lys430 is labeled by reductive methylation of intact erythrocytes (Jennings and Nicknish, 1984). Eosin maleimide also covalently binds to Lys430 (Cobb and Beth, 1990), suggesting that the

first loop is extracellular. Reductive methylation also takes place on another two lysines, one is Lys551 located next to the extracellular chymotryptic cleavage site, the other is Lys539, one of the DIDS binding sites, which further confirms their extracellular location. Modification of an extracellular arginine residue by phenylglyoxal results in labeling of the C-terminal 8 kDa chymotryptic fragment (Bjerrum et al., 1983). The only two arginine residues in this region are Arg827 and 832, thus, this region is extracellularly accessible (Tanner et al., 1988).

The membrane domain of AE1 contains three cysteine residues (Lux et al., 1989; Tanner et al., 1988). Cys479 was not reactive with membrane permeable N-ethylmaleimide, but reacted with membrane impermeable pCMBS (Solomon et al., 1983) or eosin-maleimide (Macara et al., 1983) in intact erythrocytes, suggesting it is located on the extracellular surface. Cys843 and 885 were not reactive when intact erythrocytes are treated with an impermeant maleimide but are reactive when erythrocyte ghosts are reacted with N-ethylmaleimide or when inside out erythrocyte vesicles are exposed to the impermeant maleimide (Ramjeesingh et al., 1981; Rao, 1979). Thus, Cys843 and 885 are located to the intracellular surface of the membrane. Later studies revealed that Cys843 is palmitoylated in the erythrocyte membranes, which does not allow any modification to take place (Kawano et al., 1988). This discrepancy needs to be resolved.

Lactoperoxidase-catalysed radioiodination coupled with protease fragmentation was also used to determine the location of tyrosine residues in the membrane domain of AE1 protein (Boxer et al., 1974; Jenkins and Tanner, 1977; Tanner et al., 1979; Williams et al., 1979). Application of lactoperoxidase and ^{125}I on the intact erythrocytes revealed, Tyr553, 486 and 628 were radioiodinated, suggesting their extracellular location. Tyr519

and 596 were not radioiodinated, suggesting they are located in the cytoplasmic side of AE1. Three C-terminal residues, Try818, 824 and 904 were radioiodinated, but their locations could not be resolved by this approach. They were assigned to the cytoplasmic surface of the protein, as they are located within a relative basic peptide (Tanner, 1989; Williams et al., 1979). A conflicting experimental result using the same approach surprisingly reported that Tyr486 is located on the intracellular surface of AE1 (Kalo, 1996). Jennings pointed out that this approach has a major defect. Even though the enzyme itself is membrane impermeable, the reactive iodine species readily passes through the membrane (Jennings, 1989c). Thus, it could be difficult to interpret the results.

1.6.2.3.4 Insights from immunological analyses

Although proteolytic analysis, chemical labeling and hydropathy plots have painted a clear folding picture of the first half of the membrane domain of AE1 (TM1-8), the C-terminal region is still vague. Hydropathy plots suggested that the extreme C-terminal tail is either in the cytosol or on the extracellular surface of the erythrocyte (Kopito and Lodish, 1985).

Reithmeier and co-workers first explored the location of the C-terminus of AE1 by using carboxypeptidase Y digestion (Lieberman et al., 1987). Carboxypeptidase Y sequentially removes amino acids from the carboxyl terminus of a protein. The C-terminus was observed to remain when carboxypeptidase Y was applied to the intact erythrocyte whereas it was digested when the enzyme was applied to ghost membranes or inside-out vesicles, suggesting it is on the cytoplasmic side of the erythrocyte membrane. Further immunofluorescence and competition studies using a C-terminus specific

antibody also support the intracellular location of the C-terminus (Lieberman and Reithmeier, 1988).

Tanner and co-workers mapped the topology of the C-terminus by using a series of murine monoclonal antibodies, BRIC 130, 132, 154 and 155 (Wainwright et al., 1989). All the antibodies reacted with AE1 in the western blot assay, but none of them could agglutinate intact human erythrocytes directly, or indirectly with the addition of rabbit anti-mouse immunoglobulin, suggesting that all the epitopes are not exposed on the extracellular surface of the erythrocytes. Further characterization of the epitopes by synthetic peptide mapping revealed, BRIC 130 and BRIC 154/155 recognize amino acids 899-908 and 895-901, and BRIC 132 recognizes amino acids 813-824, respectively, in the C-terminal region of AE1 (Wainwright et al., 1990a). Surprisingly, BRIC 132 epitope is completely resistant to the digestion of erythrocyte membrane by carboxypeptidase Y, whereas BRIC 130 and BRIC 154/155 epitopes can be totally destroyed, suggesting BRIC 132 epitope is located in an internal loop and the others are located on the extreme C-terminal tail. Immunoprecipitation with BRIC antibodies against intact red cells and leaky cell membranes showed only the AE1 in leaky membranes could be recognized (Wainwright et al., 1989). Thus, the C-terminal region of AE1 was proposed to be composed of an intracellular loop, a cytoplasmic tail, and a region that tranverses the membrane at least twice in between (Wainwright et al., 1990a).

1.6.2.3.5 Scanning and insertional N-glycosylation mutagenesis

Topology of the membrane domain of AE1 has been studied by scanning N-glycosylation mutagenesis, with proteins expressed in a cell free system (consisting of reticulocyte lysate and microsomal membrane) (Popov et al., 1999; Popov et al., 1997).

The approach is based on the fact that only the endoplasmic reticulum (ER) exposed acceptor site (Asn-X-Ser/Thr) can be N-glycosylated during biosynthesis. Successful N-glycosylation requires the acceptor site to be in a loop larger than 25 residues in size, with acceptor sites located greater than 12 residues away from the proceeding TM and greater than 14 residues away from the following TM (Popov et al., 1997).

To study AE1 topology, introduced glycosylation acceptor loops were introduced into an AE1 mutant otherwise devoid of acceptor sites (N642D). AE1 is N-glycosylated at Asn642, which clearly indicates the extracellular location of the putative extracellular loop 4. N-glycosylation studies of reintroduced glycosylation acceptor sites revealed that putative extracellular loop 4 was highly glycosylated, loop 3 was poorly glycosylated, and surprisingly, putative intracellular loop 5 was highly glycosylated in the cell free translation system. Further analysis using inserted extracellular loop 4 as glycosylation reporter demonstrated, that putative extracellular loops 1, 2 and 7 could also be glycosylated in the cell free system (Popov et al., 1997). A similar pattern of N-glycosylation was observed when the N-glycosylation mutants were expressed in the human embryonic kidney 293 or COS 7 cells, except the putative intracellular loop 5 was not glycosylated (Popov et al., 1999). This suggests that intracellular loop 5 may be transiently exposed to the lumen of the ER during the biosynthesis but normally folds rapidly, precluding the N-glycosylation. Thus, the studies proposed that putative extracellular loop 1, 2, 3, 4 and 7 are on the extracellular surface of the cell membrane, the putative intracellular loop 5 forms a unique re-entrant "T" loop as shown in figure 1.6. Extracellular loop 3 contains 25 residues from Phe544 to Pro568, and loop 4 contains 39 residues from Ile624 to Met663. They are the two largest loops on the

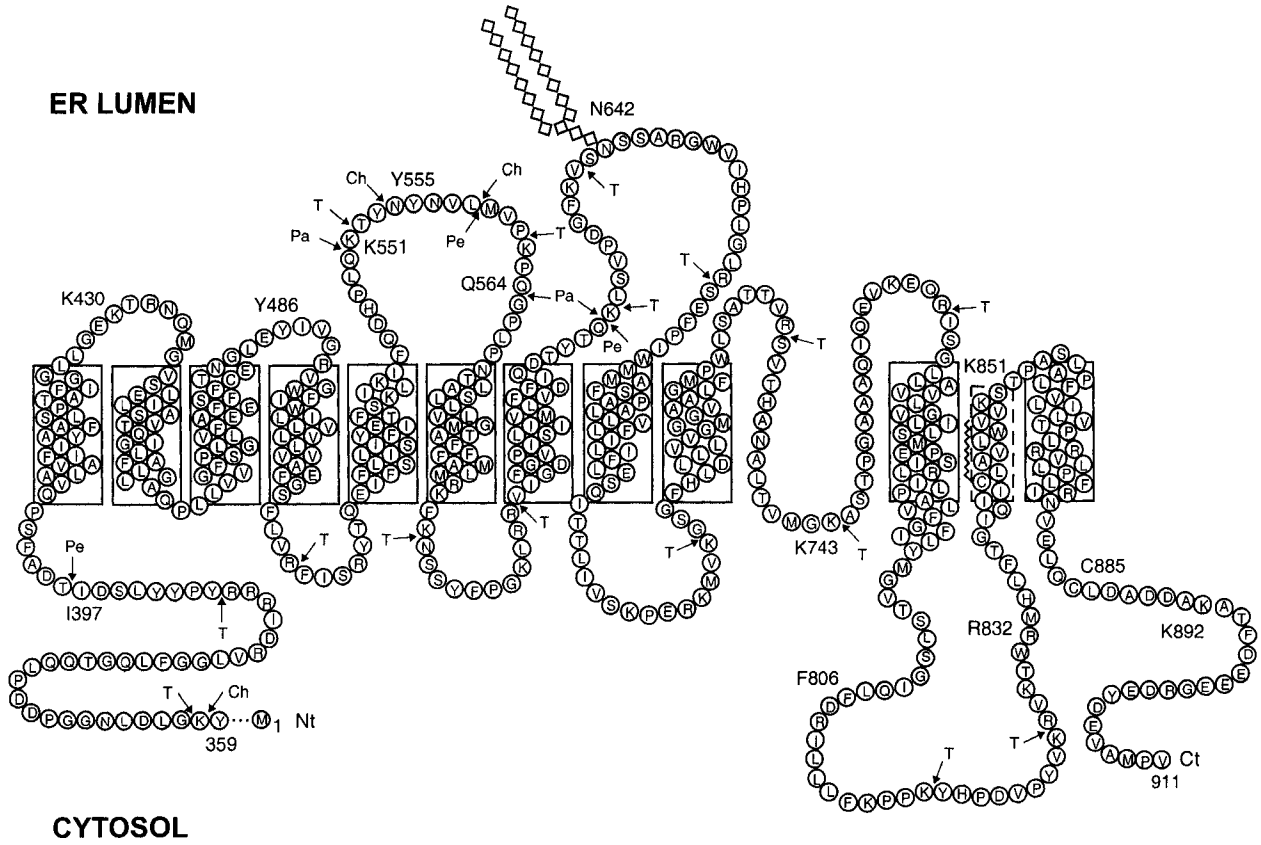


Figure 1.6 Modified topology of the membrane domain of AE1, determined by N-glycosylation scanning mutagenesis

The model contains of 12 TMs with a re-entrant loop between TM9 and 10. C843 is shown as palmitoylated. Nt, amino terminus; Ct, carboxyl terminus. Arrows represent accessible proteolytic sites under different conditions, most of which are in denatured protein. T, trypsin; Ch, chymotrypsin; Pa, papain. (Adapted from Popov et al., 1997)

extracellular surface of AE1, whereas the others are quite small. Intracellular loop 5 contains 42 residues from Pro722 to Leu764.

Topology of the C-terminal region after the "T" loop was not resolved clearly by this approach, especially the location of the long stretch of hydrophobic amino acids between TM10 and 11. Insertion of extracellular loop 4 at position of Asp821 in the putative intracellular loop 6 produced 16% glycosylation in the cell free system, whereas no glycosylation in the cultured cells. Thus, location of this loop in the protein structure is still uncertain (Popov et al., 1997; Popov et al., 1999).

The proposed "T" loop was supported by a recent study using *in situ* proteolysis on intact erythrocytes, resealed ghosts, unsealed ghosts and microsomes prepared from HEK 293 cells. Lys743, which resides in the putative intracellular loop 5, can be cleaved from the cytoplasmic side of the membrane (Kuma et al., 2002b). Hamasaki and co-workers explored the mechanism that leads to the "T" loop formation during biosynthesis (Kanki et al., 2002). N-glycosylation efficiency of loop 5 in cultured cells increased to the same level as in the cell free system when TM12-14 or TM1-3 was deleted (Kanki et al., 2002). In addition, extending the exposure time of this loop in the ER by suppressing the chain elongation rate dramatically increased the glycosylation ratio of the introduced N-glycosylation site. The level of N-glycosylation showed a dose dependent manner on cycloheximide, a reagent that suppresses the chain elogation rate (Goder et al., 2000; Ogg and Walter, 1995). Thus, the "T" loop is initially exposed to the luminal side of ER during biosynthesis, and then inserted into the membrane after TM1-12 segments are properly assembled (Kanki et al., 2002).

N-glycosylation mutagenesis coupled with cell free system was also performed to examine the orientation of the N-terminus of truncated AE1 fragments in the lipid bilayer (Groves and Tanner, 1999b). Twenty-two additional amino acids from the extracellular loop 4 containing the N-glycosylation acceptor site were added to the N-terminus of the truncated peptide as an N-glycosylation reporter. If the N-terminus of the TM starts from the extracellular surface, the peptide will be glycosylated; if it starts from the cytosol, it will not be glycosylated. Putative extracellular loops 1, 2, 3 and 4 were well N-glycosylated in the cell free system, but surprisingly, predicted intracellular loop 4, the N-terminus of TM9-14 was also strongly glycosylated. This loop is clearly located in the cytoplasmic surface, as Asn642 is the only endogenous glycosylated site of AE1 protein. Analysis of further truncated small AE1 fragments produced more intriguing results. The N-terminal end of peptide 696-755 (putative TM9-10) could not be glycosylated in the cell free system, but highly glycosylated when it was extended to Tyr824 (putative TM9-12) or Val911 (TM9-14). The interaction of TM9-10 with TM11-12 region introduces a dynamic aspect or alters the structure of TM9-10. Also, the N-terminus of fragment TM7-14 was highly glycosylated when the endogenous Asn642 mutated to Ser, whereas it was poorly glycosylated when the endogenous Asn642 was kept, which suggests glycosylation of extracellular loop 4 may have strong influence on the correct orientation of TM7 in the bilayer. Consistent with Reithmeier and co-workers' report (Popov et al., 1997), the N-terminal end of TM13-14 was poorly glycosylated, and the putative intracellular loop 5 was highly glycosylated in the cell free system. Thus, the studies supported the topology proposed by Reithmeier and co-workers (Popov et al., 1997), and

further suggested that there are intramolecular interactions among the TMs. TM1, 4, 5, 6 and 8 might act as a scaffold for the assembly of TM2-3, 7 and 9-10.

1.6.2.3.6 Cysteine scanning mutagenesis

Cysteine scanning mutagenesis coupled with sulfhydryl specific reagents is an excellent approach for probing residues that line the substrate pathway of membrane channel or transport proteins. It accurately reported the residues that form the selectivity filter of the potassium channel (Doyle et al., 1998), and the residues that form the transport site of lactose permease (Abramson et al., 2003). It has also been applied on the topology studies of AE1 (Fujinaga et al., 1999; Tang et al., 1998). Native AE1 contains five endogenous cysteine residues; two of them (Cys201 and 317) are in the cytoplasmic domain and the other three (Cys476, 843 and 885) are in the membrane domain (Lux et al., 1989; Tanner et al., 1988). Mutation of the five endogenous cysteines to serine does not affect AE1 anion transport function (Casey et al., 1995). On the basis of this construct, a consecutive cysteine scanning mutagenesis study on TM8 (Tang et al., 1998) and a widespread cysteine substitution study on the C-terminal region after TM8 were performed (Fujinaga et al., 1999). The first study clearly defined the boundaries of TM8 as Met663 to Ile684. This positions Glu681, a residue that has been implicated to be involved in the anion translocation process (Jennings and Smith, 1992), at the intracellular rim of this helix. The study also revealed the extracellular loop 4 is folded to some extent on the surface of the protein, as it is not accessible to a membrane impermeable reagent. The second study dramatically modified the topology of the region after TM8. The region of "T" loop was not only strongly labeled by a membrane impermeable reagent, but was also accessible to the extracellular medium (Fujinaga et al.,

1999). This supports the topology from N-glycosylation study that this region is formed by a loop structure, not by a helix. Surprisingly, Asp821 predicted intracellular loop was accessible to the extracellular medium, and this finding introduced a transmembrane loop between TM11 and TM12 (Fujinaga et al., 1999) (Figure 1.7). Both studies support the location of the C-terminal tail of AE1 in the cytoplasmic side of the protein.

1.6.2.3.7 Insights from blood group antigens

Single-strand conformational polymorphism analysis, AE1 genomic DNA sequencing, and sequence comparison with human AE1 cDNA have identified many blood group antigens in AE1 (Poole, 2000). Blood group antigens are created when mutations occur on the extracellular surface of AE1. Bruce and co-workers first located the Di^a antigen to the last putative extracellular loop with a point mutation of P854L (Bruce et al., 1994). Subsequently, many point mutations that create blood antigens were localized to the AE1 protein. Antigen ELO was assigned to R432W on the first putative extracellular loop; Vg^a was assigned to Y555H, Bow to P561S, Wu to G565A, BP^a to N569L, all are on the third putative extracellular loop; Sw^a was assigned R646Q, Hg^a to R656C, Mo^a to R656H, Wr^a to E658L, all are on the fourth putative extracellular loop (Jarolim et al., 1998a; Poole, 2000). These studies further support a model with the first half of the membrane domain of AE1 composed of four extracellular loops and eight TMs, which is highly consistent with the biochemical analysis.

1.6.2.3.8 Insights from malaria parasite AE1/interaction

Overwhelming evidence supports the idea that the malaria parasite, *Plasmodium falciparum*, interacts with AE1 to invade the erythrocyte (Miller et al., 1983; Okoye and Bennett, 1985; Sherman et al., 2003). The interaction site of *Plasmodium falciparum* on

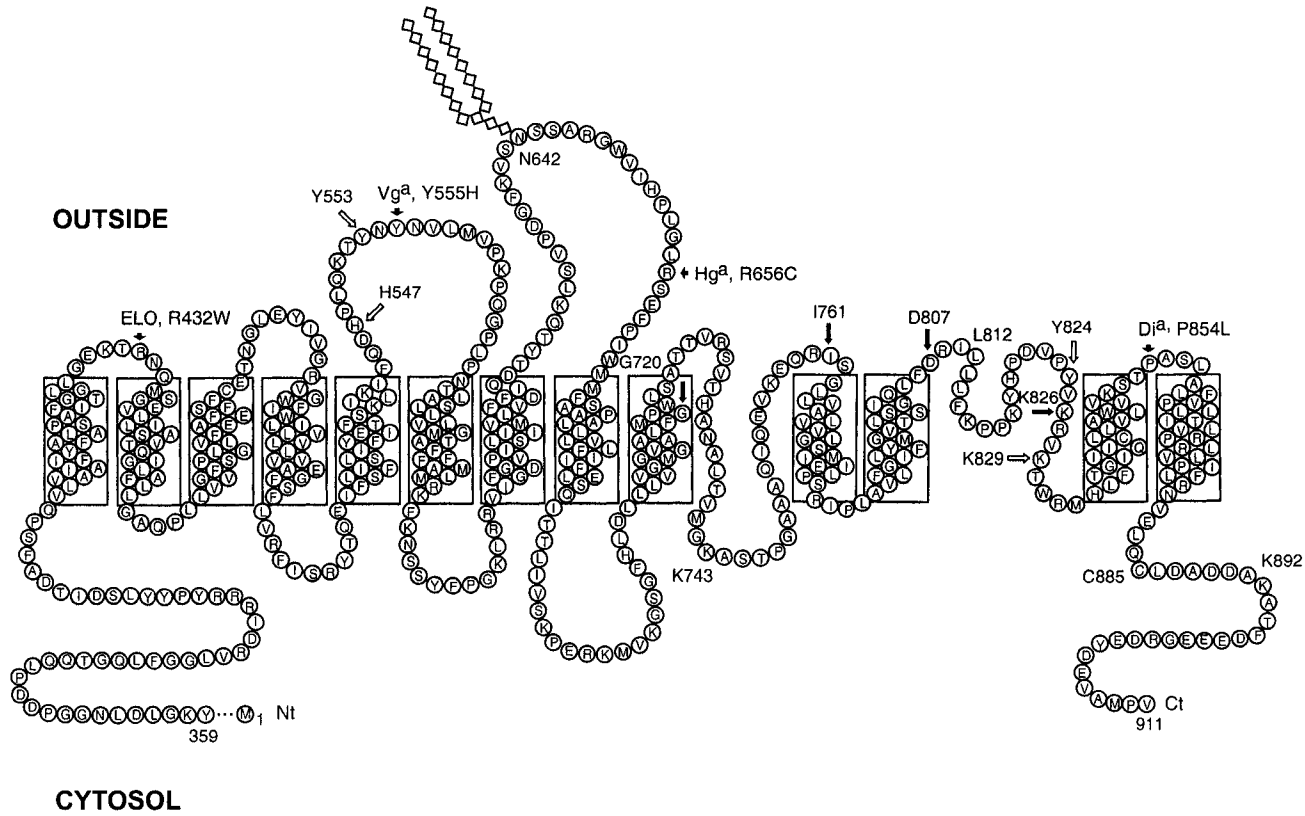


Figure 1.7 Modified topology of the membrane domain of AE1, determined by cysteine scanning mutagenesis

The model contains 13 TMs with a transmembrane loop between TM11 and 12. Four representative mutation sites that create blood group antigens are labeled, ELO, Vg^a, Hg^a and Di^a. Black arrows mark the peptides for malaria parasite invasion G720-I761, D807-K826; white arrows mark the peptides exposed result from malaria parasite infection H547-Y553, Y824-K829. Nt, amino terminus; Ct, carboxyl terminus. (Modified from Fujinaga et al., 1999; Tang et al., 1998)

AE1 protein has been explored. A series of synthetic peptides were generated and tested to assess their abilities of blocking the interaction between the parasite and AE1 (Goel et al., 2003). Among 12 tested synthetic peptides, peptides corresponding to human AE1 residues 720-761 and 807-826 dramatically inhibited *Plasmodium falciparum* invasion of the erythrocytes, suggesting these two regions in AE1 protein are accessible to the extracellular medium. Interestingly, peptide 720-761 corresponds to the "T" loop proposed by scanning N-glycosylation (Popov et al., 1997), and peptide 807-826 corresponds to the transmembrane loop proposed by cysteine scanning mutagenesis (Fujinaga et al., 1999) (Figure 1.7).

The major clinical symptom of parasite infection is sequestration, which is the attachment of *Plasmodium falciparum* infected erythrocytes to the endothelial cells lining the postcapillary venules. AE1 is involved in the cytoadherent behavior of infected erythrocytes. Indeed, synthetic peptides corresponding to the fragments of 824-829 and 547-553 of AE1 effectively blocked the adherence of *Plasmodium falciparum* infected erythrocytes to endothelial cells (Crandall et al., 1993). Therefore, these regions may be cryptic in the uninfected erythrocytes and become exposed in the *Plasmodium falciparum* infected erythrocytes upon parasite maturation. The region 547-553 in AE1 topology is generally agreed as the third extracellular loop, whereas the location of 824-829 remains controversial.

1.6.2.4 Transmembrane helix packing

1.6.2.4.1 Extensive protease digestion

An extensive proteolytic digestion of AE1 from NaOH-stripped erythrocytes coupled with HPLC resolution of peptides and peptide sequencing has been performed by

Hamasaki and co-workers (Hamasaki et al., 1997; Kang et al., 1992; Mori et al., 1995). Tryptic fragments 818-826, 731-743, 632-639, 647-656 were observed to be increasingly released from AE1 when the NaOH concentration rose from 10 mM to 100 mM (Kang et al., 1992). Release of peptides 818-826, 731-743 and 647-656 was strongly inhibited by pre-treatment of the erythrocyte membrane with DIDS, which implicates these regions as folded in the protein complex (Kang et al., 1992). As DIDS only binds to the extracellular surface of AE1 (Kaplan et al., 1976; Zaki et al., 1975), it is conceivable that these peptides are extracellularly accessible. Intensive membrane stripping and proteolysis of erythrocyte membranes revealed that TMs 1, 2, 3 and 6 of AE1 could also be released into the supernatant, which implicates that these TMs may not be strongly associated with the lipid bilayer (Hamasaki et al., 1997). Information obtained from the proteolytic analysis allowed Hamasaki to divide AE1 sequences into three classes: I) exposed loops, such as extracellular loop four and five, II) peptides that are involved in protein-protein interactions, but which can be released from the membrane by alkaline denaturation, such as regions around Lys743 and Asp824, and III) peptides that are involved in protein-lipid interaction and are therefore resistant to extraction from digested membranes, such as regions around Ala666 (Hamasaki et al., 1997). AE1 transports a variety of substrates with diameters from 3.6 Å to >10 Å, indicating that a flexible transport site may exist in the protein (Hamasaki et al., 2002). Hamasaki recently proposed that the non-helical, potentially flexible regions might form the active transport site of AE1, which highlights the importance of the class II peptides (Hamasaki et al., 2002).

1.6.2.4.2 Topogenic signals

Topogenic sequences refer to the certain sequences in the nascent polypeptides that regulate the co-translational insertion of integral membrane proteins into the ER membrane, to determine the final membrane topology of the integral membrane proteins (Blobel, 1980). Three classes of signal peptide have been defined: signal peptide and two signal-anchor sequences (type 1 and 2). Type 1 sequence initiates the translocation of the N-terminal portion of a transmembrane helix with N-terminus extracellular and C-terminus intracellular (N_{exo}/C_{cyt}) orientation, whereas signal peptide and type 2 sequence mediates the translocation of its following portion with N_{cyt}/C_{exo} orientation (High and Dobberstein, 1992). The stop-transfer sequence refers to a hydrophobic segment in a multispanning membrane protein that interrupts the ongoing protein translation initiated by signal peptide or type 2 sequences, which eventually become a TM with N_{exo}/C_{cyt} orientation.

Topogenic function of each proposed TM of AE1 was assessed in an *in vitro* system using two series of model proteins: one with each segment placed in a "stop-transfer" context (N_{exo}/C_{cyt}), the other placed in a "translocation initiation" context (N_{cyt}/C_{exo}) (Ota et al., 1998). Studies revealed that the interactions between TMs operate during membrane integration, and not all the TMs possess sufficient topogenic function for membrane integration. The TMs with weak topogenic function are given transmembrane orientations by their proceeding or following TMs. TM2 was sufficient for stop-transfer function, but in the presence of preceding TM1, it shows efficient N_{exo}/C_{cyt} topology. Further characterization found that TM2 must be in close proximity to TM1 in order to acquire its correct topology (Ota et al., 2000). In addition, TM3, 5 and 7 are not sufficient for the translocation initiation, but TM4, 6 and 8 carry strong type

1 topogenic function and thus help the preceding segments integrate into the membrane (Ota et al., 1998) (Figure 1.8). The studies concluded that the structural assembly of AE1 protein within the translocon is achieved by direct interactions among the TM segments, that synergistically work between the weak topogenic and strong topogenic TMs (Ota et al., 1998; Ota et al., 2000). A recent study also highlighted the topogenic importance of TM1 and the short link region between the AE1 cytoplasmic domain and membrane domain for the proper folding of the whole protein (Kanki et al., 2003).

1.6.2.4.3 Complementary and non-complementary fragments co-expression

1.6.2.4.3.1 Complementary fragments co-expression

Hydropathy analysis suggests that AE1 traverses the lipid bilayer 12 to 14 times. Functional importance of the loops that connect the TMs was studied by co-assembly of the complementary N- and C-terminal polypeptide fragments of AE1 in the Xenopus oocytes (Groves et al., 1996; Groves and Tanner, 1995; Groves and Tanner, 1999b; Groves et al., 1998a; Groves et al., 1998b; Wang et al., 1997). Paired complementary fragments of AE1 were expressed in Xenopus oocytes by co-injection of paired complementary cRNAs. Post-translational assembly of functional AE1 from two or more fragments requires the biosynthesis and membrane integration of each polypeptide chain similar to that of the intact protein. The integrity of extracellular loop 3 and 4, intracellular loop 3, 4, 5 and 6 is not essential for the reassembly of functional AE1, whereas the integrity of extra and intracellular loop 1 and 2 is, suggesting the first three TMs are highly compact; splitting them will disturb the proper folding of the membrane domain of the protein (Groves and Tanner, 1995; Groves and Tanner, 1999b; Wang et al., 1997). The fragmented N- and C- terminal TMs were well integrated into the microsome

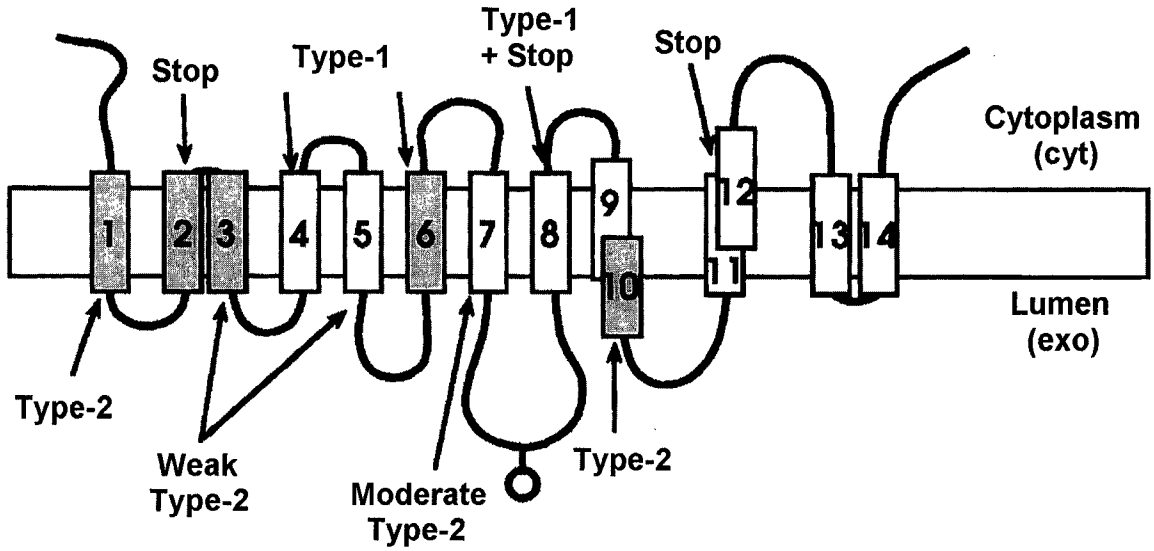


Figure 1.8 Model of topogenic functions of the TMs of AE1
The stop transfer function of TM2 is dependent on the preceding TM1. Integration of TM3 and 5 is mediated by the function of internal Type-1 topogenic sequence of TM4 and 6, respectively. TM9 and 10 functions as a single topogenic unit, and loop between TM10 and 11 locates in the lumenial side of the membrane. Some parts of the TM11 and 12 are left out of the membrane. The shadowed boxes represent the category 2 peptides (Hamasaki et al., 1997). (Adapted from Ota et al., 1998)

lipid bilayer, suggesting they contain sufficient topogenic signals for their insertion (Groves and Tanner, 1995). Interestingly, in the Xenopus oocyte, TM9-14 could not be processed to the surface in the absence of its complementary fragments TM1-8. Expression of TM9-14 alone in the Xenopus oocyte caused rapid degradation of the fragment, suggesting the peptide can not fold properly by itself (Groves and Tanner, 1995). Co-immunoprecipation of paired N- and C-terminal fragments that were expressed in the Xenopus oocyte revealed, that all of them are well associated except the pair of TM1-12 and TM13-14 (Groves et al., 1998b). As TM1-12 and TM13-14 could generate fully functional transport activity when they are co-expressed (Groves and Tanner, 1995), indicating TM13-14 may loosely interact with TM1-12 in the native protein. AE1 with truncated C-terminal 30 amino acids was retained in the oocytes, suggesting this region contains a membrane-sorting signal for the surface processing of AE1 protein (Groves and Tanner, 1995). This work strongly suggests that AE1 assembly occurs within the membrane after translation, insertion and initial folding of the individual fragments have been completed; AE1 contains poly-topogenic signals within the transmembrane region for guiding the proper folding of the protein.

1.6.2.4.3.2 Non-complementary fragments co-expression

To examine the structural importance of each single TM, functional co-assembly of non-contiguous pairs of AE1 fragments with one or more TMs omitted, or overlapped was studied (Groves and Tanner, 1999a). Data showed that no functional transport activity could be generated with omission of any single TM except TM6 and 7 in the Xenopus oocyte. This finding is somewhat contradictory to the results of extracellular pepsin and papain digestion study, where simultaneous cleavage of extracellular loop 3

and 4 totally inactivates the transport activity in erythrocytes (Brock et al., 1983; Jennings et al., 1984). Co-expression of paired fragments overlapping a single TM5, 6, 7, 8, 9-10 or 11-12 resulted in retention of a high level of functionality, whereas fragments overlapping TM regions of 2-5, 4-5, 5-8 and 8-10 only mediated some stilbene disulphonate-sensitive activity. Thus, the membrane domain has been suggested to contain sufficient flexibility in the structure that it allows the inclusion of at least one duplicated TM without losing transport function. Interactions between the N- or C-terminal fragments with intact AE1, AE1 membrane domain and other fragments were further examined by co-immunoprecipitation analysis (Groves and Tanner, 1999a). All fragments, except for TM13-14, could be co-immunoprecipitated with AE1 membrane domain. Fragments comprising TM1-6, 1-7 or 1-8 were strongly co-immunoprecipitated with fragments containing TM8-14 or 9-14. This suggested that the dimer interface of the AE1 is composed of the regions of TM1-5 and TM9-12, but not TM13-14 (Figure 1.9A).

1.6.2.4.4 Cysteine-directed cross-linking

To explore the *in situ* AE1 dimer interface, cysteine-directed cross-linking analyses were performed on the functionally active AE1 introduced cysteine mutants (Taylor et al., 2001). Single cysteine mutations were individually introduced into putative loop regions throughout cysteineless AE1 and expressed in HEK 293 cells. If the proposed loop resides close to the dimer interface, the AE1 monomers would be readily oxidatively cross-linked to homodimers, and the cross-linked homodimers could be reversed upon the addition of reducing reagent. The analyses revealed that introduced cysteine residues in extracellular loops 1-4 and intracellular loop 1 formed disulfide

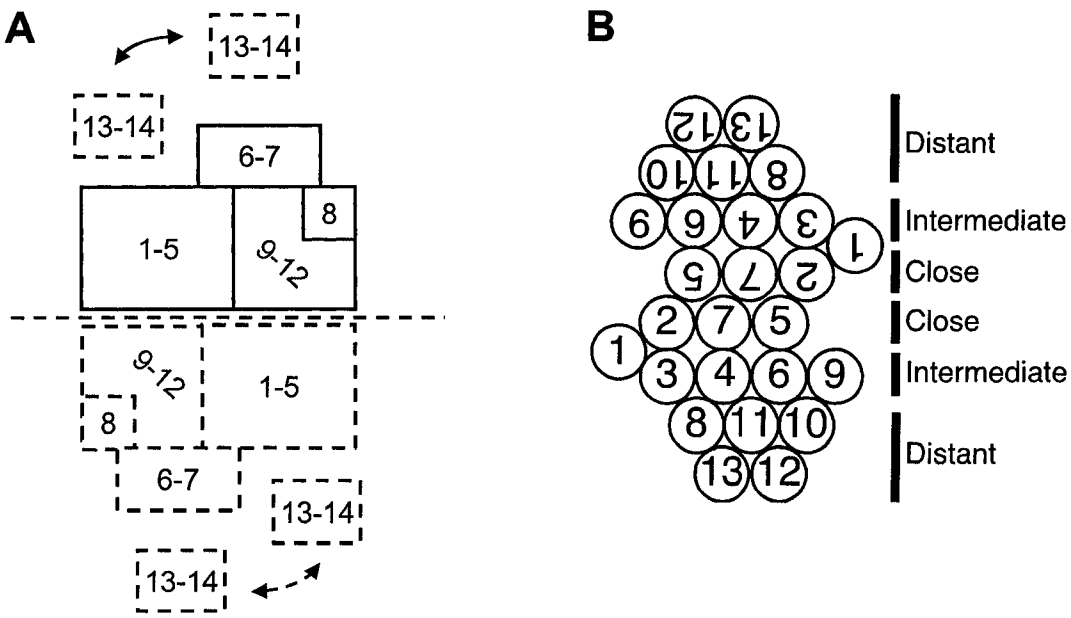


Figure 1.9 Proposed dimer interface of the membrane domain of AE1
A. The proposal is based on 14 TMs topology model. The extended short broken line indicates a likely interface between the two monomer polypeptides. The dimer interface is formed by close antiparallel interaction of two subdomains, TM1-5 and TM9-12, from each monomer. The region comprising of TM13-14 is suggested to be at the periphery of the structure and might be mobile (Adapted from Groves and Tanner, 1999a). B. The proposal is based 13 TMs topology model, determined by cysteine scanning mutagenesis.

cross-linked dimers, suggesting TMs 1-4 and 6 are relative to the dimer interface, largely consistent with the finding from non-complementary fragments co-expression study (Groves and Tanner, 1999a) (Figure 1.9B). The analyses also revealed that the loop-loop contacts across the dimer interface have no functional importance on the transport activity of AE1 protein (Taylor et al., 2001), supporting the idea that each monomer contains an anion translocation channel (Jennings et al., 1998).

1.6.2.5 Structure of the 812-827 region of AE1

One of the major disagreements on the topology of the C-terminal region is the sideness of the loop composed of amino acids 812-827. It is a unique peptide, rich in proline and positively charged residues and has thus attracted great attention about its folding structure in the protein. An NMR study was performed on a synthetic peptide composed of residues 796-841 in the 30% perdeuterated trifluoroethanol (Askin et al., 1998). Circular dichroism analysis showed the peptide forms an extended coil structure in the aqueous buffer, with an increased degree of helicity to about 38% in 30% trifluoroethanol. NMR studies revealed that, in 30% perdeuterated trifluoroethanol, region Ile803-Leu810 forms an α-helix, while Tyr824-Phe836 forms a 3_{10} helix. These helices are only approximate, as they were somewhat distorted in solution. Thus, the peptide was proposed to undergo rapid conformational exchange between a structured helical/loop and a looser extended coil in aqueous solution. The two regions are connected by an ordered short stretch (Leu811-Pro816) followed by a highly flexible loop from Lys817-Tyr824. The two consecutive proline residues introduce a sharp turn that makes the peptide a reversed "V" shape structure as shown in figure 1.10. The flexible loop has been found to be aqueous accessible (Fujinaga et al., 1999) and could be

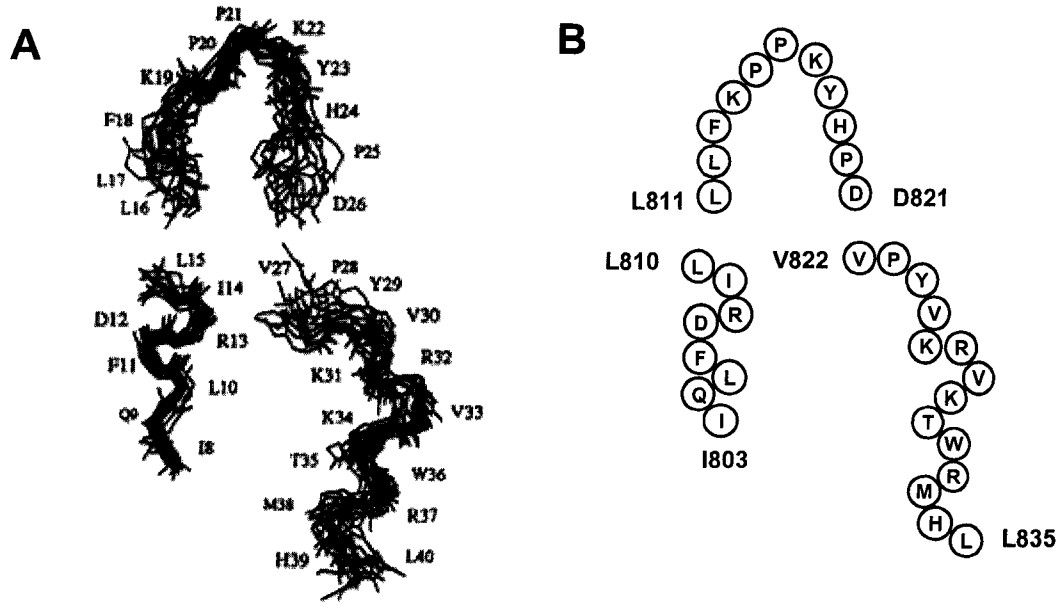


Figure 1.10 The lowest energy NMR structure of the AE1 peptide I803-L835
A. The view derives from superimposed low-energy structure (backbones only) of the peptide by NMR in 30% perdeuterated trifluoroethanol. B. I8-L15 corresponds to I803-L810, L16-D26 corresponds to L811-D821 and V27-L40 corresponds to V822-L835. (Modified from Askin et al., 1998)

recognized by BRIC 132 antibody (Wainwright et al., 1990a). The unique folding pattern of this peptide may suggest it has an important structural and functional role in AE1.

1.6.2.6 Crystal structure

A cryoelectro microscopy structure for the AE1 membrane domain has been obtained at 20 Å resolution (Wang et al., 1993). At this resolution, the overall structure of the protein is visible. The two-dimensional projection map showed that the membrane domain exists as a dimer in the detergent ($C_{12}E_8$), and each monomer consists of three subdomains (Wang et al., 1993). Two subdomains are associated forming a rectangular core and flanked by a mobile third subdomain. The mobile domain has been proposed to be the linker between the membrane domain and the cytoplasmic domain (Wang, 1994). A three dimensional structure was also constructed on the basis of the two-dimensional crystals by electron microscopy and image reconstruction (Wang et al., 1994). The three-dimensional structure formed a U-shaped structure with a membrane-embedded basal domain and two elongated protrusions. The bottom of the basal domain is quite smooth and is proposed as the extracellular surface of the dimer; the upper part, with two protrusions, is considered to be the cytoplasmic surface, which forms a canyon like structure. As the protrusions have two distinctive positions on the two-dimensional projection map, they have been proposed to be dynamic (Wang, 1994). Attempts to crystallize the AE1 membrane domain at high resolution have been extremely difficult because of the high flexibility of the protein (Lemieux et al., 2002).

1.7 Anion transport mechanism

1.7.1 Obligatory exchange and substrate specificity

AE1 acts as an obligatory exchanger (Frohlich and Gunn, 1986; Gunn and Frohlich, 1979; Jennings, 1985). The net conductive Cl^- flux mediated by the protein is at least 10^4 times slower than the exchange flux under the same conditions (Knauf et al., 1977), and this slow net flux is thought to be result from leakage of anions through the transport gate (Frohlich et al., 1983). AE1 transports its natural substrates Cl^- and HCO_3^- at a high rate of 5 x 10^4/second at 37 °C (Brahm, 1977). Many other anions, such as Br^-, F^-, NO_3^-, $HCOO^-$ are also transported by AE1 at similar rates (Brahm, 1977; Cabantchik and Greger, 1992; Dalmark, 1976; Ku et al., 1979). In addition, a variety of oxyanions, such as HPO_3^-, HSO_3^-, $S_2O_4^-$, $H_2PO_4^-$ and SO_4^{2-}, are transported by AE1 at rate that varies over four orders of magnitude less than Cl^- and HCO_3^- (Cabantchik and Greger, 1992; Labotka and Omachi, 1987; Obaid et al., 1980; Salhany and Rauenbuehler, 1983; Schnell et al., 1977). The range of transport rates is not a reflection of differences in binding affinity, since these vary little. Rather, transport rate reflects differences in the rate of transport site reorientation (Gunn and Frohlich, 1979; Milanick and Gunn, 1982; Schnell et al., 1977).

1.7.2 Ping-pong model

Kinetic studies overwhelmingly support a ping-pong mechanism for anion exchange, in which the exchanging anions take turns crossing the membrane (Frohlich and Gunn, 1986; Jennings, 1985; Jennings, 1989b; Passow, 1986). The ping-pong model proposes that AE1 has a flexible transport site that undergoes a conformational change, facing extra and intracellular transiently. In this model, an anion enters the transport site

from the extracellular surface and binds to an outward facing configuration of the protein, then the anion is transported inward by way of a conformational change of the transport site and released on the cytosolic side. Subsequently, an intracellular anion binds to the vacated site and is transported outside. When this anion is released, the transport site is available and a new cycle begins. No net charge movement occurs in the whole process, since entering of a second anion into the cell relies on the regenerated extracellular transport site. The nature of the conformational change is unknown, but could be as little as movement of an amino acid side chain.

1.7.3 Evidence for ping-pong model

Several lines of evidence support the AE1 ping-pong kinetic model (Frohlich and Gunn, 1986; Jennings, 1985; Jennings, 1989b). The ping-pong mechanism requires that the transport protein exists in either of two stable conformations, inward facing or outward facing. A simple consequence of the ping-pong mechanism is that an inward Cl^- gradient should increase the proportion of inward facing states and an outward Cl^- gradient should increase the proportion of outward facing states. Studies based on this assumption showed that when the Cl^- loaded cells are suspend in a Cl^- free medium that contains no penetrating anions except SO_4^{2-}, the outward Cl^- gradient recruits essentially all the transport sites into the outward facing conformation, and strongly enhances the inhibitory potency of DNDS, which only binds to the outward facing transport site (Jennings, 1982b). In the same study, the $K_{1/2}$ for the rapidly transported Cl^- decreased as a result of the slowly transported SO_4^{2-}, suggesting that the slow anion rather than the rapid anion is rate limiting, which also supports the ping-pong mechanism (Jennings, 1982b). Consistent with the above findings, several other competitive and non-

competitive inhibitors of anion transport, including H_2DIDS (Furuya et al., 1984), niflumic acid (Knauf and Mann, 1984), and NAP-taurine (Knauf et al., 1984), are found more potent in the presence of an outward Cl^- gradient. A recent study found that application of DIDS to erythrocytes containing limited $^{36}Cl^-$ induced a rapid release of Cl^- from the cells approximately equal to the number of AE1 molecules. The recruitment of anion binding sites to the outward facing conformation by DIDS is accompanied by a transported Cl^- flux, which offers further support the ping-pong model (Jennings et al., 1998).

1.7.4 Substrate inhibition

A decreased anion exchange rate was observed when the substrate anion was raised to concentrations exceeding 150-200 mM (Dalmark, 1975; Dalmark, 1976; Gunn and Frohlich, 1979; Schnell et al., 1977). This phenomenon is termed as the self-inhibition effect of the substrate. This observation led to the proposal that AE1 has two anion-binding sites: a transport site, and an inhibitory site, which modifies the maximum transport rate (Dalmark, 1976). The inhibition site was also observed in AE1 kinetic studies, using NAP-taurine and NIP-taurine. The two compounds competed with extracellular Cl^- for a low-affinity binding site that is separated from the transport site (Knauf et al., 1978a; Knauf et al., 1987; Knauf et al., 1978b). The relationship of the inhibition site to the transport site is not yet clear (Knauf et al., 1984; Macara and Cantley, 1983).

1.7.5 pH dependence

AE1 transports anions over a pH range of 5.0 to 9.0 in erythrocytes (Passow, 1986). The pH dependence of AE1 catalyzed anion flux has been examined over a wide range of extracellular pH at a constant intracellular pH (Wieth et al., 1982a). Acid extracellular pH inhibited Cl^- self-exchange with a pKa of 5.2, whereas alkaline pH inhibited Cl^- exchange with a pKa above 11. At extracellular pH 7-10, the Cl^- exchange rate remained constant, whereas at pH between 10 to 11, an accelerated Cl^- flux was observed (Wieth et al., 1982a). Thus, it was proposed that there are three functional groups with pKa's of about 5.2, 11 and 12 exist in the extracellular transport site. Protonation of the groups with pKa 5.2 and deprotonation of the groups with pKa 12 leads to the functional inhibition of the transport. While deprotonation of the group with pKa 11 results in the functional activation. The lowest pKa value was attributed to a carboxyl group, whereas the two higher values were attributed to arginine residues (Wieth et al., 1982a).

1.8 Transport site of AE1

The ping-pong transport kinetic model and the characteristic of broad substrate selectivity suggest that the transport site of AE1 is mobile and the substrate pathway is flexible. Extensive biochemical and biophysical analysis of the transport site of AE1 has been conducted in the past three decades (Jay and Cantley, 1986; Jennings, 1989b; Passow, 1986). Although the exact location of this site is still vague, a large amount of information is available from different approaches. The three major approaches are NMR spectroscopy, chemical modifications and site directed mutagenesis.

1.8.1 Nuclear magnetic resonance spectroscopy

^{35}Cl nuclear magnetic resonance spectroscopy (NMR) is a proven technique for probing the Cl^- binding sites in AE1 (Falke et al., 1984a). The NMR technique lies in the observation that ^{35}Cl resonance peak broadens when Cl^- is bound, as opposed to free in solution. Broadening is thus a measure of binding of Cl^- to its binding site on AE1. The observed NMR spectral width of bound Cl^- in AE1 binding site is 10^4 times larger than the line width of Cl^- in solution. Thus, rapid exchange between the free and bound Cl^- results in an averaged NMR that is directly proportional to the number of Cl^- binding sites and the rates of exchange (Falke et al., 1984a).

The ping-pong kinetic model suggests that AE1 has a single transport site. Transport kinetics at high substrate concentration also suggest there is an additional inhibition site. Indeed, ^{35}Cl NMR studies revealed AE1 has two classes of Cl^- binding sites: a high affinity and a low affinity-binding site (Falke et al., 1984a). The high affinity Cl^- binding site also has high affinities for HCO_3^-, F^- and I^-, suggesting only a single transport site exists in AE1. Binding of Cl^- to the high affinity site was inhibited by DNDS and DIDS, whereas binding of Cl^- to the low affinity site was not, leading further support to the idea that the high affinity site is the anion transport site. ^{35}Cl NMR also revealed that DNDS could recruit all the anion transport sites to the outward facing conformation, which indicates that the single transport site can be exposed alternately to either side of the membrane (Falke et al., 1984b). Thus, NMR studies fully support the data from kinetic studies that AE1 utilizes a ping-pong mechanism for transport, and AE1 contains two anion binding sites on the extracellular surface.

Transport of a substrate across the membrane by AE1 occurs in two major steps: binding and dissociation of the substrate at the transport site, and translocation of the bound substrate across the membrane. ^{35}Cl NMR data specified that the rate-limiting step is the translocation of bound Cl^- across the membrane, but not Cl^- binding or dissociation to the transport site (Falke et al., 1985a). To further define the transport site, erythrocyte membranes were extensively digested by chymotrypsin and papain and stripped by high concentrations of NaOH, to remove any portion that protrudes from the membrane. The treatment only left small fragments of AE1 in a range of 3-7 kDa in the membrane. Subsequent ^{35}Cl NMR analysis revealed that the transport site was not destroyed by extensive digestion (Falke et al., 1985b). Thus, it is proposed that the transport site of AE1 is buried in the lipid bilayer, the transmembrane segments that surrounded the transport site are held together by strong attractive forces within the membrane and the transport site is accessed by solution Cl^- *via* a channel open to the solution.

1.8.2 Chemical modifications

1.8.2.1 Stilbene disulfonates

AE1 was first identified as the anion exchanger protein by stilbene disulfonates (Cabantchik and Rothstein, 1974; Knauf and Rothstein, 1971). They are a class of potent inhibitors of AE1. These chemicals are non-penetrating compounds that inhibit anion transport at the outer surface of the membrane (Kaplan et al., 1976; Zaki et al., 1975). They bind AE1 with a 1:1 stoichiometry to inhibit the transport activity linearly at neutral pH (Lepke et al., 1976). 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate (H_2DIDS) are the two most often used

stilbene disulfonate derivatives in the anion exchanger structural and functional studies, and their interactions with AE1 have been well elucidated.

DIDS and H_2DIDS have very similar molecular structure, both contain lysine reactive isothiocyanate R-groups (Cabantchik and Greger, 1992). They interact with AE1 covalently and non-covalently; the covalent reaction does not produce further inhibition on anion exchange (Cabantchik and Rothstein, 1974; Jennings and Passow, 1979). Kinetic studies show that DIDS and H_2DIDS inhibit anion exchange with two steps. The first, rapid and reversible, leads to the inhibition of anion transport. The second, slow and irreversible depending on pH and temperature (Passow, 1986), consists of covalent reaction of the two isothiocyanate groups with two lysine residues in the adjacent segments of the peptide chain. One lysine residue has been identified as Lys539, which is on the extracellular end of TM5; the other is Lys851 at the extrafacial end of the putative second last TM (Bartel et al., 1989a; Okubo et al., 1994). Further studies suggested that at neutral pH H_2DIDS first interacts with AE1 protein to induce a local conformational change, and then one isothiocyanate group covalently reacts with Lys539. At pH 9.5, covalent reaction of the other isothiocyanate group and Lys851 takes place, and forms an intramolecular cross-linking (Jennings and Passow, 1979; Kampmann et al., 1982; Verkman et al., 1983). 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) is another often used stilbene disulfonate derivative (Cabantchik and Greger, 1992). It contains two nitro R-groups, and thus inhibits AE1 transport activity reversibly. Binding of DIDS, H_2DIDS or DNDS to AE1 increased when the intracellular substrate concentration was greater than the extracellular, suggesting they bind to an extracellular facing transport site (Furuya et al., 1984; Jennings, 1982b). Indeed, Cl^- competed with

DIDS, H_2DIDS and DNDS binding to the transport site of the protein (Frohlich, 1982; Shami et al., 1978). Surprisingly, NMR studies revealed that binding of DIDS to AE1 does not fully prevent the binding of Cl^- to the transport site, whereas binding of DNDS completely blocks the Cl^- binding, suggesting that the lysine that DIDS reacts with may not offer the fully positive charge in the transport site (Falke and Chan, 1986a; Salhany, 2001).

The interaction site of stilbene disulfonates and AE1 was examined by fluorescence resonance energy transfer revealing the DIDS site as a cavity that extends some distance from the outer surface of the membrane (Rao et al., 1979). Binding of DIDS to AE1 was also observed to stabilize the membrane domain against thermal denaturation (Appell and Low, 1982), which further supports that the binding site is in a cleft of the protein.

1.8.2.2 Eosin 5-maleimide

Eosin 5-maleimide (EMA) is a fluorescent reagent that has been used to study the rotational dynamics (Cherry, 1979) and conformational changes of AE1 (Wyatt and Cherry, 1992). EMA completely inhibits anion transport at a molar ratio of 1:1 by binding to the extracellular surface of AE1. The binding can be prevented by pre-incubation of AE1 with DIDS (Nigg and Cherry, 1979b), suggesting the binding site is located in the anion transport pocket (Macara and Cantley, 1981). Indeed, bound EMA is buried within the membrane in a hydrophobic environment (Rao et al., 1979). The reactive site of EMA in AE1 has been localized to Lys430 (Cobb and Beth, 1990), which is on extracellular loop 1 connecting the first and second transmembrane helices (Reithmeier, 1993). Although EMA reacts with Lys430 to irreversibly inhibit anion

transport activity, ^{35}Cl NMR studies revealed that the binding of EMA to AE1 does not affect Cl^- binding to the transport site (Liu et al., 1995a), and mutation of this residue does not inhibit anion exchange (Passow et al., 1992), also, EMA analogous compounds can inhibit anion exchange reversibly (Knauf et al., 1993), which indicates that Lys430 is not responsible for the inhibitory effect of EMA. Recent studies suggested that EMA is located in the wall of the anion access channel such that it does not inhibit anion binding (Pan and Cherry, 1995), but its location is accessible to both sides of the membrane (Pan and Cherry, 1998).

1.8.2.3 Reductive methylation

Reductive methylation was developed as a lysine specific protein modification under mild conditions (Means and Feeney, 1968). The products of reductive modification are ϵ-N-mono- and ϵ-N-dimethyllysine residues. Thus, the modification does not alter the charge, but only introduces a minor steric perturbation. However, the modified lysine residue is no longer reactive with the isothiocyanate groups of H_2DIDS. Reductive methylation of AE1 inhibits the transport activity by 70-80%. Inhibition did not result from altered substrate binding, as Br^- still binds with high affinity after methylation. Further analysis revealed both H_2DIDS covalent reactive lysine residues, 539 and 851, are modified, with 539 partially and 851 completely. After methylation, H_2DIDS could still bind reversibly to the modified AE1 producing inhibitory effect, but the covalent reaction is blocked. Transport inhibition by modification was linear with the methylation of Lys851, suggesting this residue resides in the anion translocation site. Methylation of Lys851 was proposed to affect the rate of the conformational changes associated with anion translocation (Jennings, 1982a; Okubo et al., 1994).

1.8.2.4 Pyridoxal 5'-phosphate

Pyridoxal 5'-phosphate (PLP) is an amino group specific reagent that can be transported slowly by AE1 (Cabantchik et al., 1975a). Treatment of AE1 with PLP inhibits transport activity, and the inhibition becomes irreversible after reduction with $NaBH_4$ (Cabantchik et al., 1975a; Cabantchik et al., 1975b). Identification of the PLP reactive sites has been quite interesting, as PLP serves as a substrate for the protein. PLP reacts with lysine residues on both the 17 and 35 kDa chymotryptic fragments of AE1 (Matsuyama et al., 1983). Further characterization found that only the PLP-labeling site in the 35 kDa fragment could be protected by pre-treatment with DNDS and DIDS, suggesting that the site in the 17 kDa fragment does not contribute to the PLP inhibition (Nanri et al., 1983). Eventually, amino acid sequencing of PLP labeled C-terminal peptide of AE1 localized the binding site to Lys851 (Kawano et al., 1988), which is one of the H_2DIDS reactive site. This residue was later found accessible from both side of the membrane (Bar-Noy and Cabantchik, 1990), indicating the region around this residue forms the transport site of AE1.

1.8.2.5 Bis(sulfosuccinimidyl)suberate

Bis(sulfosuccinimidyl)suberate (BSSS) is a membrane impermeant reagent (Staros, 1982) that covalently reacts with AE1. The reaction cross-links AE1 both inter- and intramolecularly from the extracellular surface (Staros and Kakkad, 1983). The intermolecular cross-linking does not affect anion transport, whereas the intramolecular cross-linking causes significant functional change of AE1 (Jennings et al., 1985). Treatment of AE1 by BSSS produces intriguing results. At extracellular pH above 8, anion exchange is inhibited at least by 90%. However, when the extracellular pH is

lowered to 5-6, anion exchange is activated to the similar level as without BSSS treatment. Further lowering the extracellular pH inhibits the anion transport again (Jennings et al., 1985). Thus, BSSS-treated AE1 shifts the alkaline branch of its extracellular pH dependence by 5 pH units. In addition, BSSS treatment also eliminates the self-inhibition effect of AE1 that are produced by its substrates (Jennings et al., 1985). The intramolecular cross-linking sites of BSSS overlap with the H_2DIDS cross-link sites, which are lysine 539 and 851 (Jennings et al., 1985; Okubo et al., 1994). As reaction of BSSS abolishes lysine positive charges, the explanation for the inhibition is that the positive charge provided by one of the lysines has a necessary role in the transport and this positive charge can be replaced by lowering the extracellular pH.

1.8.2.6 Phenylglyoxal

Phenylglyoxal (PG) is a chemical reagent that reacts preferentially with the nonionized form of arginine residues in proteins in alkaline solution (Cheung and Fonda, 1979; Wieth et al., 1982c). It reacts with the guanidine group of arginine with 2:1 stoichiometry (Takahashi, 1968). The observation that one or more positively charged groups with an apparent pKa of 12 may form part of the exofacial anion binding site of AE1, leads to the hypothesis that arginine residues may be essential for the transport site (Wieth and Bjerrum, 1982). Indeed, modification of erythrocyte membranes with PG inactivated 90% of anion transport activity (Bjerrum et al., 1983). Analysis of the ratio of incorporated PG to AE1 molecule revealed that only two arginine residues are modified at pH 10.3, suggesting these two residues are functionally important (Bjerrum et al., 1983). Localization of the labeled residues by chymotryptic digestion determined that they are exclusively in the C-terminal 35 kDa membrane bound fragment (Bjerrum et al.,

1983). Further digestion of the 35 kDa fragment localized one residue to the 9 kDa membrane bound fragment (Bjerrum, 1989), possibly either Arg827 or Arg832 (Tanner et al., 1988).

1.8.2.7 Diethyl pyrocarbonate

Diethyl pyrocarbonate (DEPC) is a membrane permeant reagent that reacts principally with unprotonated histidine residues as well as amino groups of proteins (Miles, 1977). Incubation of DEPC with erythrocyte ghosts inhibits anion transport (Izuhara et al., 1989), in a pH dependent manner. At pH 6.0, no inhibition is observed; at pH 7.4, there is 90% inhibition; at pH 8.0, inhibition is about 100% (Izuhara et al., 1989). This suggests the residues that react with DEPC become increasingly deprotonated as pH increases from 6 to 8. The apparent pKa of the DEPC-sensitive residue is 6.6, which is similar to the pKa of the imidazole group of histidine, indicating the modified amino acids are histidine residues (Izuhara et al., 1989; Matsuyama et al., 1986). Further studies found that the modified functional important histidine residue is in the intracellular surface of AE1, specifically His834 (Jin et al., 2003). Interestingly, pre-binding of DNDS to AE1 from the extracellular surface greatly reduced the DEPC modification on His834, whereas pre-modification of AE1 with DEPC abolishes the extracellular H_2DIDS binding (Izuhara et al., 1989). This indicates that His834 undergoes significant conformational changes, close to the anion translocation site. Also, DEPC modification inhibits Cl^- binding to the transport site (Hamasaki et al., 1990). As DNDS could induce the transport site to an outward facing conformation (Falke et al., 1984b; Jennings, 1982b), modification of His834 has been suggested to be available only when the

transport site is in the inward facing conformation, indicating an essential role of this residue in AE1 conformational changes.

1.8.2.8 Woodward's Reagent K

Woodward's reagent K (WRK, N-ethyl-5-phylisoxazolium 3'-sulfonate) is a negatively charged membrane impermeant reagent that reacts rapidly with carboxyl groups in proteins (Dunn and Anfinsen, 1974). Titration studies showed that decreasing extracellular pH at a constant intracellular pH leads to the inactivation of Cl^- self-exchange with a pKa of 5.2-5.4, suggesting that a carboxyl group must be deprotonated for the translocation to take place (Wieth et al., 1982a). A carboxyl group with similar pKa was also found to be protonated for the translocation of the divalent anion SO_4^{2-} to occur (Milanick and Gunn, 1984). To identify this functionally important group, WRK was applied to modify AE1, followed by reductive cleavage with BH_4^- to produce alcohol (Jennings and Anderson, 1987). Exposure of intact erythrocytes to WRK and BH_4^- at 0°C inhibits Cl^--Br^- exchange by about 80% (Jennings and Anderson, 1987), but accelerates Cl^--SO_4^{2-} exchange considerably (Jennings and Al-Rhaiyel, 1988), and H_2DIDS-sensitive Cl^- conductance about ten times (Jennings et al., 1988). This suggests that the negative charge on the carboxyl group acts as part of the permeability barrier to conduct anion movement; removal of this charge by converting the side chain to an alcohol lowers the barrier and raises the conductance. Other than an extensive labeling of AE1, no intracellular proteins were labeled by the treatment (Jennings and Anderson, 1987). Biochemical characterization revealed the modified residue as Glu681 (Jennings and Anderson, 1987; Jennings and Smith, 1992), which is located toward the C-terminal end of the TM8 (Tang et al., 1998). As the C-terminal end of TM8 is clearly in the

cytoplasmic side of the membrane, and WRK is membrane impermeant, Glu681 lies along the transport pathway and is accessible from outside to the permeability barrier (Jennings and Smith, 1992). Further analysis with WRK reagent suggested that Glu681 could traverse most of the transmembrane electric field to transport SO_4^{2-} in the erythrocytes (Jennings, 1995). Recent mutagenesis studies also support functional importance of this residue, as replacement of Glu681 by Cys abolished the Cl^-/HCO_3^- exchange (Tang et al., 1999). Taken together, Glu681 is functionally involved in anion exchange events and resides at the permeability barrier of AE1.

Table 1.1 summarises the modification effects of all the chemical reagents described above.

1.8.3 Site directed mutagenesis

Chemical modification studies suggested that lysines, arginines, histidines and carboxylate groups are required for the normal function of AE1. To test the functional roles of the proposed important residues, a series of site directed mutagenesis studies have been performed. Lys539 is one of the DIDS binding sites and has been proposed to be functionally important, however, mutation of this residue to Gln, Pro, Leu or His does not affect the transport activity, and DIDS can still bind covalently to the mutant protein (Garcia and Lodish, 1989). Further, simultaneous mutation of both Lys539 and 542 to Asn also did not affect the transport activity, except that covalent reaction of H_2DIDS with protein was blocked (Bartel et al., 1989b). These data suggest that Lys539 is not important for either the protein function or DIDS binding. Mutagenesis of Lys851 produced intriguing results. Simultaneous mutation of Lys539 to Asn and Lys851 to Met did not show any effect on the transport function, but H_2DIDS could covalently react

Table 1.1 Summary of the modification effects of chemical reagents on human AE1.

Chemical reagents	Functionally important reactive sites	Modification effects
DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate), H_2DIDS (4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate) (Okubo et al., 1994)	Lys539 Lys851	Inhibits AE1 activity; intramolecularly cross-link AE1 protein; may lock AE1 transport site in the outward facing conformation
EMA (eosin 5-maleimide) (Cobb and Beth, 1990)	Lys430	Blocks AE1 substrate access channel
Methylation (Jennings, 1982a; Okubo et al., 1994)	Lys851	Retains lysine positive charge; may affect protein conformational change
PLP (pyridoxal 5'-phosphate) (Kawano et al., 1998)	Lys851	Inhibits AE1 substrate translocation
BSSS (bis(sulfosuccinimidyl)suberate) (Jennings et al., 1985)	Lys851	Abolishes lysine positive charge; shifts the alkaline branch of AE1 extracellular pH dependence by 5 pH units
PG (phenylglyoxal) (Bjerrum, 1989; Tanner et al., 1988)	Arg827 Arg832	Inhibits AE1 transport activity when applied extracellularly, mechanism unknown
DEPC (diethyl pyrocarbonate) (Jin et al., 2003)	His834	Completely inhibits AE1 transport activity at pH 8.0; may lock AE1 transport site in the inward facing conformation
WRK (Woodward's reagent K) (Jennings and Anderson, 1987; Jennings and Smith, 1992)	Glu681	Inhibits Cl^- transport, activates SO_4^{2-} transport

with the mutant protein at pH 9.5 (Wood et al., 1992), suggesting a lysine residue similar as Lys851 still exists. It may be that a lysine residue other than Lys851 was knocked out in the study. Mutagenesis studies have also been performed on the histidine residues. Replacement of His703, 819 and 834 by Gln completely inhibited the transport function (Müller-Berger et al., 1995b). Interestingly, mutation of His734 to Ser, or Glu681 to Asp shifts the transport pKa from 6.0-9.2 to a narrow range of 6.8-6.9, suggesting these two residues may form a hydrogen bond that is responsible for the decrease of transport activity at low pH (Müller-Berger et al., 1995a). Substitution of Arg490 with Lys, Thr, Cys, or Arg730 with Lys, Gln did not produce functional proteins, possibly as a result of misfolding (Karbach et al., 1998).

AE1 contains five endogenous cysteine residues. Substitution of all the cysteines to serine yielded a functional transporter (Casey et al., 1995). Cysteine scanning mutagenesis studies found 8 cysteine replaced residues, Ala666, Ser667, Leu669, Leu673, Leu677, Leu680, Ile684 and Ile688, that line along one face of TM8 are sensitive to the inhibition by sulfhydryl reagents, suggesting these form part of the anion translocation pore (Tang et al., 1999). Two cysteine mutant residues in the last two TMs, Ser852 and Ala858, also could be inhibited by sulfhydryl reagents, highlighting the functional importance of the C-terminal region of AE1 (Fujinaga et al., 1999).

1.8.4 Transport site structure

AE1 undergoes significant conformational changes during the anion translocation process (Canfield and Macey, 1984). Intriguingly, AE1 has an extremely high transport rate (5 x 10^4/second at 37 $^{\circ}$C), only one order of magnitude less than an ion channel protein (Brahm, 1977; Hille, 1991). To explain the structural basis of the nature of the

transport site, several models have been proposed (Brock et al., 1983; Falke and Chan, 1986b; Jay and Cantley, 1986; Wieth et al., 1982b). The "thin-barrier" model postulates that there is a hydrophilic transport pathway in AE1 interrupted by a thin permeability barrier in the middle of the membrane with unspecified nature. The translocation event takes place when the barrier moves toward to the position of the anion or a gate opens in front of a bound anion and another gate closes behind the anion (Jennings, 1989b). Several lines of evidence favor this model. The AE1 inhibitor, DIDS, lies in a deep cavity on the extracellular surface of AE1 (Landolt-Marticorena et al., 1995; Rao et al., 1979). Another inhibitor, EMA, when bound to the outer surface of AE1, could partially extend through the permeability barrier as judged by quenching by Cs^+ from the cytosolic surface (Macara et al., 1983). NMR analysis found that an anion-binding site exists in the middle of the lipid bilayer and accessed by channels from both side of the membrane (Falke et al., 1985b). Also, conductance of anions through AE1 was observed to involve the tunneling of the anion through a permeability barrier (Frohlich, 1984; Knauf et al., 1983). In addition, a functionally important residue, Glu681 could move across the permeability barrier and be exposed alternately to the intracellular and extracellular media (Jennings and Al-Rhaiyel, 1988; Jennings and Smith, 1992), suggesting a thin barrier exists in the AE1 protein. Glu681 has been located as close as 5 Å from the inner surface of the membrane, which further suggests that a channel structure goes through the AE1 protein, leading to the transport site, close to the cytosolic surface (Tang et al., 1998). The recent crystal structure for the KcsA potassium (Doyle et al., 1998) and the ClC chloride channels (Dutzler et al., 2002), as well as lactose permease (Abramson et

al., 2003) and glycerol-3-phosphate transporters (Huang et al., 2003) all contained substrate channels and permeability barriers.

1.9 Cysteine scanning mutagenesis and sulfhydryl specific chemistry

Cysteine scanning mutagenesis, also called substituted-cysteine accessibility method (SCAM), is a powerful technique that uses the specificity of sulfhydryl chemistry (Akabas et al., 1994a; Akabas et al., 1992; Karlin and Akabas, 1998). Cysteine residues contain sulfhydryl groups that react with a variety of sulfhydryl-specific reagents. By using site directed mutagenesis coupled with neutral or charged sulfhydryl reagents, SCAM can precisely locate the position of the introduced cysteine residue and evaluate the functional importance of the replaced amino acid (Karlin and Akabas, 1998). Thus, SCAM has been widely used to determine integral membrane protein topologies (Loo and Clarke, 1995; Zhu et al., 2003), identify residues that line the substrate channel (Akabas et al., 1994b), and locate gates and selectivity filters of ion channel and transport proteins (Cheung and Akabas, 1997).

1.9.1 Sulfhydryl reaction and cysteine substitution

SCAM is based on certain observations. In aqueous media sulfhydryl reagents react with ionized sulfhydryls about 5 x 10^9 times faster than with un-ionized –SH (Roberts et al., 1986). Thus, if a sulfhydryl group is in the lipid-accessible surface or in the protein interior, it will not be reactive, because ionization of –SH is suppressed due to the low dieletric constant of the environment (Karlin and Akabas, 1998). The sulfhydryl group of a cysteine residue in a membrane embedded transport or channel protein could be in one of three environments: the water accessible surface, the lipid accessible surface,

or the protein interior. Residues lining a channel or pore, or residues on the protein surface should be water accessible, and further the channel or pore lining should be the only water accessible surface inside the protein complex. In the presence of sulfhydryl specific reagents, sulfhydryls in the water accessible surface will be much more reactive than in the lipid accessible surface or in the protein interior, and thus will be covalently modified by the reagents and may lead to the irreversible alteration of the function of the protein (Akabas et al., 1992).

SCAM needs a background that is free of sulfhydryl reactive groups. Any reactive native cysteines must be suitably mutated and the mutation should not affect the function of the protein. In probing of a series of cysteine mutant function with a reagent, some may be affected, and some may not. The lack of an effect could indicate the lack of a reaction, or a reaction could have no detectable functional effect. A number of factors should be taken into consideration when interpreting negative results, as the reaction depends not only on the simple accessibility to reagent, but also depends on the acid dissociation of the cysteine-SH, the steric constraints on the formation of an activated complex, and for charged reagents, the electric fields along the path to the residue. Thus, more tests should be conducted by reaction of reagents with the same reaction mechanism but differ in charge or differ in size (Karlin and Akabas, 1998).

1.9.2 Sulfhydryl-specific reagents

1.9.2.1 Maleimide and bimane derivatives

Maleimides are excellent reagents for sulfhydryl-specific modification. The most used derivatives are N-ethylmaleimide (Kimura et al., 1998) and biotin maleimide (Loo and Clarke, 1995). Both are membrane permeant reagents, which react with sulfhydryl

groups on proteins to give thioether-coupled products. At a pH above 8, maleimides can also react with protein amines and hydrolyze to a mixture of isomeric nonreactive maleamic acids. Thus, the optimal pH for the reaction of maleimides is 7.0-7.5. In this pH range sulfhydryl groups are sufficiently nucleophilic and reactive, whereas the protein amines are protonated and relatively unreactive.

The bimane derivatives are another important class of sulfhydryl-derivatization reagents that form blue-fluorescent adducts with sulfhydryl-containing proteins. They are essentially nonfluorescent until conjugated, and are used as blue-fluorescent reactive tracers. Monobromotrimethylammoniobimane (qBBr) is one of the derivates that contains a positive charge, which makes it a non-penetrating bimane, and thus useful for probing cell surface sulfhydryl groups. The reaction of qBBr with sulfhydryls can be performed at a physiological range of pH (Kosower and Kosower, 1995).

1.9.2.2 Mercurial compounds and methanethiosulfonates

pCMBS is a small membrane impermeant sulfhydryl-specific reagent that has been used for a long time to probe the sulfhydryls in proteins. It carries a negatively charged sulfonate group and a bioactive mercurial group, which reacts with the sulfhydyls in protein at a moderate rate in physiological solution. pCMBS has been widely used in probing the extracellular medium accessible sulfhydryls in the membrane channel or transport proteins (Mueckler and Makepeace, 2002).

Methanethiosulfonates are a class of sulfhydryl-specific reagents, including three charged derivatives: $MTSEA^+$, $MTSET^+$ and $MTSES^-$ (Karlin and Akabas, 1998). They are small, charged reagents that react rapidly and selectively with sulfhydryls under physiological condition. The high selectivity is due to the fact that amino groups are

completely protonated at the physiological pH. MTSET and MTSES are permanent positively and negatively charged, so that they are membrane impermeant. MTSEA has a pKa of 8.5 and higher, a significant fraction of the reagent is unprotonated and neutral at physiological pH, thus, it is membrane permeant and readily crosses the cell membrane (Karlin and Akabas, 1998).

1.10 Fidelity of cysteine scanning mutagenesis to protein crystallography

Crystallization of membrane channel and transporter proteins has proved very difficult for many years, until recent dramatic advances made by McKinnon and co-workers (Doyle et al., 1998). A number of channel proteins have been crystallized since 1998, including the KcsA potassium channel (Doyle et al., 1998), the ClC chloride channel (Dutzler et al., 2002), water channel (Sui et al., 2001) and glycerol channel (Fu et al., 2000). Recently, two membrane co-transport proteins, lactose permease (Abramson et al., 2003) and glycerol 3-phosphate transporter (Huang et al., 2003), were also crystallized. Most of the crystallized channel and transport proteins have been characterized by cysteine scanning mutagenesis before their crystallization. The structural information obtained from cysteine scanning mutagenesis showed very high fidelity to the protein crystallography (Doyle et al., 1998; Abramson et al., 2003).

The recently crystallized lactose permease is a membrane symporter that co-transports galactosides and protons (Abramson et al., 2003). This protein was extensively analyzed by cysteine scanning mutagenesis by Kaback and co-workers (Frillingos et al., 1998). The mutagenesis studies observed that residues Glu126 on TM4 and Arg144 on TM5 are crucial for the substrate binding; Glu269 on TM8 is involved in

both substrate bonding and proton translocation; Arg302 on TM9, His322 and Glu325 on TM10 are all essentially needed for proton translocation. All these functionally important residues were located close to the middle of the transmembrane segments (Frillingos et al., 1998). Indeed, the crystal structure of lactose permease shows that the substrate-binding site is in a hydrophilic cavity in the middle of the membrane formed by residues Glu126, Arg144, Trp151 and Glu 269. Also, residues Arg302, His 322 and Glu 325 are shown involved in the proton translocation and coupling (Abramson et al., 2003). Thus, it is clear that cysteine scanning mutagenesis is a valuable technique that provides authentic information about protein structure.

1.11 Significance of the C-terminal region of AE1

The C-terminal region (Phe806-Val911) of AE1 has attracted great attention in studying the transport mechanism. Experimental evidence from different approaches implicates the C-terminus of AE1 as part of the anion translocation site. Two AE1 inhibitors, PLP and DIDS, both react with Lys851 in the C-terminal region (Kawano et al., 1988; Okubo et al., 1994). Mutagenesis and methylation studies also highlighted the functional importance of Lys851 (Garcia and Lodish, 1989; Jennings, 1982a). PLP is slowly transported by AE1, indicating that Lys851 may lie on the anion translocation pathway (Cabantchik et al., 1975a). Cys843 is the only palmitoylated residue of five endogenous cysteines in AE1 (Okubo et al., 1991). Fatty acylation is proposed to play important functional roles of the membrane proteins (Resh, 1999). The naturally occurring P868L mutation in this region substantially increased anion exchange activity (Bruce et al., 1993). His834 has been identified as undergoing conformational changes, close to the anion translocation site (Jin et al., 2003). Also, the Asp821 to His834 region

is involved in *Plasmodium falciparum* invasion of erythrocytes and the adhesion of infected erythrocytes to endothelial cells (Crandall et al., 1993; Goel et al., 2003). This stretch was also identified as the senescence antigen of aged erythrocytes (Kay et al., 1990). Some proteolytic sites in the AE1 C-terminal region were accessible only after treatment of erythrocytes with high concentrations of NaOH, suggesting that those regions normally are folded into the AE1 structure (Hamasaki et al., 1997). Recently, carbonic anhydrase II was shown to bind the L886DADD890 motif in the cytoplasmic C-terminal tail of AE1 to form a transport metabolon (Reithmeier, 2001), and deletion of the C-terminal last 11 amino acids abolished processing of the protein to the plasma membrane (Toye et al., 2002). Taken together, the C-terminal region of AE1 has an important functional role and may form the anion transport apparatus of the protein.

1.12 Thesis objectives

The aim of this thesis was to determine the topology of the C-terminal region of AE1 (Phe806-Cys885) and identify the residues in the region that involve in forming the anion translocation site. Eighty cysteine mutations were individually introduced into an otherwise cysteineless human AE1 at the position between Phe806 to Cys885, which covers the putative transmembrane loop and the last two TMs. Structural and functional importance of each mutant was characterized by transport assays and sulfhydryl specific chemical reagents. The findings reported here paint a clear picture of the folding structure of the Phe806-Cys885 region and defined the amino acids that form the anion transport site.

Chapter 2

Materials and methods[1]

[1]**Portions of this chapter have been previously published and are reproduced with permission from:**
Zhu, Q., Lee, D.W. and Casey, J.R. (2003) Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1. *J Biol Chem*, **278**, 3112-3120
Zhu, Q. and Casey, J.R. (2003) The substrate anion selectivity filter in the human erythrocyte Cl^- /HCO_3^- exchange protein, AE1, submitted to *J Biol Chem*

Materials and methods

2.1 Materials and reagents

Restriction endonucleases were from New England Biolabs. Pwo DNA polymerase was from Roche Diagnostics. Plasmid preparation kits were from Qiagen. T_4 DNA ligase, Dulbecco's modified Eagle's medium (DMEM) and all cell culture reagents were from Invitrogen Corporation. 3-(N-maleimidylpropionyl) biocytin (Biotin maleimide) and lucifer yellow iodoacetamide (LYIA) were from Molecular Probes. Monobromotrimethylammoniobimane (qBBr), parachloromercuibenzenesulfonate (pCMBS), (2-Aminoethyl)Methanethiosulfonate (MTSEA) and [2-(Trimethylammonium)ethyl]Methanethiosulfonate (MTSET) were from Toronto Research Chemicals (Toronto, Ontario, Canada). Protein A Sepharose-CL4B, streptavidin/biotinylated-horseradish peroxidase complex, sheep anti-mouse IgG conjugated horseradish peroxidase and enhanced chemiluminescent (ECL) reagent were from Amersham Pharmacia Biotech. Hypersensitive chemiluminescent reagent was from PerkinElmer Life Sciences. SDS-PAGE reagents were from Bio-Rad. PVDF membrane was from Millipore. Glass coverslips were from Fisher Scientific products (Nepean, Canada). Hexadecyltrimethylammonium bromide (CTAB), Igepal, poly-lysine, nigericin and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) were from Sigma. Monoclonol human AE1 antibody IVF12 was a gift from Dr. Michael Jennings. All other chemicals used in the experiments were from Sigma.

2.1.1 Cell line

Human embryonic kidney (HEK) 293 cells were employed for all tissue culture procedures throughout the studies.

2.2 Molecular biology

2.2.1 Cloning strategy

To express human AE1 in the eukaryotic cells, AE1 cDNA on an Acc I-Hind III fragment was cloned into the Hind III and EcoR I sites of mammalian expression vector pRBG4 (Lee et al., 1991) using an Acc I/EcoR I linker (Casey et al., 1995). The recombinant plasmid pJRC26, which is a construct of human AE1 cDNA with all five endogenous cysteine codons mutated to serine ($AE1C^-$), was used as the template for the site-directed mutagenesis (Casey et al., 1995). Two silent restrict digestion sites, BstE II and Mlu I, were first introduced into $AE1C^-$ cDNA at codons 804 and 824, respectively, to facilitate cloning and screening of mutant DNAs. Cloning fragments were amplified on the template containing Mlu I site, and then inserted into the vector that does not contain an Mlu I site by using BstE II and EcoN I sites. Colonies were screened by Mlu I digestion, to test if the fragments have been cloned.

2.2.2 Mutagenic oligonucleotide design

All the mutagenic oligonucleotides were designed using the primer design program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) from the Whitehead Institute for Medical Research. Selection of mutagenic primers was based on the following criteria:

1. Melting temperature close to 60°C.
2. No more than 8 bp self complementarity, or 4 bp 3' complementarity.
3. Subtract 4°C from T.M. for each mismatch.
4. At least 9 bases of wild type sequence on either side of the mutation site.
5. The 5' and 3' ends of the oligo are both chosen to have a G/C.

The mutagenic oligonucleotides were designed as forward primers and synthesized by Invitrogen Inc. and Operon Inc.

2.2.3 Site-directed mutagenesis

Two-step polymerase chain reaction (PCR) was used to generate the point mutations (Colosimo et al., 1999). The first round of PCR occurred between the mutageneic oligonucleotides and a reverse primer 5'-AAGGTGGGGATGTGGAATG-3' that flanks the EcoN I site. This round of PCR generated a megaprimer that contains the point mutation. In the second round of PCR, the products of the first round PCR were diluted and supplemented with two flanking primers 5'-AGCGCAAGATGGTCAAGG-3' and 5'-AAGGTGGGGATGTGGAATG-3'. PCR was performed using an ERICOMP thermal cycler and Pwo DNA polymerase. Eighty cysteine codons were individually introduced in AE1C$^-$ cDNA at each position corresponding to amino acid Phe806-Cys885, at BstE II and EcoN I sites. Each mutant cDNA contains only a single cysteine codon. All mutants were verified by DNA sequencing.

2.2.3.1 CTAB treatment of PCR product

All PCR products were treated by CTAB prior to the endonuclease digestion (Zhang and Weiner, 2000). 25 μl 5% (w/v) CTAB (prepared in 0.5 M NaCl) was added into 100 μl PCR product, mixed, and left at room temperature for 5 min. After centrifugation at full speed for 3 min, the pellet was washed with TE buffer (1 mM EDTA, 10 mM Tris-Cl, pH 8.0) and dissolved with 5 μl 1.2 M NaCl. The dissolved DNA was precipitated by 95% ethanol, washed once by 70% ethanol, and then was used for restriction digestion.

2.2.4 Preparation of plasmid DNA

All plasmid DNA for transfection was prepared using Qiagen columns (Qiagen Inc., Mississauga, Canada), and verified by DNA sequencing, performed by the Core Facility in the Department of Biochemistry, University of Alberta with a Beckman Instruments CEQ2000 DNA sequencer.

2.3 Cell culture

Human embryonic kidney (HEK) 293 cells were grown at 37 °C, in a 5% CO_2 atmosphere in DMEM, supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 5% (v/v) fetal bovine serum (FBS) and 5% (v/v) calf serum (Life Technologies). Two days before experiments, HEK 293 cells were plated onto 60 mm dishes in 4 ml culture medium and seeded for six to eight hours. Cells were then transfected with mutant plasmids, using the calcium phosphate precipitation method.

2.3.1 Poly-lysine coating of dishes

In some experiments, dishes were pre-coated with poly-lysine, to increase cell adhesion. Poly-lysine was dissolved in distilled water at 0.1 mg/ml, and sterilized by filtering through a 0.22 μm membrane filter (Millipore). Culture dishes were incubated with poly-lysine solution for 30 min in the tissue culture hood. Dishes were washed with distilled water and dried under UV light overnight.

2.3.2 Trypan blue staining of cells

Trypan blue staining was used to ensure the cell integrity during some of the chemical labeling experiments. HEK 293 cells in 60 mm dishes before and after chemical labeling were incubated with 2 ml 0.2% (w/v) trypan blue solution (made in

PBS solution (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4)) for 5 min at room temperature, and checked under a microscope for stained cells. Stained cells represent cells with leaky membranes.

2.4 Calcium phosphate transfection

HEK 293 cells were transiently transfected using the calcium phosphate method (Graham and van der Eb, 1973; Ruetz et al., 1993). 4.0 μg of mutant cDNA was diluted in 250 mM $CaCl_2$ to a final volume of 590 μl. The diluted DNA was added dropwise to the same volume of 2 x HEPES-buffered saline (275 mM NaCl, 30 mM Na_2PO_4, 55 mM HEPES, pH 7.0) and left at room temperature for 5 minutes until a precipitate had formed. The precipitate was then mixed by pipetting up and down and added dropwise to a dish of cells plated at 20-30% confluency (316 μl to a 60 mm dish and 890 μl to a 100 mm dish) and cells were left for two days before experiment.

2.5 Cysteine-specific labeling assay

2.5.1 Biotin maleimide labeling

Transfected HEK 293 cells were washed with 5 ml PBS and allowed to deplate in 2 ml PBS for 10 min, at room temperature. Cells were collected and sedimented by centrifugation for 5 min at 800 x g. Cells were then resuspended in 1 ml of PBSCM (PBS, containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.0) and incubated with a final concentration of 0.2 mM biotin maleimide (added from a 20 mM stock in dimethyl sulfoxide). After 10 min incubation at room temperature, the reaction was stopped by the addition of 0.5 ml of stop solution (2% (v/v) 2-mercaptoethanol in DMEM medium (containing 10% (v/v) FBS)) and incubated at room temperature for 10 min. Cells were

sedimented by centrifugation and washed with 1 ml PBSCM. Cells were then lysed with 500 µl of IPB buffer (1% (v/v) Igepal, 5 mM EDTA, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5), containing 2% (w/v) BSA, 200 µM TPCK, 200 µM TLCK and 2 mM PMSF, on ice for 10 min.

2.5.2 qBBr and LYIA accessibility assay

Experiments were performed with cells on the tissue culture dish to prevent cell lysis. HEK 293 cells were grown and transfected on 60 mm poly-lysine coated tissue culture dishes. Twenty-two to twenty-four hours later, cells were washed twice with 5 ml PBS. One cell sample was incubated with 1 mM qBBr (freshly prepared in PBS), at room temperature for 10 min, while the second sample was incubated in parallel with PBS alone. The qBBr was removed by aspiration and cells were washed once with PBS. Both cell samples were then incubated with biotin maleimide (prepared in DMSO, final concentration 0.2 mM), for 20 min at room temperature. Reactions were stopped by adding 0.5 ml of stop solution. Cells were then washed, collected and lysed in IPB buffer. As a control for the labeling procedure, the intactness of cells was assessed by trypan blue exclusion. Exclusion of trypan blue following the labeling protocol ensured that cells were intact during the labeling procedure. LYIA experiments were performed with LYIA replacing qBBr and incubation time was 20 minutes.

2.6 Cell lysis and immunoprecipitation

Cell lysates were centrifuged at 16,000 x g for 15 min to sediment any insoluble material. The supernatant was transferred to a fresh microcentrifuge tube containing 1.5 µl pre-immune rabbit serum and 50 µl protein A Sepharose resin. The tubes were

incubated for 2 hours at room temperature, with constant rotation. Resin was then removed by centrifugation at 7,500 x g, 5 min. The procedure was repeated, using anti-AE1 polyclonal 1658 antibody (Tang et al., 1998) in place of pre-immune serum and the mixture was incubated at 4 °C for 12-16 hours. After incubation, resin was collected by centrifugation and washed consecutively with wash buffer 1 (0.1% Igepal, 1 mM EDTA, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5), wash buffer 2 (2 mM EDTA, 0.05% SDS, 10 mM Tris-HCl, pH 7.5), and wash buffer 3 (2 mM EDTA, 10 mM Tris-HCl, pH 7.5). Resin was then mixed with SDS sample buffer containing 2% (v/v) 2-mercaptoethanol and heated at 65 °C for 4 min.

2.7 SDS-PAGE and Immunoblotting

Protein samples were cooled to room temperature and centrifuged at 7,500 x g for 1 min to sediment insoluble material. Samples were resolved by SDS-PAGE on 8% polyacrylamide gels using a Bio-Rad mini-protein II apparatus. The separated proteins were transferred from polyacrylamide gels to PVDF membranes by electrophoresis for 60 min at 100 V at room temperature in the transfer buffer (20% (v/v) methanol, 25 mM Tris and 192 mM glycine). PVDF membranes were blocked by incubation for 30 min at room temperature in TBST buffer (TBST buffer (0.1% (v/v) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 5% (w/v) non-fat dry milk powder (Carnation) and then were washed 3 times by TBST buffer before being further probed by antibodies or reagents.

2.8 Immunoblot analysis and antibodies

Biotinylated proteins were detected by incubation of blots with 10 ml of 1:2500 diluted streptavidin-biotinylated horseradish peroxidase (Amersham) in TBSTB buffer

(TBST, containing 0.5% (w/v) bovine serum albumin). After 1.5 hours incubation, blots were washed 3 times with TBST buffer and visualized using ECL reagent and Hyperfilm (Amersham), or using a KODAK image station 440CF.

After analyzing each sample for the incorporation of biotin, blots were stripped by incubation in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 at 50 °C for 20 min. Blots were then washed with TBST and incubated in 10 ml TBSTM (TBST, containing 5% (w/v) non-fat dry milk powder (Carnation)), containing 3 µl monoclonal anti-AE1 antibody, IVF12, for 12-16 hours at 4 ^{o}C. After washing, blots were probed with 10 ml of 1:3000 diluted horseradish peroxidase conjugated to sheep anti-mouse IgG in TBSTM and subsequently processed with ECL reagent.

2.9 Anion exchange activity assay

HEK 293 cells were grown on poly-lysine coated glass coverslips in 60 mm tissue culture dishes, and transfected as described. Two days post-transfection, cells were rinsed with serum-free DMEM medium (Invitrogen Corporation) and incubated with 4 ml of serum-free DMEM medium containing 2 µM BCECF-AM (37 °C, 30 min). Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, pH 7.4), containing either 140 mM sodium chloride (chloride buffer) or 140 mM sodium gluconate (chloride-free buffer). Both buffers were bubbled continuously with air containing 5% carbon dioxide. Intracellular pH was monitored by measuring fluorescence changes at excitation wavelengths 440 and 502 nm and emission 520 nm, in a Photon Technologies International RCR/Delta Scan spectrofluorometer. Intracellular

pH was calibrated, using the nigericin- high potassium method (Thomas et al., 1979), with three pH values between 6.5 and 7.5. Transport rates were determined by linear regression of the initial linear rate of change of pH, using Sigma plot software.

2.10 Pore-lining residue determination

Anion exchange activity was measured by: 1. perfusion with Cl^- containing Ringer's until pH was stable, 2. perfusion with Cl^- free Ringer's, until stable alkalinization was reached, 3. perfusion with Cl^- containing Ringer's until pH stabilized and 4. perfusion with Cl^- free Ringer's, until stable alkalinization was reached. Cells were then perfused with 10 ml Cl^- free Ringer's buffer containing either 500 μM pCMBS, 5 mM MTSEA or 5 mM MTSET and incubated for 10 min, followed by washing with Cl^- free Ringer's buffer for 10 min. A shutter was placed in the light path to prevent possible decomposition of pCMBS under high intensity light. Cells were then perfused with Cl^- containing, Cl^- free and Cl^- containing Ringer's buffer to re-assess transport rates. Inhibition of anion exchange was determined by comparison of the initial 100 second rates of alkalinization and acidification before and after incubation with sulfhydryl reagent.

2.11 Image analysis and data analysis

Films from immunoblots and chemical biotinylation blots were scanned with a Hewlett Packard Scanjet 4C scanner, calibrated with a Kodak gray scale. Scanned film images were quantified using NIH Image 1.60 software. Images obtained using KODAK image station 440CF were quantified by KODAK ID image analysis software. Biotinylation levels were calculated as:

$Biotinylation_{norm}$= Pixels biotin signal/ Pixels AE1 immunoblot.

Each mutant was normalized to the biotinylation level of mutant Y555C, which was treated in parallel and electrophoresed on the same acrylamide gel. Normalized data was calculated as:

Relative Biotinylation= ($Biotinylation_{norm\ mutant}$/ $Biotinylation_{norm\ Y555C}$) x 100%.

qBBr and LYIA accessibility were calculated as (for LYIA, LYIA replaced qBBr):

Accessibility= $Biotinylation_{norm\text{-}qBBr}$/$Biotinylation_{norm+qBBr}$.

2.12 Statistical analysis

Standard errors were calculated with Kaleidagraph 3.5 software (Synergy Software). Statistical analysis was performed using Kaleidagraph 3.5 software. Paired T-test was used to assess statistical significance, with $p< 0.05$ considered significant.

Chapter 3

Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1[2]

[2]Portions of this chapter have been previously published and are reproduced with permission from:

Zhu, Q., Lee, D.W.* and Casey, J.R. (2003) Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1. *J Biol Chem*, **278**, 3112-3120

* Lee, D.W. was an AHFMR supported summer student who helped with some of the anion transport assays.

Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1

3.1 Introduction

Human AE1, also called Band 3, is the most abundant integral membrane protein of the erythrocyte membrane (50% of integral membrane protein, 1.2 x 10^6 copies per cell) (Fairbanks et al., 1971). AE1 facilitates the electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane. AE1 also maintains the flexible biconcave disc shape of the erythrocyte through interaction with the spectrin/actin cytoskeleton, mediated by ankyrin (Jay, 1996). In aged erythrocytes, AE1 serves as the senescence antigen for clearance from the circulation (Beppu et al., 1990; Kay et al., 1994). In addition, AE1 participates in the adhesion of malaria-infected erythrocytes to endothelial cells (Crandall et al., 1993). Mutation or deletion a part of the AE1 gene induces blood group antigens, variant erythrocyte morphologies and human diseases, including the $Diego^a$ blood group antigen (Bruce et al., 1994), southeast Asian ovalocytosis (Jarolim et al., 1991) and erythroid spherocytosis (Bruce and Tanner, 1999). AE1-deficient mice had retarded growth, hemolytic anemia and a high rate of neonatal death (Peters et al., 1996).

Human AE1 belongs to a multi-gene family consisting of three members. AE1 is found in the erythrocytes and an N-terminal truncated form is present in the kidney; AE2 is found in a variety of tissues and AE3 is found in the brain, retina and heart. AE1 is 110 kDa protein composed of 911 amino acids. AE1 has two-domains: a 55 kDa membrane domain, which is highly conserved with AE2 and AE3, and a 45 kDa cytoplasmic domain. The membrane domain is responsible for anion exchange activity and is predicted to span the lipid bilayer 12-14 times (Kopito and Lodish, 1985). The

structure of the AE1 cytoplasmic domain was recently determined by X-ray crystallography (Zhang et al., 2000).

The C-terminal portion of the AE1 membrane domain is implicated in the anion translocation process. Two AE1 inhibitors, pyridoxal phosphate (PLP) and DIDS, both react with Lys851 in the C-terminal region (Kawano et al., 1988; Okubo et al., 1994). Mutagenesis and methylation studies also highlighted the functional importance of Lys851 (Garcia and Lodish, 1989; Jennings, 1982a). The naturally occurring P868L mutation in this region substantially increased anion exchange activity (Bruce et al., 1993). Also, the Asp821 to His834 region is involved in the adhesion of malaria-infected erythrocytes to endothelial cells (Crandall et al., 1993). This stretch was also identified as the senescence antigen of aged erythrocytes (Kay et al., 1990). Some proteolytic sites in the AE1 C-terminal region were accessible only after treatment of erythrocytes with high concentrations of sodium hydroxide, suggesting that those regions normally are folded into the AE1 structure (Hamasaki et al., 1997). In addition, carbonic anhydrase II binds to the LDADD motif in the cytoplasmic C-terminal tail to facilitate HCO_3^- transport activity (Reithmeier, 2001).

Determination of the topology of AE1 is important because of the role of AE1 as a cell surface protein and in the anion translocation process. Although topology of the membrane domain of AE1 has been studied extensively, a clear picture of the C-terminal region remains elusive. Hydropathy analysis of the region yields ambiguous results, with unclear transmembrane segments (Kopito and Lodish, 1985). Topology models have been proposed for this region based on experimental evidence including, epitope mapping (Wainwright et al., 1989), N-glycosylation scanning mutagenesis (Popov et al., 1999;

Popov et al., 1997), protease accessibility (Kuma et al., 2002b) and cysteine scanning mutagenesis (Fujinaga et al., 1999; Tang et al., 1998). In this paper, we examined the topology of the C-terminal region of AE1, using introduced cysteine scanning mutagenesis and sulfhydryl-specific chemical labeling. Cysteine scanning mutagenesis has been applied widely to study membrane transporters (Kimura-Someya et al., 2000; Loo and Clarke, 2000). Mutation of individual amino acids to cysteine represents a minor structural modification. Therefore, it possesses some potential advantages over other approaches, as most mutant proteins remain functional. We constructed 80 introduced cysteine mutants at each position between amino acid Phe806 and Cys885, spanning the C-terminal quarter of the membrane domain. Accessibility of each individual cysteine mutant protein to membrane permeant and impermeant chemical reagents was assayed. On the basis of these results, we propose a topology model for the C-terminal region of Human AE1.

3.2 Results

3.2.1 Construction of introduced cysteine mutants at the C-terminus of AE1

Eighty consecutive introduced cysteine mutants were constructed between Phe806 and Cys885 in the C-terminal region of AE1, of which 12 mutants were reported previously (Fujinaga et al., 1999). Each cysteine mutant was cloned into AE1C$^-$, an AE1 mutant with all five endogenous cysteine codons mutated to serine (Casey et al., 1995). Each mutant protein contains only a single cysteine residue. Some mutants outside the C-terminal region were used as controls for the labeling protocol. Y555C was used as the extracellular surface control, since it is adjacent to the two chymotryptic cleavage sites in

intact erythrocytes (Tanner, 1989); K892C was used as intracellular surface control because it is located in the C-terminal tail of the protein, which previously mapped to the cytoplasm (Lieberman and Reithmeier, 1988; Wainwright et al., 1989). Cys201, an endogenous cysteine residue located in the N-terminal cytoplasmic domain of AE1, was also cloned into AE1C$^-$ as an intracellular surface control.

3.2.2 Labeling of introduced cysteines with biotin maleimide

Biotin maleimide, a membrane permeant chemical reagent, reacts with the sulfhydryl groups of cysteine residues to introduce a biotin group into the labeled protein through a thioether bond. Biotin can be detected by streptavidin conjugated horseradish peroxidase on blots. Since biotinylation occurs only in the aqueous environment, cysteine residues in the aqueous medium can react with biotin maleimide, whereas cysteine residues in the TMs cannot (Tang et al., 1998). We labeled intact HEK cells that expressed a single cysteine mutant AE1 protein with biotin maleimide. Figure 1 presents representative data from the biotin maleimide labeling experiments. AE1C$^-$ was very slightly biotin-labeled, consistent with the absence of cysteine residues in the construct and the limited reactivity of biotin maleimide toward primary amines. Extra and intracellular control mutants Y555C and K892C, were both strongly biotinylated. All mutant proteins were expressed to a similar level as shown in figure 3.1. The reaction of each individual cysteine mutant with biotin maleimide varied greatly, due to varying degrees of exposure to the aqueous medium.

The majority of AE1 expressed in transfected HEK cells is retained in intracellular membranes (Tang et al., 1998). This intracellular protein may be mis-folded, although intracellular-retained AE1 has previously been shown to be functionally

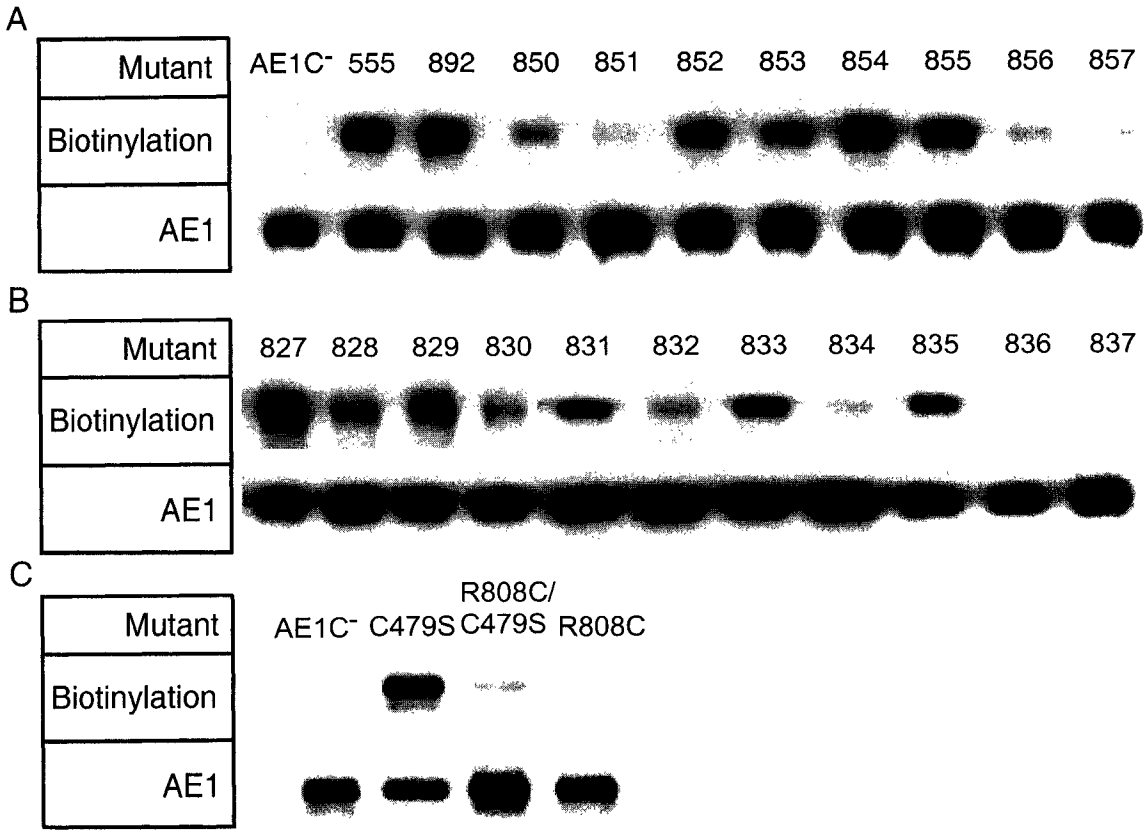


Figure 3.1 Representative data of labeling human AE1 introduced cysteine mutants with biotin maleimide

HEK cells were transiently transfected with human AE1 cDNAs, as indicated in the figure. Cells were harvested and incubated with 0.2 mM biotin maleimide for 10 min, at room temperature. After solubilization, samples were immunoprecipitated with anti-AE1 antibody, subjected to electrophoresis on 8% acrylamide gels and transferred to PVDF membrane. Incorporated biotin was detected by HRP-streptavidin and ECL, as indicated. Blots were stripped and probed with monoclonal anti-AE1 antibody to detect the amount of AE1 in each sample, as indicated. A and B, Individual cysteine codons were introduced into AE1C$^-$ background at amino acid positions indicated at the top of each panel. C, Biotin maleimide labeling of intracellular AE1 was assessed. Mutant R808 was cloned into the AE1C$^-$ background. C479S and R808C/C479S were both constructed in a wildtype AE1 background and therefore have four endogenous cysteine residues.

active (Ruetz et al., 1993). Thus, it is important to determine if any intracellular AE1 is labled by biotin maleimide, as this protein may not have native structure. The R808C mutation has previously been shown to cause retention of AE1 in intracellular membranes (Quilty and Reithmeier, 2000). To determine whether intracellular AE1 is accessible to labeling by biotin maleimide we constructed the R808C/C479S double mutant in a wildtype AE1 background. C479S was used as the cloning background for R808C because of availability of restriction sites. C479S AE1 (with 4 endogenous cysteine residues) and R808C/C479S AE1 were treated with biotin maleimide, in the same way as each of the introduced cysteine mutants (Figure 3.1, A and B). AE1C$^-$ and R808C were not significantly labeled (Figure 3.1C), consistent with the absence of cysteine residues in AE1C$^-$. While the four endogenous Cys residues in C479S were strongly labeled by biotin maleimide, R808C/C479S incorporated <10% as much biotin. Therefore, the R808C mutation causes intracellular retention of AE1, which causes greatly reduced labeling of the protein's cysteine residues. We conclude that in the analysis of the Arg806-Cys885 introduced cysteine mutants (Figure 3.1, A and B), intracellular-retained AE1 contributed <10% of the biotinylation signal.

The ability to label each introduced cysteine mutant in the Phe806-Cys885 region with biotin maleimide is quantified in figure 3.2. The biotin signal of each introduced cysteine mutant was quantified by densitometry of the biotinylation blot and the corresponding anti-AE1 immunoblot. Data was then normalized to the Y555C mutant, which was used as an internal standard in each experiment. Three regions (Phe815-Leu827, Ser852-Ala855, and Val876-Cys885) stood out as labeled in a background of otherwise unlabeled, or weakly labeled mutants. The strong labeling of these three

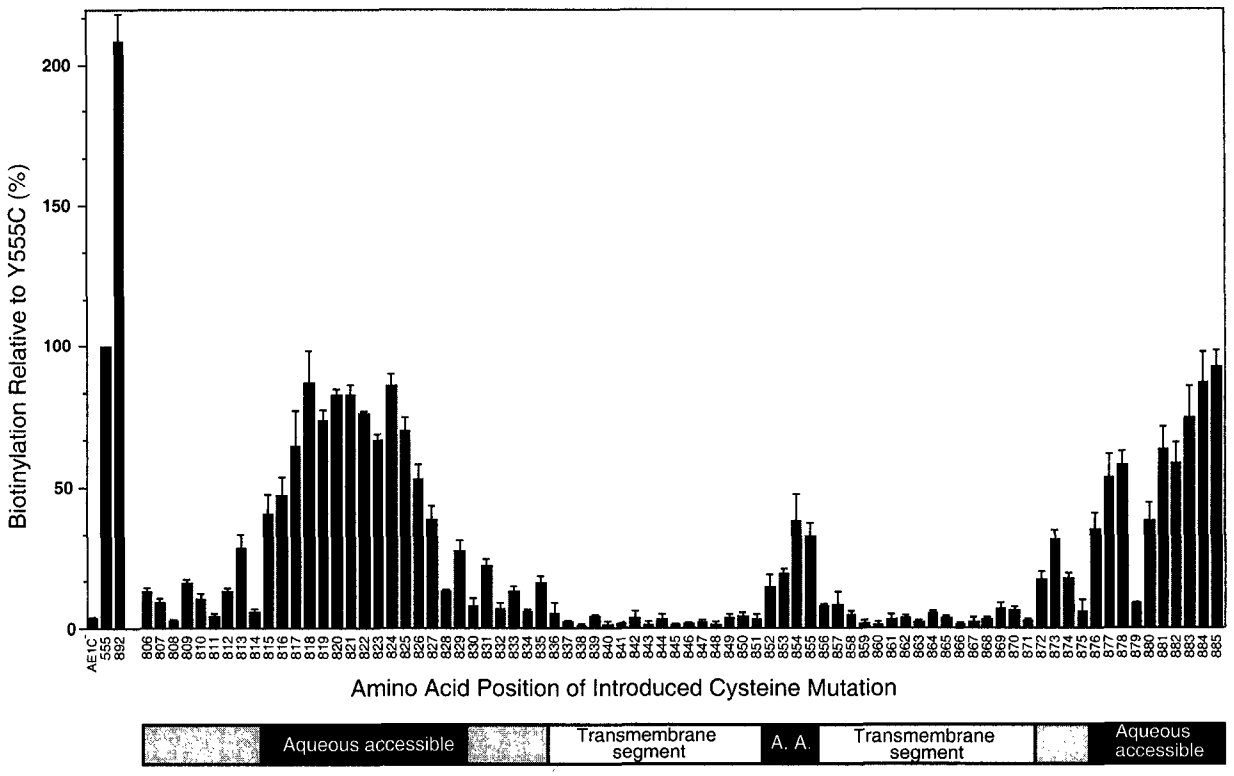


Figure 3.2 Summary of labeling of introduced cysteine mutants by biotin maleimide
Each introduced cysteine mutant was treated with biotin maleimide, as described. The level of biotin incorporation was quantified by densitometry and this signal was normalized to the amount of AE1 present in the sample. In each experiment the level of biotinylation was compared to that of the Y555C mutant, whose labeling was set to 100%. Data represents mean of 3-6 determinations ± standard error. Beneath the figure is a bar representation of the structural interpretation of the biotinylation data. A.A. stands for aqueous accessible.

regions is consistent with aqueous-accessible localization (Figure 3.2, bottom). Each of these regions had a consistent pattern of labeling, with a labeling maximum and a decrease moving away from that maximum. On the margins of the strongly labeled regions were the weakly labeled stretches, Phe806-Lys814, Val828-Leu835 and Val872-Pro875. Notably Phe836-Lys851 and Ser859-Arg871 did not label with biotin maleimide to an extent greater than AE1C$^-$, consistent with two aqueous-inaccessible TMs, as identified previously in TM8 of AE1 (Tang et al., 1998). Two mutants (P875C and R879C) were not biotinylated, yet were found in regions that were otherwise strongly labeled. Interestingly, Phe806-Lys814 and Val828-Leu835 had labeling patterns, with a suggestion of three-fold and two-fold periodicity, respectively.

3.2.3 Accessibility of biotinylated cysteine residues to qBBr

The intracellular or extracellular location of introduced cysteine residues was determined by differential labeling with the membrane impermeant compound, qBBr. The bimane compound, qBBr, has a positively charged quaternary amine group, which will not penetrate erythrocytes and cultured V79 cells with up to one hour incubation under physiological condition and was not toxic (Kosower et al., 1979; Newton et al., 1992).

The protocol to determine the location of introduced cysteine requires integrity of the cells, since permeabilized cells would allow access of qBBr to intracellular sites. To assess whether the plasma membranes of transfected HEK cells were intact, we measured the ability of the membrane impermeant dye, trypan blue, to enter the cytosol. We unexpectedly found that significant fraction (>50%) of transfected HEK cells could be stained by trypan blue after 48 hours expression of AE1 protein, which indicates that

some cells were permeable to the extracellular medium. Expression of AE1 started 12 hours post-transfection and the amount of the expressed protein increased with time. But, less than 0.5% cells were stained with trypan blue up to 22 to 24 hours post-transfection. Thus, experiments were performed on cells 22 to 24 hours post-transfection.

Accessibility to membrane impermeant qBBr was measured by the ability to block cysteine labeling by biotin maleimide. Data could not accurately be collected for mutants that labeled weakly with biotin maleimide, as the labeling became even weaker when the experiments were performed on HEK cells attached to plates. Therefore, only mutants that had >30% biotin maleimide labeling relative to Y555C were assayed. Figure 3.3 shows the degree of biotinylation of representative mutants and the effect of qBBr on biotinylation. The lower blot shows that similar amounts of AE1 were expressed in each sample. The extracellular control mutant, Y555C, shows strong competition of biotinylation by qBBr. In contrast, the intracellular control, Cys201, shows little effect of qBBr on biotin maleimide labeling.

Figure 3.4 quantifies the qBBr accessibility results. Data represent the relative biotinylation in the absence relative to presence of qBBr. Thus, an intracellular site should be unaffected by qBBr and have a ratio close to unity. An introduced cysteine residue labeled by qBBr would have access to the extracellular medium and a ratio >1.0. Introduced cysteine mutants P815C-K829C (excluding V828C) and P854C-A855C showed strong competition by qBBr labeling and therefore lie outside the permeability barrier that restricts qBBr movement across AE1. Significantly, among L873C-C885C mutants that labeled with biotin maleimide, none were affected by qBBr pre-labeling, consistent with an intracellular localization of these sites.

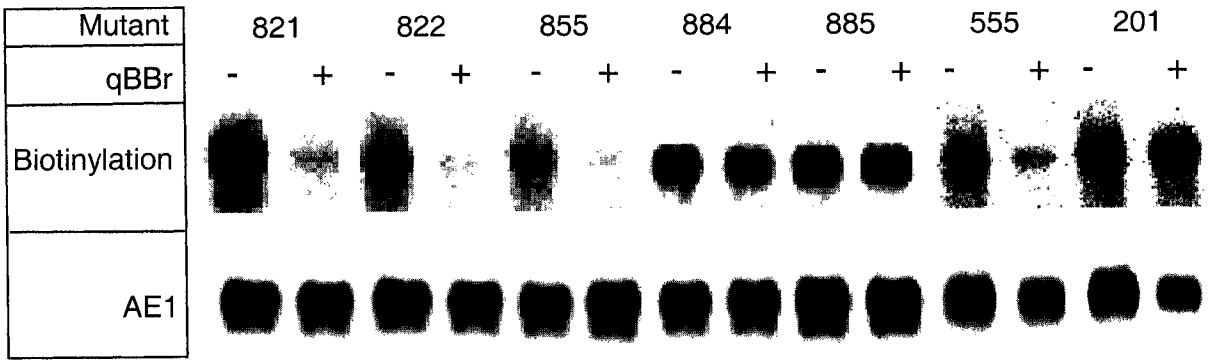


Figure 3.3 Representative data of accessibility of introduced cysteine residues to qBBr

HEK cells, transfected with human AE1 introduced cysteine mutants, were incubated for 10 min at room temperature with (+) or without (-) 1 mM qBBr. Cells were then washed with PBS and treated with 0.2 mM biotin maleimide for 20 min at room temperature. The amount of biotin incorporated into each introduced cysteine mutant was detected with HRP-streptavidin and ECL reagent. Blots were stripped and probed with anti-AE1 antibody, as indicated.

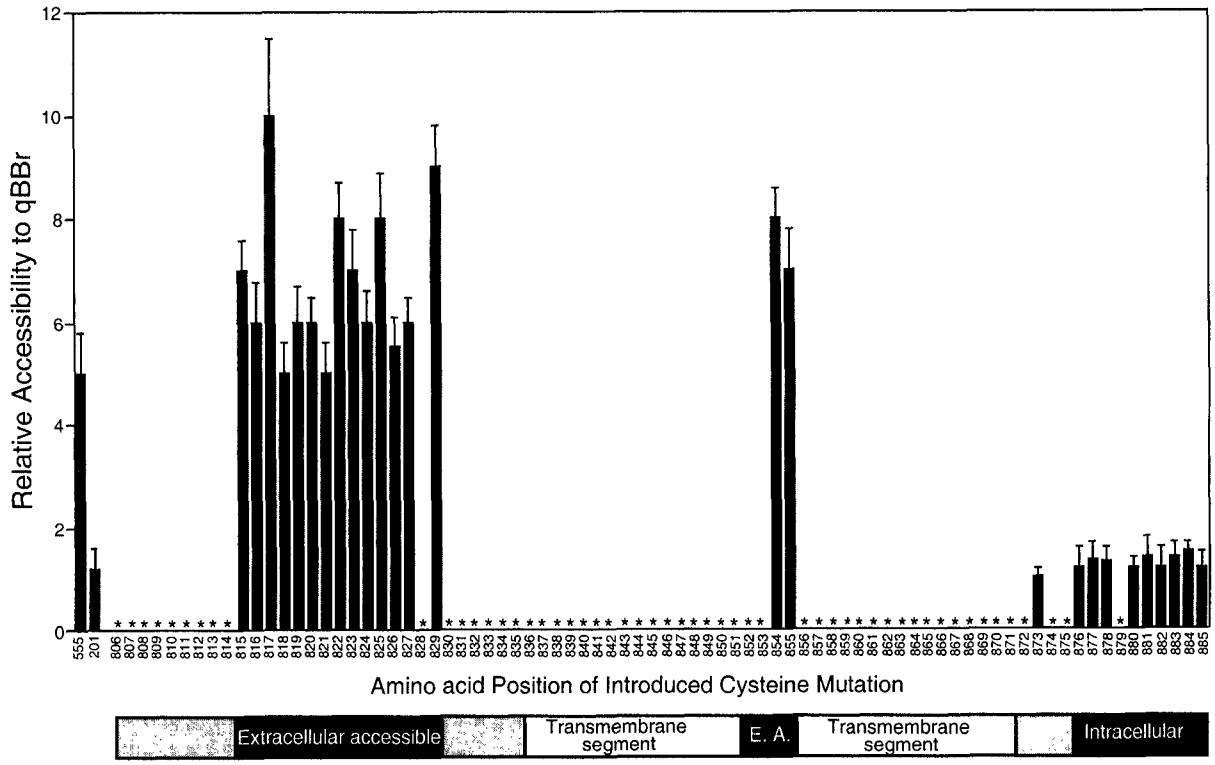


Figure 3.4 Summary of accessibility of introduced cysteine mutants to qBBr
Human AE1 introduced cysteine mutants were pre-incubated with or without qBBr, then treated with biotin maleimide, as described. Biotin incorporation was quantified by densitometry and was normalized to the relative expression level of AE1. For each mutant, the normalized level of incorporation of biotin without qBBr pre-labeling was divided by biotinylation after pre-labeling with qBBr. This ratio represents the relative accessibility of each introduced cysteine site to qBBr. Data represents the mean of 3-4 determinations ± standard error. Each asterisk represents a mutant that was not analyzed because the level of biotinylation was too low to allow analysis. Beneath the figure is a bar representation of the structural interpretation of the qBBr accessibility data. E.A. stands for extracellular accessible.

3.2.4 Accessibility of biotinylated cysteine residues to LYIA

To confirm the topology data obtained with qBBr, we determined the ability to label introduced cysteine mutants with the sulfhydryl compound, LYIA. LYIA, which is anionic and larger (Mr=620) than qBBr (Mr=409), has been previously used as a membrane-impermeant probe of AE1 topology (Fujinaga et al., 1999; Tang et al., 1998). Cysteine residues in the N-terminal cytoplasmic domain (Cys201) and cytoplasmic C-terminal tail had little labeling with LYIA, since they had only 1.1-1.2 fold more biotinylation in the absence than presence of LYIA (Figure 3.5). In contrast, extracellular control mutants Y555C and S657C were strongly accessible to LYIA. Experiments with qBBr suggested that the Pro815-Lys829 region was accessible to the extracellular medium (Figures 3.3, 3.4). To confirm this result, we examined the accessibility of introduced mutants at 817, 819, 821, 823 and 825 to labeling with LYIA. Each of these mutants was strongly labeled by LYIA (Figure 3.5). Thus, we conclude that the Pro815-Lys829 region is in an environment that is sufficiently accessible to the extracellular medium to allow access to both cationic qBBr and anionic LYIA.

3.2.5 Anion exchange activity of AE1 mutants

Mutation of structurally important residues could impair transport activity of AE1. To determine if the mutant proteins retained native structure, transport activity of each introduced cysteine mutant was measured by monitoring intracellular pH changes associated with Cl^-/HCO_3^- exchange in a whole cell assays. Transport rates were determined from the initial 100 seconds of alkalinization and acidification (Figure 3.6). Negative control cells transfected with pRBG4 vector alone had 10% of $AE1C^-$ transport activity (Figure 3.6, A and C). Transport data was corrected for this background activity

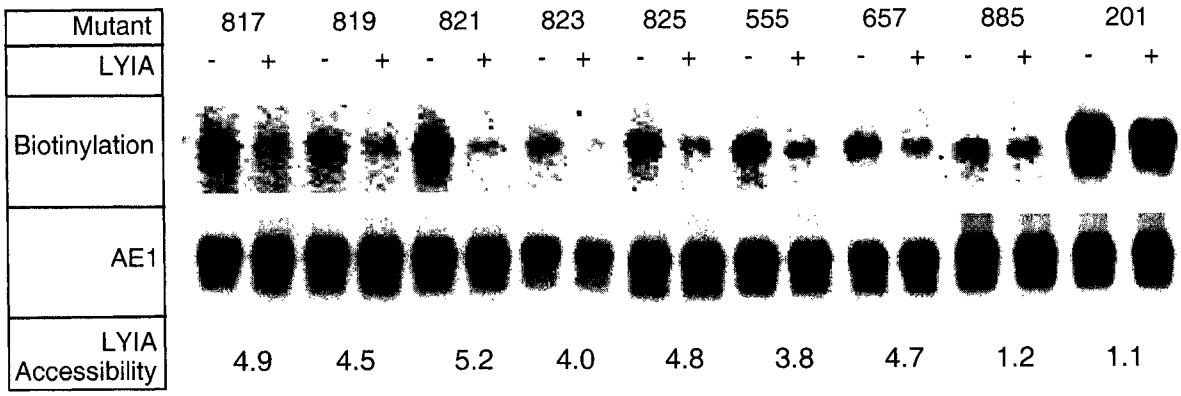


Figure 3.5 Accessibility of introduced cysteine mutants to LYIA
HEK cells, transfected with human AE1 introduced cysteine mutants, were incubated for 20 min at room temperature with (+) or without (-) 1 mM LYIA. Cells were then washed with PBS and treated with 0.2 mM biotin maleimide for 20 min at room temperature. The amount of biotin incorporated into each introduced cysteine mutant was detected with HRP-streptavidin and ECL reagent. Blots were stripped and probed with anti-AE1 antibody, as indicated. The ratio of biotinylation with or without prior LYIA treatment, normalized to expression level of AE1, is quantified beneath the blots (accessibility).

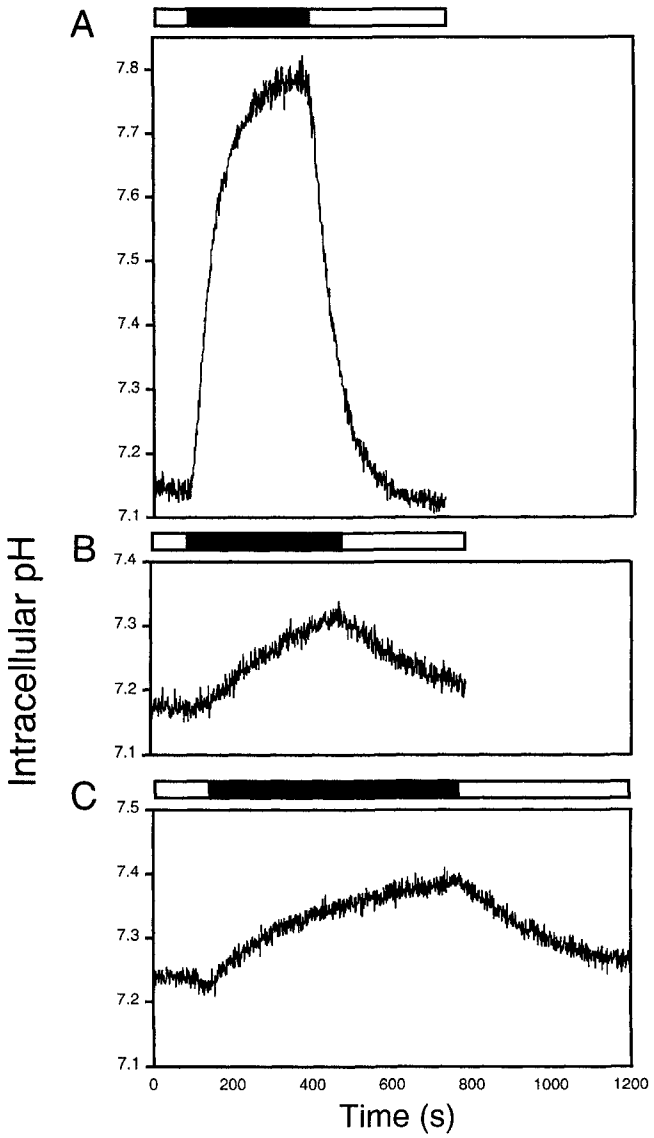


Figure 3.6 Assay of AE1 anion exchange activity
HEK cells transfected with cDNA encoding A, AE1C$^-$, B, introduced cysteine mutant R808C and C, pRBG4 vector alone were grown on glass coverslips, then loaded with pH sensitive dye BCECF-AM. Coverslips were suspended in a fluorescence cuvette and perfused alternatively with chloride-containing Ringer's buffer (open bar) and chloride-free Ringer's buffer (solid bar).

Table 3.1 Summary of anion exchange activity of introduced cysteine mutants
Transport rates were corrected for the background rate of vector alone (pRBG4) transfected cells. Rates are expressed as a percentage of the rate of AE1C$^-$. Biotinylation levels were expressed as % relative to Y555C. -, background biotinylation level; +, 10-20%; ++, 20-30%; +++, 30-50%; ++++, >50%.

Introduced cysteine mutation	Transport activity relative to AE1C$^-$ (%)	Biotin maleimide labeling	Introduced cysteine mutation	Transport activity relative to AE1C$^-$ (%)	Biotin maleimide labeling
F806C	74	+	V846C	96	-
D807C	27	+	L847C	55	-
R808C	1	-	W848C	26	-
I809C	79	+	V849C	47	-
L810C	57	+	V850C	86	-
L811C	74	-	K851C	9	-
L812C	46	+	S852C	83	+
F813C	78	++	T853C	65	+
K814C	63	-	P854C	76	+++
P815C	32	+++	A855C	53	+++
P816C	53	+++	S856C	72	-
K817C	75	++++	L857C	86	-
Y818C	60	++++	A858C	65	-
H819C	22	++++	L859C	10	-
P820C	33	++++	P860C	4	-
D821C	70	++++	F861C	105	-
V822C	90	++++	V862C	142	-
P823C	89	++++	L863C	77	-
Y824C	15	++++	I864C	103	-
V825C	57	++++	L865C	81	-
K826C	92	++++	T866C	86	-
R827C	50	+++	V867C	90	-
V828C	89	+	P868C	50	-
K829C	40	++	L869C	111	-
T830C	72	-	R870C	4	-
W831C	44	++	R871C	109	-
R832C	115	-	V872C	80	+
M833C	9	+	L873C	54	+++
H834C	11	-	L874C	36	+
L835C	61	+	P875C	20	-
F836C	1	-	L876C	96	+++
T837C	1	-	I877C	53	++++
G838C	89	-	F878C	1	++++
I839C	54	-	R879C	82	-
Q840C	17	-	N880C	83	+++
I841C	107	-	V881C	93	++++
I842C	98	-	E882C	3	++++
C843	78	-	L883C	9	++++
L844C	54	-	Q884C	65	++++
A845C	120	-	C885	74	++++

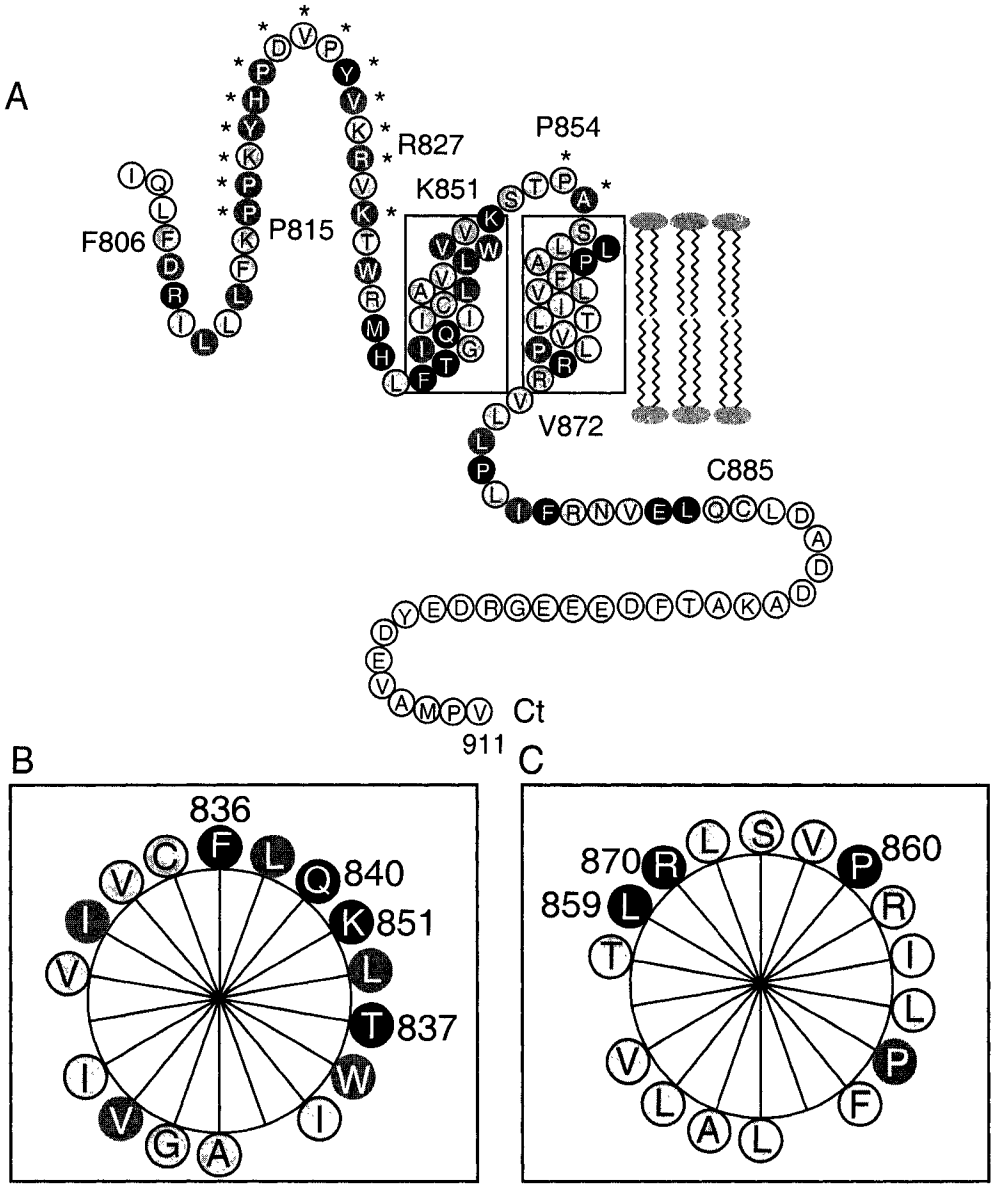


Figure 3.7 Relative anion exchange activity of introduced cysteine mutants and protein structure

Residues are colored to indicate effect of mutation on transport activity: unfilled, not determined; light gray, activity >60% of AE1C$^-$; dark gray, 20-60% activity relative to AE1C$^-$; black, inactive mutants with <20% activity of AE1C$^-$. A, topology model of AE1 based on the data presented in this paper. B, C helical wheel models with 3.6 residues/turn for B, helix from 836-851 and C, 856-871. The first residue in each sequence is at the top of the wheel and sequence proceeds clockwise. Numbers indicate residue positions of functionally inactive mutants. Asterisks indicate residues that were accessible to labeling by qBBr.

(Table 3.1). Some mutants had little or no functional activity, as illustrated by R808C (Figure 3.6B). Among the eighty introduced cysteine mutants analyzed, fifteen were functionally inactive, defined as <20% activity of AE1C$^-$ (R808C, Y824C, M833C, H834C, F836C, T837C, Q840C, K851C, L859C, P860C, R870C, P875C, F878C, E882C and L883C) (Table 3.1).

Figure 3.7 shows the locations of functionally inhibited mutants on topology and helical wheel models of the last two TMs. Helical wheel analysis shows that each of the last two TMs has one helical face that was highly sensitive to mutation.

3.3 Discussion

The recent determination of a high resolution structure for a ClC Cl$^-$ channel revealed a complex protein with transmembrane segments of varied length and tilt angle (Dutzler et al., 2002). If the ClC channel is a guide, we can expect the AE1 anion transporter to have a complex topology, requiring substantial biochemical characterization. In the present study, we examined the topology of the functionally important C-terminal region of AE1 (Phe806-Cys885), using the established method of introduced cysteine scanning mutagenesis and sulfhydryl specific chemistry (Tang et al., 1998). Labeling of an introduced cysteine mutant with biotin maleimide indicates that the residue is accessible to the aqueous environment. Sites that cannot be labeled may be in the plane of lipid bilayer, or folded into an inaccessible conformation. Data on the accessibility of eighty cysteine mutants to the membrane-permeant compound, biotin maleimide paint a clear picture of two large aqueous-accessible regions separated by two transmembrane segments and an intervening small extracellular loop. Labeling with the membrane-impermeant compounds, qBBr and LYIA, leads to the conclusion that

Pro815-Arg827 is readily accessible to the extracellular medium and the C-terminal tail region is cytosolic. The observation that Pro815-Arg827 is extracellular-accessible presents interesting implications for the folding of AE1. The data reported here were combined with findings obtained from proteolytic mapping (Hamasaki et al., 1997), glycosylation scanning mutagenesis (Popov et al., 1999; Popov et al., 1997) and other introduced cysteine accessibility studies (Fujinaga et al., 1999; Tang et al., 1998) to develop a topology model of the C-terminal portion of human AE1 membrane domain (Figure 3.8).

The second half of the AE1 membrane domain has been the subject of many topology studies. Topology of the TM8 region was established using a substituted cysteine accessibility approach similar to this study (Tang et al., 1998). The large re-entrant loop (T-loop) between TM9 and TM10 (Figure 3.8) was proposed on the basis of glycosylation scanning mutagenesis results (Popov et al., 1999; Popov et al., 1997). The inaccessibility of G790C and S801C to labeling by biotin maleimide led to the proposal that these sites form part of a TM (Fujinaga et al., 1999). However, we have subsequently found that introduced cysteine mutants may not label with biotin maleimide because they are folded into an inaccessible conformation, so that the identification of a TM can be made only with a sequence of biotin maleimide-inaccessible sites. Glycosylation scanning mutagenesis showed that Phe785 could not be glycosylated, but was unable to establish for certain the number of transmembrane segments between TM10 and TM12 (Popov et al., 1997). The paucity of topology data between TM10 and Phe806 make it difficult to draw conclusions on the topology here.

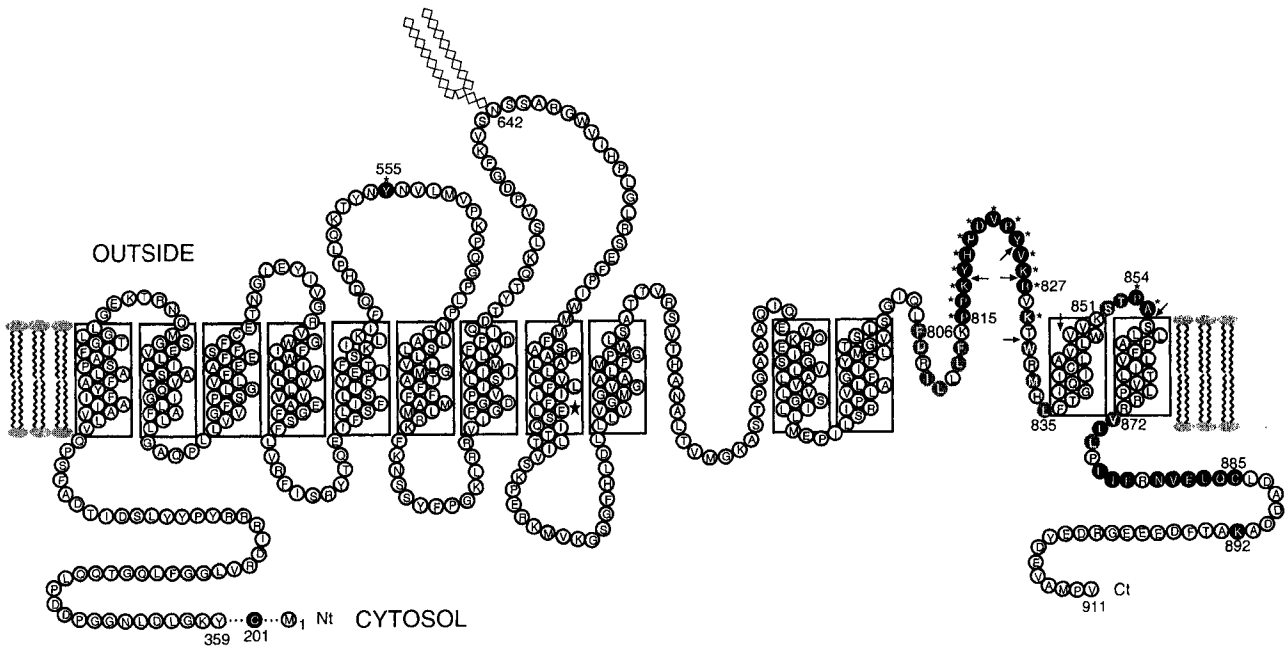


Figure 3.8 Proposed topology model for the membrane domain of human AE1
The branched structure at N642 represents N-linked glycosylation. The model summarizes previous investigations (Popov et al., 1999; Popov et al., 1997; Tang et al., 1998; Wainwright et al., 1989) and work from the present report. Arrows represent proteolytic sites found following treatment with NaOH (Okubo et al., 1994). Residues were colored to indicate the degree of labeling with biotin maleimide: unfilled, not determined; light gray, no significant labeling; dark gray, <30% labeling of Y555C; black, above 30% labeling of Y555C. Asterisk marks mutants that were accessible to qBBr, indicating that the residue was accessible to the extracellular medium. The amino (Nt) and carboxyl (Ct) termini are marked. E681, which defines the permeability barrier, is marked with a star. P815-L835 has unusual structure. We could not rule out the possibility that this region is intracellular, but that P815-R827 is able to reorient to access the extracellular medium.

In the present study we found that Phe806-Lys814 has a three-fold periodicity of accessibility to biotin maleimide (Figures 3.2, 3.8), which is suggestive of a helical region. The NMR structure of a synthetic peptide corresponding to Gly796-Ile841 revealed Ile803-Leu810 is in α-helical conformation (Askin et al., 1998). The R808C mutation, found in this region, causes hereditary erythroid spherocytosis due to a failure to process AE1 to the cell surface (Quilty and Reithmeier, 2000). Consistent with that finding, R808C AE1 was not functional and was not biotinylated in the present study.

The next distinct region, Pro815-Lys829, was strongly labeled by biotin maleimide, qBBr and LYIA, indicating that the region was readily accessible to the extracellular medium. Consistent with our biotinylation data, other studies have localized Phe813-Tyr824 (Wainwright et al., 1990b), Asp821-His834 (Crandall et al., 1993), Leu812-Arg827 (Kay and Lin, 1990) and either Lys814 or Lys817 (Kuma et al., 2002a) as accessible to aqueous medium. However, the accessibility of the Pro815-Lys829 region to extracellular medium is controversial. Glycosylation scanning mutagenesis studies showed that position 820 could be partially glycosylated when translated *in vitro*, but not glycosylated when expressed *in vivo* (Popov et al., 1999). These findings suggest that under some circumstances the region can be induced to face outside the cell, but normally the region is cryptic. The BRIC 132 antibody, with epitope Phe813-Tyr824 (Wainwright et al., 1990b), can bind erythrocytes only following detergent treatment, suggesting that the epitope is not readily accessible at the extracellular surface. In contrast, binding of malaria parasite infected erythrocytes to the endothelium is dependent on the region Asp821- His834 (Crandall et al., 1993), suggesting that parasitic invasion can induce exposure to the extracellular surface. Similarly, the Leu812-Arg827

region has been identified as an extracellular antigen, produced when erythrocytes age (Kay and Lin, 1990). Taken together, the malaria infection and cell senescence antigen data suggest that Asp821-Val828 is normally cryptic, but can be extracellular-accessible under some circumstances. Our data is not sufficient to resolve the structure of the Pro815-Lys829 region, except that it can access extracellular solution.

Figure 3.8 depicts Thr830-Leu835 as an extended structure in the plane of the lipid bilayer. The two-fold periodicity of labeling by biotin maleimide (Figures 3.2, 3.8) and effect of mutation on transport activity (Figure 3.7) are consistent with a β conformation. However, as discussed above we have previously observed that transmembrane regions are not accessible to labeling by biotin maleimide. Therefore, we are left with two remarkable possibilities regarding AE1 structure: 1. Pro815-Lys829 resides on the extracellular portion of the membrane, allowing it to access membrane-impermeant reagents. Val828-Leu835 therefore forms an extended structure (as depicted in Figure 3.8) that is remarkable for its ability to be labeled by the aqueous reagent, biotin maleimide or 2. Pro815-Lys829 and Val828-Leu835 are intracellular, yet Pro815-Lys829 is sufficiently mobile to be able to move to the outer part of the membrane to access extracellular reagents. Although our data are unable to differentiate these possibilities, it points to Pro815-Leu835 as a region with high flexibility and unusual structure. Two-dimensional crystallography of the membrane domain of AE1 revealed a mobile subunit in the protein complex (Wang et al., 1993), which may be the last two TMs (Groves and Tanner, 1999a). Pro815-Leu835 may therefore form a flexible connection between the mobile last two TMs and the rest of the protein.

Two regions, Phe836-Lys851 and Ser856-Arg871, were not labeled with biotin maleimide, consistent with two aqueous-inaccessible TMs. The 16 amino acid length of the regions would allow them to extend 24 Å in α-helical conformation, which is shorter than the 30 Å thickness of the mammalian bilayer (Reithmeier and Deber, 1992). A helical conformation for the Phe836-Lys851 and Ser856-Arg872 regions is also supported by the marked clustering of functionally important residues on one face of helical wheel plots (Figure 3.7, B and C). In both helices the functionally important residues localize to a helical face that is much more polar than the opposing hydrophobic face. This suggests that the sensitive helical surfaces may form a part of the anion translocation pore, while the opposing face interacts with the rest of the protein. Interestingly, Lys851, which reacts covalently with DIDS to inhibit transport (Okubo et al., 1994) and is implicated in anion translocation (Jennings, 1982a), is in the center of the sensitive face of the Phe836-Lys851 helix (Figure 3.7B).

Transport assays revealed a cluster of important amino acids at the N-terminal end of the second last TM (Met833, His834, Phe836, Thr837, Gln840) (Figure 3.7 and Table 3.1). Human mutations H834P and T837M both cause erythrocyte spherocytosis, due to a failure of AE1 to be processed to the cell surface (Quilty and Reithmeier, 2000). In the present study, mutation to cysteine at each of these sites resulted in inactive protein, which was not labeled by biotin maleimide. The intolerance of positions 833, 834, 836, 837 and 840 to mutation suggests a critical role of this region in protein folding. AE1 variant P868L increased AE1 transport activity (Bruce et al., 1993), but here P868C decreased AE1 activity by 50%, indicating that it is more than loss of proline that causes the increased transport rate of P868L AE1.

A short biotin maleimide labeled region, Ser852-Ala855, lies between the two TMs. This region was also sensitive to membrane impermeant, qBBr, consistent with an extracellular location. Our results therefore indicate that Ser852-Ala855 forms a short aqueous-accessible loop, connecting the last two TMs. Glycosylation-scanning mutagenesis had previously identified Pro854 as an extracellular location (Popov et al., 1999). An extracellular location for this residue is also supported by naturally occurring mutation, P854L, which induces the Diego^a blood group antigen (Bruce et al., 1994). Our data shows for the first time that Pro854 resides in a very small extracellular loop.

The extreme C-terminal region (Val872-Cys885) was strongly labeled by biotin maleimide, but was not very accessible to the membrane impermeant reagent qBBr, indicating an exposed intracellular location of the tail. This is consistent with results from epitope mapping (Wainwright et al., 1989) and carboxypeptidase Y digestion (Lieberman and Reithmeier, 1988). The observed increase of biotinylation from V872C to C885 provides a high resolution determination of the last TM of AE1, which ends at Arg871. The cytoplasmic C-terminal region (Val872- Cys885) becomes progressively more accessible to the aqueous medium, moving away from the membrane.

The presence of four functionally inactive mutants in the cytoplasmic C-terminal tail is somewhat surprising. All of these mutations are close to the identified binding motif for carbonic anhydrase II on the C-terminal tail (L886DADD890) (Reithmeier, 2001). Since binding of CAII is essential for full AE1 activity, mutation at adjacent sites could reduce transport activity. Alternatively, the C-terminal tail of AE1 has been implicated in protein trafficking (Toye et al., 2002), so that these sites may be required for processing AE1 to the cell surface.

In summary, we individually assessed topology of each residue in the C-terminal region of the membrane domain of human AE1. We defined the last two short transmembrane segments and identified the small loop that connects them and which forms the Diego[a] blood group antigen. The last two TMs each contain one helical face that is sensitive to introduced cysteine mutations, possibly because these faces form part of the transmembrane pore lining. Finally, we have presented evidence that Pro815-Arg827 is accessible to the extracellular medium. This finding combined with the biotin maleimide accessibility of Val828-Leu835 leads to the conclusion that the Pro815-Leu835 region of AE1 has unconventional structure that may be linked to the catalytic mechanism.

This thesis presents a number of topology models, which were altered progressively as we collected more data.

Chapter 4

The substrate anion selectivity filter in the human erythrocyte Cl^-/HCO_3^- exchange protein, AE1[3]

[3]**Portions of this chapter have been submitted for publication and previously published in abstract form:**

Zhu, Q. and Casey, J.R. (2003) The substrate anion selectivity filter in the human erythrocyte Cl^-/HCO_3^- exchange protein, AE1, submitted to *J Biol Chem*

Zhu, Q. and Casey, J.R. (2003) Identification of pore-ling residues in the membrane domain of human anion exchanger, AE1. *FASEB J,* 17, A456

The substrate anion selectivity filter in the human erythrocyte Cl^-/HCO_3^- exchange protein, AE1

4.1 Introduction

Respiration and circulation of blood are the two essential physiological functions. Removal of waste CO_2 from the body is equally important as the uptake of O_2. High CO_2 levels cause tissue acidosis and depress central nervous system function (Silverthorn, 2001). CO_2 is transported mainly as HCO_3^- in the blood (about 70%). Conversion of CO_2 to HCO_3^- benefits the body in two ways: enhancement of blood capacity to carry CO_2 and pH buffering to stabilize the body's pH. The human anion exchanger 1 (AE1, also called SLC4A1 or Band 3) plays a critical role in the CO_2 transport system. In the systemic capillaries, the high CO_2 partial pressure drives CO_2 diffusion into the erythrocyte where it is converted to HCO_3^-. HCO_3^- is then transported out of the erythrocyte by AE1 in exchange for Cl^- to prevent the alkalisation of the erythrocyte. When the blood reaches the lung, the process is reversed and CO_2 is exhaled from the body. With a turnover rate of 5×10^4 anions S^{-1}, AE1 rapidly performs tightly coupled one to one electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane, placing AE1 among the fastest membrane transport proteins (Brahm, 1977). Transport is especially rapid since AE1 is the most abundant integral membrane protein of the erythrocyte membrane (1.2×10^6 copies per cell and 50% of integral membrane protein) (Fairbanks et al., 1971).

AE1 has additional roles in erythrocyte biology. AE1 interacts with the cytoskeleton via ankyrin to maintain the flexible biconcave disc shape of the erythrocyte (Jay, 1996). In aged erythrocytes, AE1 serves as the senescence antigen for clearance from the circulation (Kay et al., 1994; Low et al., 1985). AE1 also participates in the

adhesion of malaria-infected erythrocytes to endothelial cells (Crandall et al., 1993), as well as a host receptor for the *Plasmodium falciparum* invasion of erythrocytes (Goel et al., 2003). Mutation or deletion of the AE1 gene induces blood group antigens, variant erythrocyte morphologies, and human diseases, including the Diego^a blood group antigen (Bruce et al., 1994), Southeast Asian Ovalocytosis (Jarolim et al., 1991), and erythroid spherocytosis (Bruce and Tanner, 1999). AE1 deficient mice display retarded growth, haemolytic anemia and a high rate of neonatal death (Peters et al., 1996).

Human AE1 belongs to a multi-gene family with three members. AE1 functions in erythrocytes and an N-terminally truncated form is in the kidney; AE2 is the housekeeping anion exchanger found in a variety of tissues; AE3 is expressed in heart, brain and retina. The three AE isoforms have high sequence conservation in their membrane domains and lower conservation in their cytoplasmic domains. Human AE1 is a 110 kDa glycosylated integral membrane protein composed of 911 amino acids. It exists as a homo-dimer in the lipid bilayer, but each monomer performs anion exchange activity independently (Jennings and Passow, 1979). The 45 kDa cytoplasmic domain of AE1, whose structure has been determined by X-ray crystallography, forms the anchoring sites for cytosolic proteins, including haemologlobin, glycolytic enzymes and ankyrin, which anchors AE1 to the underlying cytoskeleton (Zhang et al., 2000). The membrane domain performs anion transport alone (Lepke and Passow, 1976). Erythrocytes from the AE1 knock out mouse showed continuous loss of the plasma membrane by forming rod-like blebs (Peters et al., 1996). Recently, AE1 was shown to be part of a large macromolecular complex in the erythrocyte membrane, with Rh protein and other membrane proteins (Bruce et al., 2003).

AE1 functions by a ping-pong mechanism to exchange Cl^- and HCO_3^- across the plasma membrane (Frohlich and Gunn, 1986). The Ping-Pong model proposes that AE1 has a flexible transport site that undergoes conformation change to alternatively face the extra and intracellular environment (Frohlich and Gunn, 1986). Extensive studies of the anion transport site demonstrate that it has distinct inward and outward facing conformations (Jennings et al., 1998). Three-dimensional (Wang et al., 1994) and two-dimensional (Wang et al., 1993) cryoelectron microscopy structures are available for the membrane domain of AE1 at 20 Å resolution.

The C-terminal region of AE1 has been proposed to be involved in the anion translocation mechanism for many years. Two anion exchange inhibitors, pyridoxal phosphate and DIDS, both react with Lys851 in this region to inhibit the transport activity (Kawano et al., 1988; Okubo et al., 1994). The mutation, P868L, causes acanthocytosis and substantially increases anion exchange activity (Bruce et al., 1993). Histidine 834 has been identified as undergoing conformational changes, close to the anion translocation site (Jin et al., 2003). Carbonic anhydrase binds to the C-terminal cytoplasmic region via the L886DADD890 motif, forming a metabolon to facilitate HCO_3^- transport (Sterling et al., 2001; Vince and Reithmeier, 2000). These studies implicate the C-terminal region as the anion translocation site. We recently determined the topology of the C-terminal region of human AE1, using a panel of individual introduced cysteine mutants, probed with permeant and impermeant sulfhydryl reagents (Zhu et al., 2003). The last two TMs were shorter than a standard TM, composed of only 16 amino acids each and were connected by a small extracellular loop. In the present paper, we have assessed the sensitivity of AE1 introduced cysteine mutants to functional

inhibition by small, hydrophilic sulfhydryl reagents to identify the residues in this region that form the anion selectivity filter and that line the anion translocation pore.

4.2 Results

4.2.1 Effect of introduced cysteine mutations on AE1 transport activity

To identify amino acids involved in the AE1 anion translocation process, we constructed 80 individual cysteine mutations consecutively introduced into a cysteineless human AE1 cDNA, at all positions between Phe806 and Cys885 in the C-terminal region of human AE1. Cysteineless AE1 cDNA was constructed previously and mutant protein is fully functional (Casey et al., 1995). Mutant AE1 proteins were expressed in HEK 293 cells by transient transfection. All proteins were well expressed to similar levels as shown on immunoblots probed with IVF12 antibody, which recognizes the extreme C-terminal tail (Jennings et al., 1986) (Figure 4.1). Transport activity of each mutant protein was assessed by monitoring intracellular pH changes associated with Cl^-/HCO_3^- exchange in a whole cell assay (Zhu et al., 2003). As shown in Figure 4.2, introduced cysteine mutations impaired transport activity to varying degrees. Among 80 introduced cysteine mutant protein, 15 had less than 20% transport activity compared to the cysteineless AE1 protein, and were thus defined as functionally inactive (Zhu et al., 2003). Interestingly, there are five functionally inactive residues (M833C, H834C, F836C, T837C and Q840C) clustered at the N-terminal end of the second last transmembrane helix, which suggested that this region is critical for the proper folding of the last two transmembrane helices. Mutation of Lys851, which reacts with both

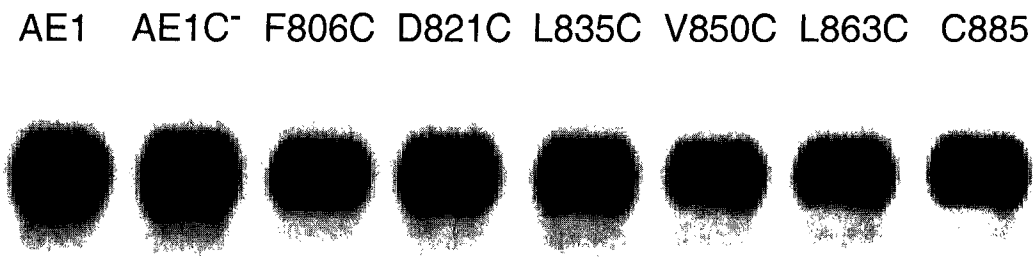


Figure 4.1 Expression of introduced cysteine mutants in HEK 293 cells
Representative AE1 introduced cysteine mutants were expressed in HEK 293 cells. Mutant proteins on the PVDF membrane were probed on immunoblots with IVF 12 antibody, which recognizes the C-terminal tail of AE1 protein.

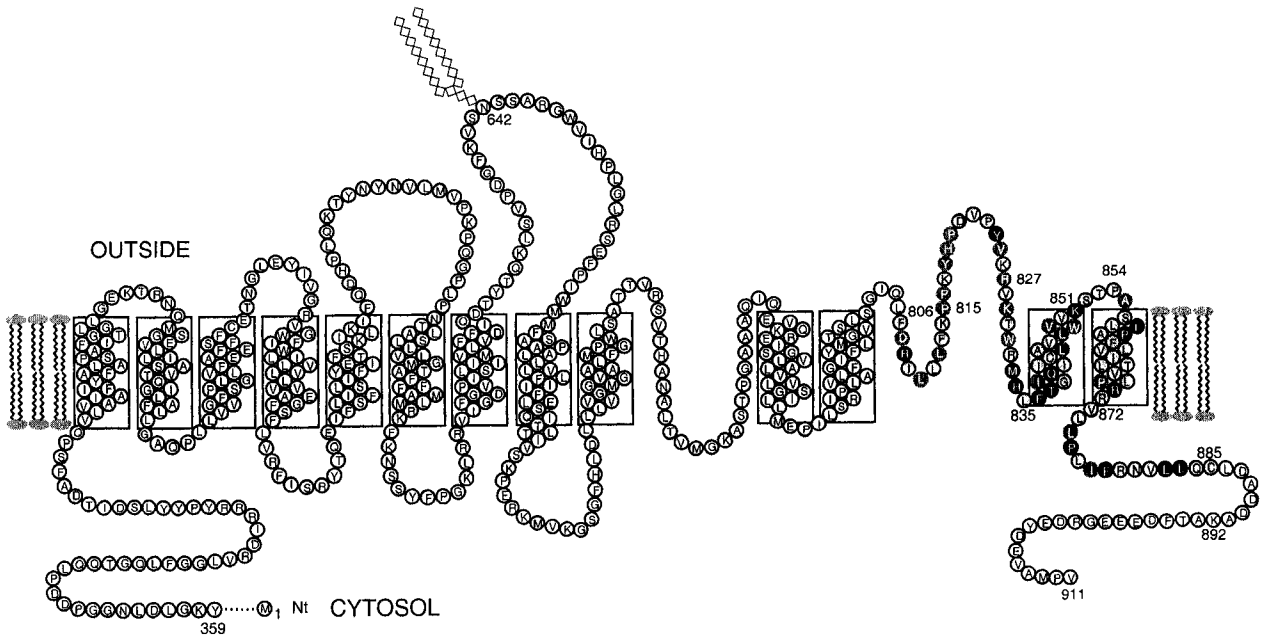


Figure 4.2 Position of introduced cysteine mutations in the topology model of human AE1 and their relative anion exchange activity

The model was recently proposed on the basis of cysteine scanning mutagenesis (Zhu et al., 2003). Transport activity of each mutant protein was assayed by measurement of pH changes associated with bicarbonate transport. Transport activity is expressed as activity relative to the human AE1C$^-$ mutant. Black, <20% of AE1C$^-$ activity; Dark gray, 20-60% of AE1C$^-$ activity; Light gray, >60% of AE1C$^-$ activity (Zhu et al., 2003).

pyridoxal phosphate and DIDS to inhibit anion transport, also inactivated transport function, highlighting the importance of this residue.

4.2.2 Eight AE1 mutant proteins were functionally inhibited by the membrane-impermeant reagent, pCMBS

Data from many studies has implicated the C-terminal portion of AE1 membrane domain as critical for ion translocation (Jennings, 1989b; Passow, 1986). To identify the residues that form the anion translocation pore in this region, we examined the sensitivity of all the introduced cysteine mutants to small hydrophilic, covalently-acting sulfhydryl-reagents (Figure 4.3). If an introduced cysteine residue is in the anion translocation pore, covalent modification with any of these compounds will block the substrate pathway, and thus sterically inactivate the transport activity. To determine the effect of sulfhydryl reagents, transport assays were performed before and after treatment with sulfhydryl reagents.

Among the three compounds, pCMBS is a negatively-charged, membrane-impermeant reagent that has been widely applied to probe membrane transport protein structure (Mueckler and Makepeace, 2002). Figure 4.4 provides an example of the effects of pCMBS on AE1 proteins. AE1C$^-$ was insensitive to pCMBS, as expected since it does not contain any cysteine residues. In contrast, L863C transport activity was greatly reduced by pCMBS, as seen by the reduction in the rate of pH change associated with HCO_3^- transport, following pCMBS treatment. Among the 65 functionally active introduced cysteine mutants analysed, only eight mutants (V849C, V850C, T853C, L857C, A858C, F861C, V862C, L863C) were inhibited by pCMBS (Figure 4.5). V850C and L863C achieved the maximum effect with inhibition by pCMBS >70%, suggesting

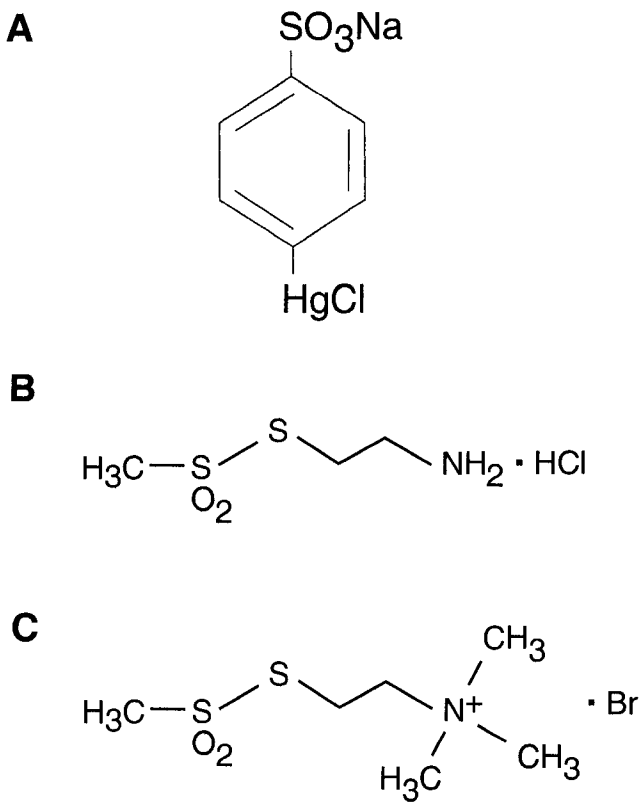


Figure 4.3 Structures of cysteine-reactive reagents
A, para-Chloromercuribenzenesulfonic acid (pCMBS). B, (2-Aminoethyl) Methanethiosulfonate Hydrochloride (MTSEA). C, [2-(Trimethylammonium)ethyl] Methanethiosulfonate Bromide (MTSET).

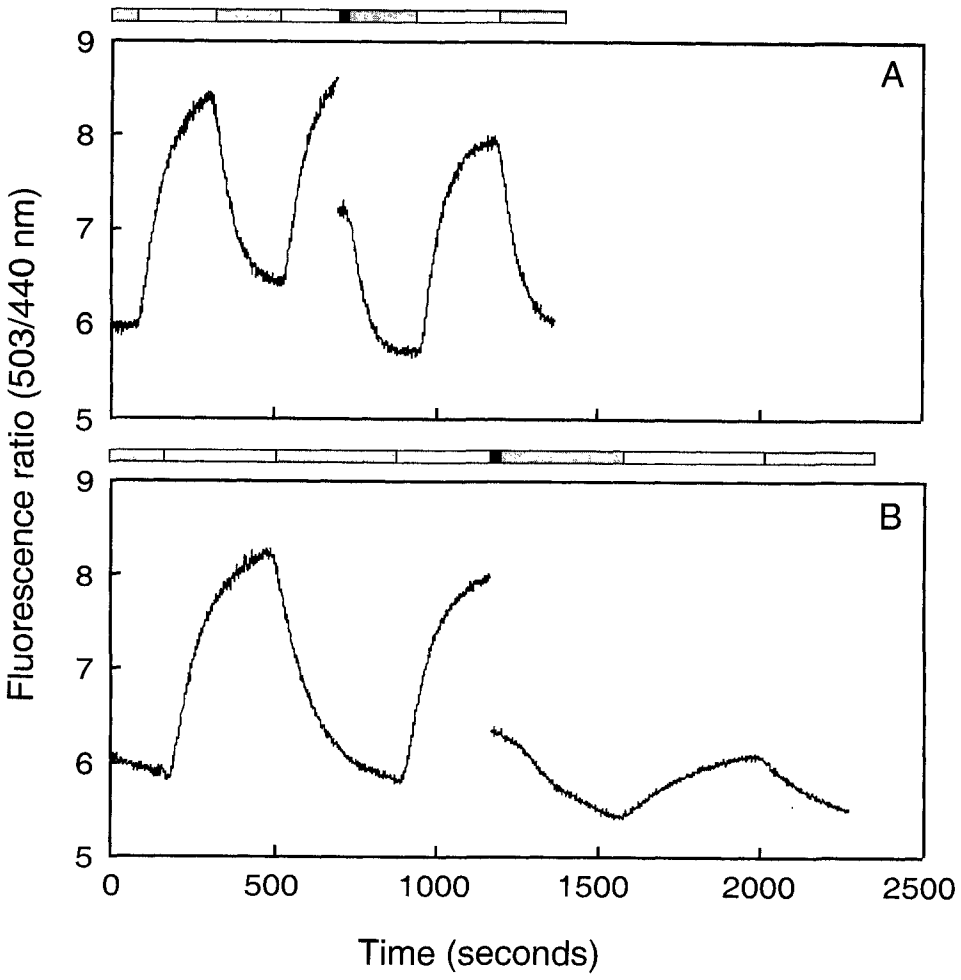


Figure 4.4 Representative Cl^-/HCO_3^- exchange assays with the cysteine-reactive reagent, pCMBS

HEK293 cells grown on coverslips were transiently transfected with cDNA encoding A, AE1C$^-$ and B, L863C. Two days post-transfection, cells were loaded with the pH sensitive dye, BCECF-AM, and placed in a fluorescence cuvette in a fluorimeter to monitor fluorescence. Transport rates were measured by perfusion of cells alternately with Cl^- free (white), Cl^- containing (gray) Ringer's buffer. Cells were then perfused with Cl^- free Ringer's buffer, containing 500 μM pCMBS (black bar) and incubated for 10 min, followed by wash-out with Cl^- free Ringer's buffer for 10 min. Curves are broken because the light path was blocked to prevent possible decomposition of pCMBS under high intensity light (black). Cells were then perfused with Cl^- containing, Cl^- free and Cl^- containing Ringer's buffer to re-assess transport rates. Inhibition of anion exchange was determined by comparison of the initial 100 second rates of alkalinization and acidification before and after pCMBS incubation. Fluorescence ratio (503 nm/440 nm) is a measure of intracellular pH.

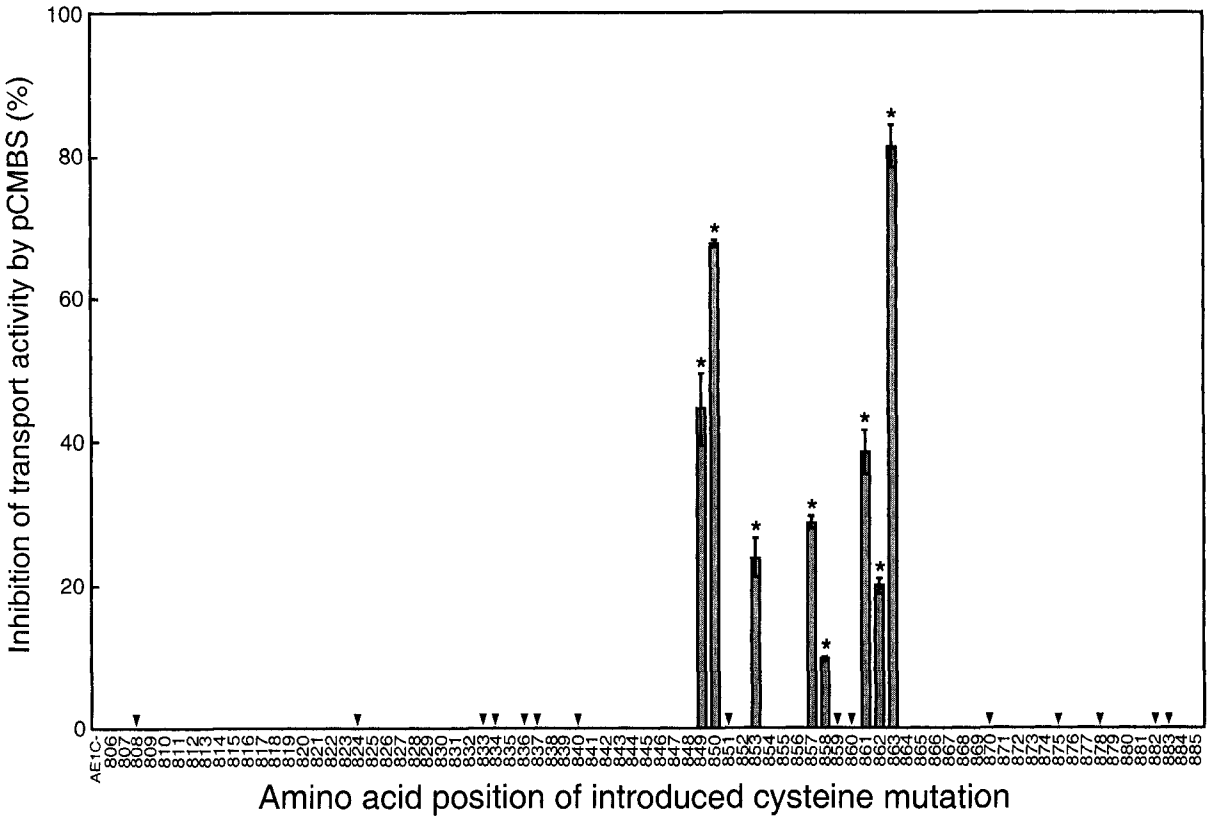


Figure 4.5 Effect of pCMBS on AE1 transport activity

Individual AE1 introduced cysteine mutants were expressed in HEK293 cells grown on coverslips. Transport rates were measured before and after incubation with 500 μM pCMBS (n=1-5). Background transport activity of mock-transfected cells was subtracted from each assay. Arrowheads mark mutants not analyzed due to low transport activity. Asterisks mark mutants whose transport rate was significantly ($p<0.01$) inhibited by pCMBS.

that these residues line the anion translocation pore. All the sensitive residues are hydrophobic, consistent with the previous findings on TM8 of AE1 (Tang et al., 1999). Interestingly, these residues lie at the extracellular ends of the last two transmembrane helices and the small extracellular connecting loop (Figure 4.2). In addition to the pCMBS-inhibited mutants, some other mutants had anion exchange activity that was slightly increased by pCMBS. Small increases in activity following treatment with sulfhydryl reagents have been reported in studies of introduced cysteine mutants of both transporters and ion channels (Cheung and Akabas, 1996; Frillingos et al., 1998) and may reflect effects on cells rather than the protein itself.

4.2.3 The V849 and L863 region is sensitive to inhibition by both pCMBS and MTSEA

To explore further if any residues close to the intracellular side of the protein may line the translocation pore, we scanned all the functional cysteine mutants for sensitivity to the sulfhydryl reagent, MTSEA. The pKa of MTSEA is 8.5 (Karlin and Akabas, 1998), so that a significant fraction of the reagent is unprotonated and neutrally-charged in the pH range of transport assays (pH 7.2 to 7.8). Unprotonated MTSEA is thus membrane-permeant (Holmgren et al., 1996). As found for pCMBS, eight residues in the Val849 to Leu863 region were inhibited by MTSEA (Figure 4.6). Among these six were also inhibited by pCMBS (V849C, V850C, T853C, L857C, A858C and I863C). Two other mutants (S852C and A855C) were inhibited by MTSEA, but not pCMBS. Conversely, F861C and V862C were inhibited by pCMBS, but insensitive to MTSEA. Strikingly, in the entire region scanned for sensitivity to sulfhydryl reagents, Phe806 to Cys885, all pCMBS and MTSEA-sensitive mutants clustered narrowly between Val849

and Leu863. The maximum transport inhibition by MTSEA was less than for pCMBS, only about 30%. This may be explained by the covalent moiety of MTSEA, which is smaller than that of pCMBS. Alternatively, anionic pCMBS may have greater access to these sites than neutral/cationic MTSEA.

4.2.4 Positively-charged MTSET inhibits mutants only in the extracellular loop region

Human AE1 will accept a wide range of substrates, but all of these are anionic (Jennings, 1989b). In the ClC Cl^- channel, whose structure may serve as a guide for an anion transporter like AE1, the anion selectivity filter is formed by four residues at the ends of different helices (Dutzler et al., 2002). To examine if the Val849-Leu863 region similarly forms the anion selectivity filter, we tested the effect of a cationic sulfhydryl reagent, MTSET (Figure 4.3), on the transport activity of these residues. MTSET has a quaternary amine group, with a fixed positive charge (Karlin and Akabas, 1998). Figure 4.7 shows that MTSET did not inhibit cysteineless AE1C$^-$. In contrast, mutants S852C, T853C, A855C, L857C and A858C were inhibited by MTSET to varying degrees, suggesting that this reagent can reach and react with most of the residues in the loop region. However, MTSET did not inhibit mutants at the ends of the transmembrane segments: V849C, V850C, F861C, V862C and L863C. Notably all five of these mutants were inhibited by pCMBS or MTSEA.

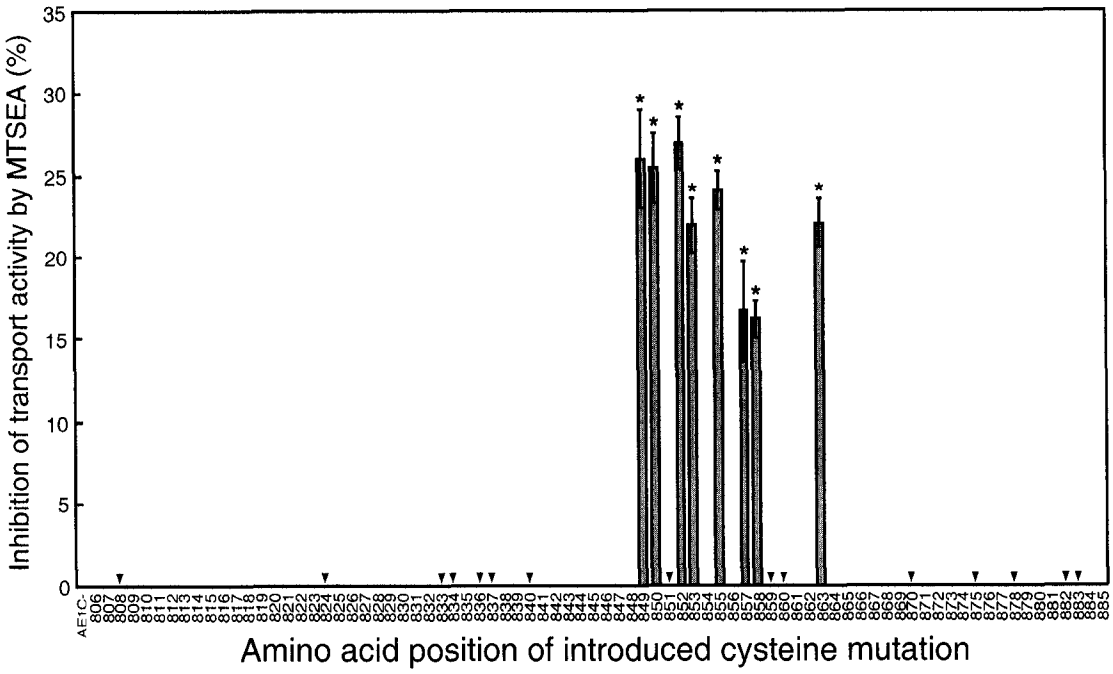


Figure 4.6 Effect of MTSEA on AE1 transport activity
Transport rates were measured before and after incubation with 5 mM MTSEA (n=1-5). Background transport activity of mock-transfected cells was subtracted from each assay. Arrowheads mark mutants not analyzed due to low transport activity. Asterisks mark mutants whose transport rate was significantly ($p<0.01$) inhibited by MTSEA.

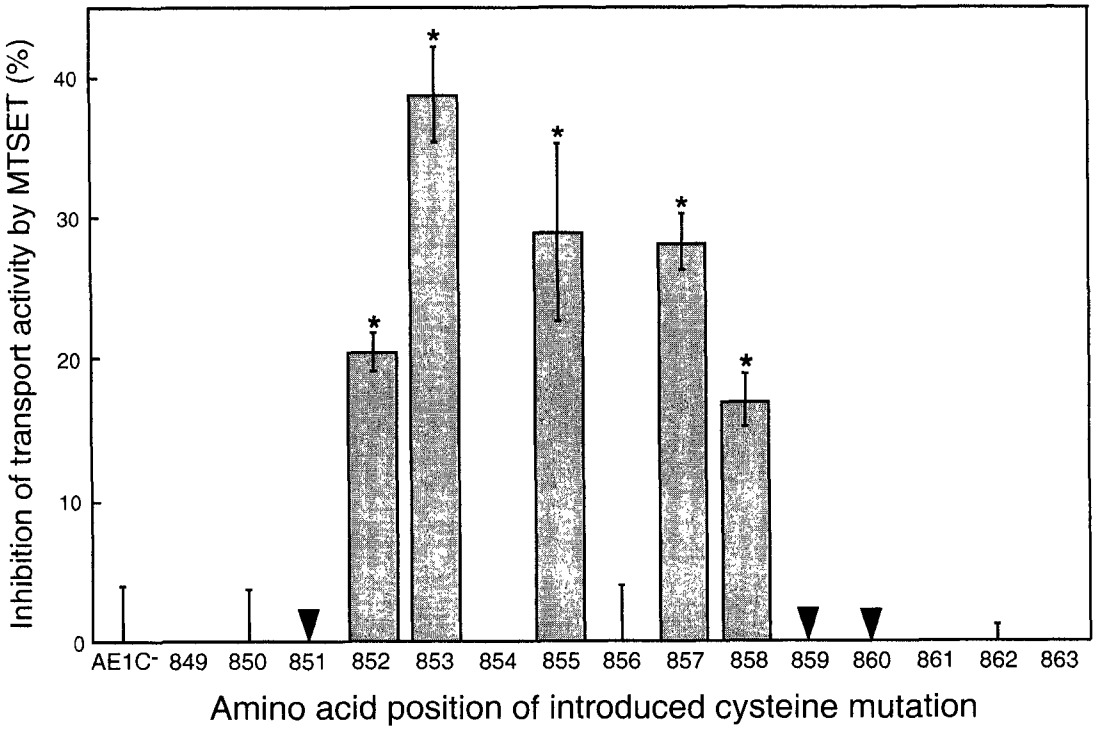


Figure 4.7 Effect of MTSET on AE1 transport activity
Introduced cysteine mutants between V849 and L863 were incubated with 5 mM MTSET (n>3). Transport rates were measured before and after MTSET treatment. Arrowheads mark mutants not analyzed due to low transport activity. Asterisks mark mutants whose transport activity was significantly ($p<0.01$) inhibited by MTSET.

4.2.5 Cl^- reduces the inhibitory effect of pCMBS on mutants V849C and L863C

Kinetic and $^{35}Cl^-$ NMR studies revealed that Cl^- competes with AE1 inhibitors, PLP, DNDS and DIDS, when binding to the anion transport site (Falke and Chan, 1986a; Frohlich, 1982; Salhany, 2001; Salhany et al., 1987; Shami et al., 1978). To examine competition between pCMBS and Cl^-, we measured the pCMBS sensitivity of mutant transport activity in the presence and absence of 140 mM NaCl in Ringer's buffer. All of the pCMBS-inhibited mutants in the Phe806-Cys885 region were examined. Some mutants (T853C, L857C, F861C and V862C) were not significantly influenced by Cl^- (Figure 4.8A). However, Cl^- significantly reduced the inhibitory efficacy of pCMBS for mutants V849C, V850C, A858C and L863C. The magnitude of the Cl^- effect can be quantified from the difference in pCMBS inhibition in the absence and presence of Cl^- (Figure 4.8B). A clear trend is evident, where the ends of the Val849-Leu863 region are most affected by Cl^- and the centre least. This pattern of Cl^- sensitivity strongly suggests that the last two transmembrane segments of AE1 form the anion selectivity filter.

Analysis of the inhibition data in the context of AE1 structural models provides additional insight (Figure 4.9). Measurements of accessibility of introduced cysteine mutants to biotin maleimide suggested that the last two transmembrane segments are short and connected by a very short extracellular loop (Zhu et al., 2003). In the present study, the only introduced cysteine mutants that are susceptible to inhibition by sulfhydryl reagents cluster at the extracellular ends of the last two transmembrane segments of AE1. In spite of the capacity of MTSEA to permeate the membrane, mutants sensitive to MTSEA to extend only about half way across the membrane (L863C). The short loop connecting the last two transmembrane segments also featured

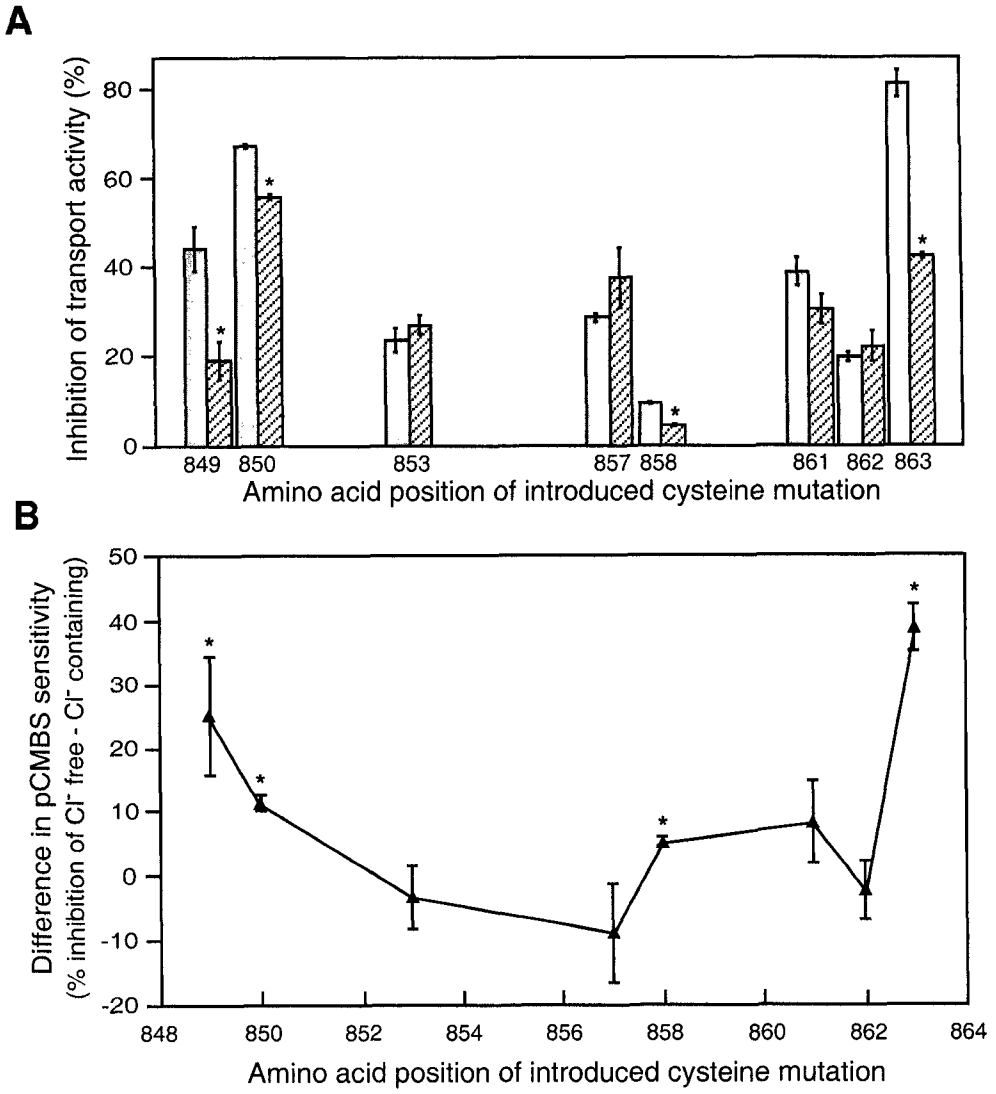


Figure 4.8 Charge filtering in the last two transmembrane segments
Transport activity was measured for AE1 introduced cysteine mutants before and after incubation with 500 μM pCMBS. Incubation with pCMBS was performed either in the absence (gray bars) or presence (hatched bars) of 140 mM NaCl. A, the inhibitory effect of pCMBS was measured as the % difference in activity in the absence and presence of pCMBS. B, the effect of Cl^- on pCMBS sensitivity was measured from the difference in pCMBS sensitivity when pCMBS was incubated in the absence or presence of Cl^-. Error bars represent standard error (n=3~5). Asterisks show statistical difference ($p<0.01$).

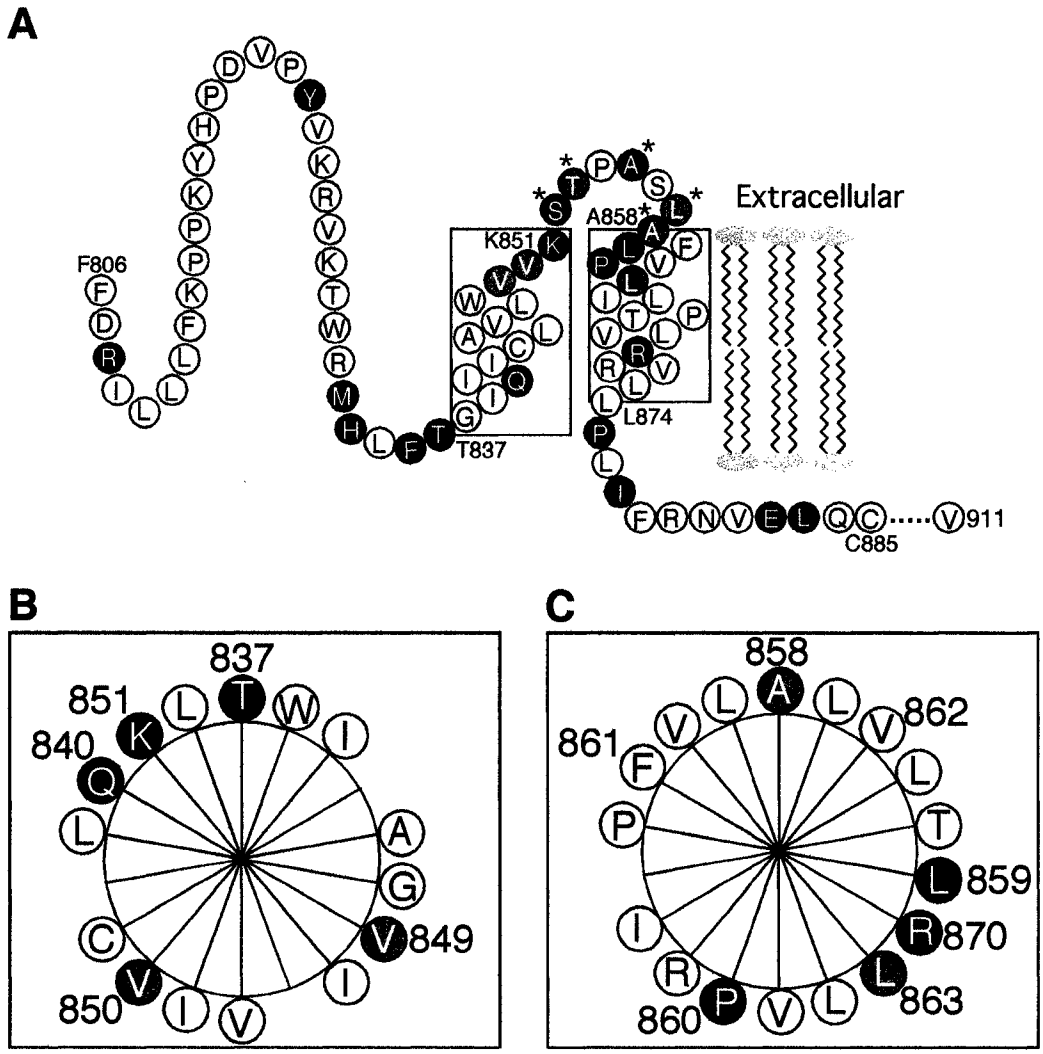


Figure 4.9 Location of sulfhydryl reagent-sensitive mutants
A, topology of the C-terminal portion of AE1. B, C helical wheel models with 3.6 residues/turn for B, sequence spanning amino acids 837-851 and C, 858-874. The first residue in each sequence is at the top of the wheel and sequence proceeds clockwise. Residues are coloured to indicate: Black, functionally inactive mutants; yellow, pCMBS sensitive mutants; red, MTSEA sensitive mutants; orange, pCMBS and MTSEA inhibitable residues. Asterisks mark mutants inhibited by MTSET. Unfilled circles represent mutants, which were not inhibited by pCMBS or MTSEA, except for C-terminal residue V911, which was not mutated and is only shown to indicate the relative position of the protein's C-terminus.

mutants sensitive to both MTSEA and pCMBS. Interestingly, although one might expect the loop to be highly accessible to labeling by sulfhydryl compounds, S852C and A855C were inhibited by MTSEA, but not pCMBS. Helical wheel analysis reveals that, in the Thr837-Lys851 region, mutants functionally inactivated when mutated are organized on one face of the helix (Figure 4.9B). Directly opposite this face is a hydrophobic surface on which two valine residues sensitive to both pCMBS and MTSEA are found. In the Ala858-Leu874 region a similar pattern is found (Figure 4.9C). Residues intolerant to cysteine mutation are on one helical face; opposite is a hydrophobic face where the pCMBS and MTSEA-inhibited sites lie. At odds with the pattern is L863C, which is sensitive to both pCMBS and MTSEA and is found on the same helical face as the mutation-sensitive residues.

4.3 Discussion

The human AE1 Cl^-/HCO_3^- exchanger has an anion translocation rate about 10 fold lower than an ion channel (Brahm, 1977; Hille, 1991). Although the substrate translocations rates of AE1 and chloride channels are similar, mechanistically AE1 differs because coupling of inward and outward ion translocation events is obligatory (Frohlich and Gunn, 1986). As well, the rigid structures found for ion channels (Doyle et al., 1998) differ from AE1, which has been shown to undergo large conformational changes during anion transport (Canfield and Macey, 1984). The present study, focussed on the C-terminal portion of the AE1 membrane domain (Phe806-Cys885), analysed the sensitivity of AE1 introduced cysteine mutants to sulfhydryl reagents. The fidelity of the substituted cysteine accessibility method (SCAM) has recently been shown by comparison of SCAM results to the crystal structures for the bacterial K^+ channel and

lactose permease (Abramson et al., 2003; Doyle et al., 1998). Here SCAM data has allowed identification of amino acids forming the AE1 anion selectivity filter. The last two transmembrane segments of AE1 are also revealed to form part of an ion translocation pathway unlike those found in ion channels.

Substrate Ion Charge Filtering

Like ion channels, AE1 is exquisitely selective toward the charge of its substrates, transporting only anions (Jennings, 1989b). The data presented here paint a picture of the substrate anion charge filtering in AE1. In the scan of the Phe806-Cys885 region, only introduced cysteine mutants in the Val849-Leu863 sequence were susceptible to inhibition by three different sulfhydryl reagents. The basis for inhibition by these compounds is their ability to permeate aqueous regions, to form a covalent adduct and to sterically block the confined space of the ion translocation pathway. Val849-Leu863 is therefore the only part of the C-terminal portion of the membrane domain, which meets these criteria.

The charge filter is cleanly delineated in these experiments (Figures 4.9 and 4.10). The Ser852-Leu857 region is modelled as an extracellular loop on the basis of accessibility to the aqueous reagent, biotin maleimide, which has the greatest ability to label extramembraneous regions and which does not label transmembrane segments (Zhu et al., 2003). In this loop four mutants (S852C, T853C, A855C and L857C) and A858C on the edge of the loop are inhibitable by MTSET, which has a fixed positive charge. MTSET has no effect on AE1 transport activity in regions deeper into the last two transmembrane segments. The failure to inhibit deeper sites does not represent a lack of

accessible or inhibitable residues, since five mutants in this deeper region were inhibited by pCMBS and or MTSEA. Exclusion of MTSET is consistent with charge filtering, to exclude cations. A second line of evidence demonstrating powerful substrate charge filtering was provided by experiments with pCMBS in the absence and presence of extracellular Cl^- (Figure 4.8). A clear trend in the effect of Cl^- was evident across the region. Cl^- maximally blocked pCMBS at the ends of the Val849-Leu863 region and had the least effect in the middle. That is, the effects of MTSET and Cl^- were reciprocal; Cl^- was most potent in the region where MTSET had no effect and least effective at sites where MTSET inhibited transport. The ability of Cl^- to reduce pCMBS efficacy at sites deeper in the membrane is consistent with charge filtering to allow anionic Cl^- into the deeper sites, where Cl^- competes with pCMBS for site occupancy. We conclude that MTSET has defined the boundaries of the cation exclusion charge filter in AE1. In the last two TMs cations cannot permeate deeper into the membrane than Ser852 and Ala858, respectively. As modelled in Figure 4.10, the cation exclusion filter is likely formed from two elements: the positive charge on Lys851 and the δ^+ charge helical dipole at the N-terminus of the last TM (Lehninger et al., 1993). Support for a role of Lys851 in charge filtering also comes from studies of Lys851, modified by reaction with bisulfosuccinimydyl suberate (Jennings, 1989a; Jennings et al., 1985). These studies led to the conclusion that loss of charge on Lys851 results in loss of AE1 transport function, but that this could be compensated at acid pH, where the net charge on the AE1 surface was increased.

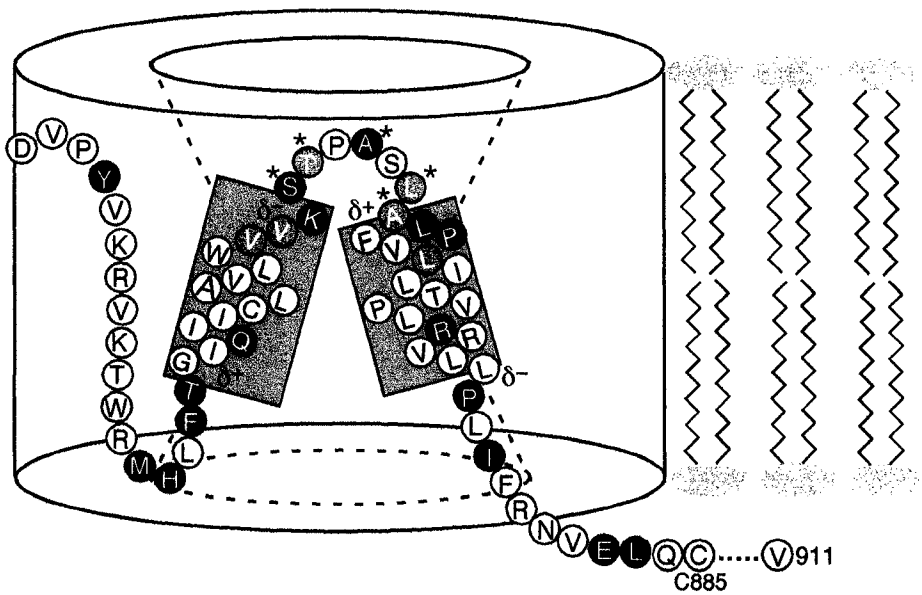


Figure 4.10 Model for the AE1 charge filter and anion binding site
Charges at the N and C-termini of helices are indicated by δ^+ and δ^-, respectively. Residues are coloured to indicate: Black, functionally inactive mutants; yellow, pCMBS sensitive mutants; red, MTSEA sensitive mutants; orange, pCMBS and MTSEA inhibitable residues. Asterisks mark mutants inhibited by MTSET. Unfilled circles represent mutants, which were not inhibited by pCMBS or MTSEA, except for C-terminal residue V911, which was not mutated and is only shown to indicate the relative position of the protein's C-terminus. Lipid molecules have been added on one edge of the model to indicate the proposed boundaries of the lipid bilayer.

Structure and function of extracellular loop Ser852-Leu857

Extracellular loop Ser852-Leu857 is dense with sites where sulfhydryl reagents will cause AE1 transport inhibition. The loop is thus localized to a region where addition of the covalent moiety of MTSET, pCMBS and MTSEA will cause inhibition. The pattern of inhibition with sulfhydryl reagents is consistent with the loop in a confined space, at the entry to the anion translocation channel. Figure 4.10 depicts the loop in this way, beneath the plane of the lipid bilayer, in a confined space. Further evidence for the localization of this loop in an inaccessible location comes from several sources. The classical anion exchange inhibitor, DIDS, covalently reacts with Lys851 (Okubo et al., 1994). An anti-DIDS antibody was unable to bind DIDS, when covalently attached to native AE1, but able to bind to DIDS on denatured AE1, leading to the conclusion that DIDS is buried in the AE1 structure (Landolt-Marticorena et al., 1995). Similarly, fluorescence energy transfer experiments indicated that the distance between stilbene disulfonates and the cytoplasm was less than the thickness of the bilayer, suggesting that stilbene disulfonates bind beneath the plane of the lipid bilayer (Rao et al., 1979).

The loop is likely to be in an extended β-conformation. In a β-conformation amino acid side-chains alternate in their orientation. Consistent with this, P854C and S856C could not be inhibited by any of the sulfhydryl reagents, suggesting that their side chains point away from the pore. In contrast, Thr853, Ala855 and Leu857 were inhibitable by sulfhydryl reagents, indicating that these side-chains are arranged to face into the anion translocation pathway.

The anion translocation pore

The last two TMs form a portion of the AE1 anion translocation pore. In these TMs Val849, Val850, Ala858, Phe861, Val862 and Leu863 were all positions where inhibition by sulfhydryl reagents occurred. Val849 and Thr837 are found on one helical face, consistent with being on one wall of the translocation pore (Figure 4.9B). Interestingly, other residues on this face are hydrophobic (isoleucine and valine). Pore linings formed of hydrophobic side chains have been a common feature of channels and have been proposed to reduce interaction of substrates with the pore linings (Doyle et al., 1998; Schirmer et al., 1995). Interestingly, on the face opposite Val849 and Thr837 are three residues whose mutation to cysteine results in loss of function. We propose that these residues localize to the face opposite the anion translocation pore. We previously speculated that Thr837 is required for proper insertion of the TM into the bilayer during biosynthesis (Zhu et al., 2003). The role of Gln840 may be in interactions between transmembrane segments. Importantly, Lys851 is on the helical face directly opposite the pore lining. As discussed above we have proposed an essential role of Lys851 in charge filtering. Lys851 is likely remote form the pore so that the fixed positive charge on the residue does not impede ion flow, but its electric field can contribute to charge filtering. Consistent with this model, K851C is functionally inactive, suggesting an essential role for the residue. Also, covalent modification of Lys851 with DIDS or PLP did not block the ability of Cl^- to bind AE1 (Falke and Chan, 1986a; Salhany, 2001), consistent with a location of Lys851 remote from the Cl^- interacting pore.

On the last TM Ala858, Phe861 and Val862 were sulfhydryl reagent inhibitable (Figure 4.9C). As seen in the other TM, this helical face is formed of hydrophobic amino

acids (phenylalanine, valine, leucine, alanine), again consistent with a hydrophobic pore lining. Leu863 stands apart from the other sulfhydryl-inhibitable mutants, on the opposite face of the helix. If Ala858, Phe861 and Val862 form the pore lining of AE1, then Leu863 would be directly opposite the pore and it would be difficult to understand how sulfhydryl reagents could affect L863C activity. Pro860 could induce a kink in the TM, altering the helical phase so that Ala858, Phe861 and Val862 are on the same helical face. Alternatively the presence of Leu863 on the opposite helical face from Ala858, Phe861 and Val862, suggests the possibility that the last TM of AE1 is conformationally mobile such that Leu863 can alternately face the pore or not, perhaps as part of the transport cycle. Recent studies on the ability to label His834 with diethyl pyrocarbonate provided compelling evidence for large conformational flexibility in the last two TMs of AE1 (Jin et al., 2003).

One dramatic feature of the sulfhydryl reagent transport inhibition data is the marked clustering of sensitive mutants at the extracellular ends of the last two TMs (Figure 4.9). Surprisingly, pCMBS and MTSEA greatly inhibited transport activity of mutants localized close to the extracellular end of these helices, but had no effect on the remainder of the two TMs. Inhibition by sulfhydryl reagents extended only 3 and 6 residues from the ends of the last two TMs, respectively. In a helical conformation each residue translates 1.5 Å, so 3 and 6 residues corresponds to a depth of only 4.5 and 9 Å respectively from the end of the helix, which is only a fraction of the distance across the bilayer. The observation is also unique in comparison to related studies of ion channels in which clear "stripes" of inhibitable mutants were observed down the length of the pore lining TM (Tang et al., 1999). The data is also distinguished from the results in a study

of TM8 of AE1, in which a clear "stripe" of pore lining residues running the length of the TM were identified (Tang et al., 1999). In that study pCMBS was able to inhibit introduced cysteine mutants until a point just before Glu681, which has been defined as the permeability barrier. MTSEA was effective beyond Glu681, as a result of its membrane permeability. The last two TMs therefore differ significantly from the structure of ion channel pores and the pore structure contributed by TM8. It may be that the last two TMs are kinked in such a way that before Val849 and after Leu863 the TM does not form the pore lining. There are two other possible explanations for the observation: 1. The uninhibited mutation sites are inaccessible to MTSEA and pCMBS, a possibility, which seems unlikely and 2. These sites are in a large open structure (Figure 4.10), which is sufficiently open that addition of the sulfhydryl reagent's covalent adduct does not occlude the translocation pore.

The AE1 plasma membrane Cl^-/HCO_3^- exchanger has a remarkably high ion translocation rate. However, the basis for the anion translocation event has not been established. Here we have presented evidence that the last two transmembrane segments of AE1 are intimately involved in the translocation process. These last two short (16 residue) helices are connected by a small extracellular loop. The loop region is required for selection of substrate ions on the basis of charge. We also presented evidence that at least the outer portions of these last two transmembrane segments form a portion of the transmembrane anion translocation pathway. Identification of this significant portion of the anion translocation pathway paves the way to identify the site of anion exchange, which is likely nearby this region, close to the centre of the bilayer.

Chapter 5

Summary and future directions

Summary and future directions

Human AE1 has served as a model for our understanding of the structure and mechanism of membrane transport proteins for decades, because of its high abundance in the erythrocyte membrane and its easy availability. Kinetic studies overwhelmingly support the idea that AE1 uses a ping-pong mechanism to transport anions (Frohlich and Gunn, 1986; Jennings, 1989b). However, the protein structure that underlies the ping-pong mechanism is largely unknown. The C-terminal region of the membrane domain has been implicated as involved in the anion translocation process, suggesting it may form part of the anion translocation site (Jennings, 1989b). Although topology of the membrane domain of AE1 has been studied extensively, a clear picture of the C-terminal region remains elusive. Determination of the topology and identification of the functionally important residues in the C-terminal region of AE1 is prerequisite to our understanding of the anion transport mechanism.

5.1 Topology of the C-terminal region of the membrane domain of AE1

In **chapter 3**, we determined the topology of the AE1 C-terminal region using cysteine scanning mutagenesis coupled with sulfhydryl-specific chemistry. Eighty cysteine residues were individually introduced into an otherwise cysteineless AE1 mutant between Phe806 and Cys885, which covers the last two putative transmembrane segments and the proposed transmembrane loop. Mutant proteins were expressed by transient transfection of HEK 293 cells, and topology of the region was determined by comparing cysteine labeling with the membrane-permeant cysteine-directed reagent BM, with or without prior labeling with the membrane-impermeant reagents, qBBr and LYIA.

BM only reacts with unprotonated sulfhydryls in the aqueous environment. Thus, BM labeled residues are considered to be located in an aqueous accessible region. Unlabeled residues are considered to be located in the lipid bilayer or protein interior. qBBr and LYIA are membrane impermeable and thus only label residues on the extracellular surface of the protein.

We found three regions, Phe806-Leu835, Ser852-Ala855 and Ile872-Cys885, were labeled by BM, suggesting their location in an aqueous environment. In contrast, Phe836-Lys851 and Ser856-Arg871 were not labeled by BM and therefore localize to the plane of the bilayer, as TM. Labeling by qBBr revealed that Pro815-Lys829 and Ser852-Ala855 are accessible to the extracellular medium. Pro815-Lys829 mutants were also labeled with LYIA. Mutants Ile872-Cys885 were inaccessible to the extracellular medium and thus localize to the intracellular surface of AE1. Functional assays revealed that 15 cysteine mutations inactivate the transport activity, and one face of each of two AE1 TMs was sensitive to mutation. On the basis of these results, we proposed a novel topology model for the C-terminal region of the membrane domain of human AE1.

5.2 Identification of pore-lining residues in the membrane domain of AE1

The unusual topology of the C-terminal region revealed in **chapter 3** suggests it may have an important functional role. In **chapter 4**, we further analyzed each introduced cysteine mutant protein in the functional assay after treatment with three small sulfhydryl reactive reagents, membrane impermeant anionic pCMBS, cationic MTSET and membrane permeant MTSEA, to identify residues involved in anion substrate selection and translocation. Functional activity of all mutants, before and after treatment

with the reagents, was characterized using a whole cell florescence assay by monitoring intracellular pH changes associated with bicarbonate transport.

Among the 80 introduced cysteine mutants, sulfhydryl reagents only inhibited those in the region of Val849-Leu863, suggesting the direct involvement of these residues in anion transport. Most residues inhibited by pCMBS were also sensitive to MTSEA, but to a lesser extent, as the covalent moiety of MTSEA is smaller than that of pCMBS. Sensitivity to cationic MTSET and effects of Cl^- identified the substrate charge filter is formed by residues Ser852-Ala858. Residues Val849, Val850, Phe861, Val862 and Leu863, which are clustered at the extracellular ends of the last two TMs, are not accessible to MTSET, fitting well with the observation that ion selectivity filters are formed by the helical dipole. Lys851 may offer an important positive charge to counteract the negative helical dipole, consistent with mutation or neutralization of this residue inactive the transport function (Jennings, 1989a). We propose the extracellular ends of the last two TMs form the anion selectivity filter and are conformationally mobile with intracellular ends that are widely open.

5.3 Future directions

The molecular basis of Diego^a blood group antigen is a single mutation (P854L) on the extracellular surface of the AE1 protein (Bruce et al., 1994). Results from **chapter 3** suggested that proline 854 is located in a small extracellular loop composed of four amino acids. Proline is a special amino acid and usually forms β-turn structures on the surface of the protein. To test if this mutation alters the local structure of the protein that generates the antigen, we mutated proline 854 to leucine in a cysteineless AE1 cDNA to recapitulate the Diego^a antigen. On the basis of this construct, fifteen cysteine residues

have been individually introduced into either side of the P854L mutation. Biotin maleimide (BM) will be used to determine local structural alterations of each of the eighteen mutant proteins. This work will offer us the first direct evidence if the Diego^a antigen is formed by a local conformational change, or a wide spread conformational change on the extracellular surface of the protein. It may also offer us an alternative way to determine the structural alteration of a protein that associated with mutations.

Pro814-Lys829 in AE1 has been proposed as a transmembrane loop in **chapter 3**, because of its labeling by BM, qBBr and LYIA from the extracelular medium. However, this region is also proposed as a cytoplasmic loop, as it is recognized by an antibody only after the membrane is permealized (Wainwright et al., 1989). Intriguingly, this loop is not accessible to protease in the native AE1 conformation, only after the protein is treated by high concentration of NaOH (Hamasaki et al., 1997). The discrepancy between the two proposals may be caused by a cryptic location of this loop in an extracellular protein cleft, as the size of chemical reagents is much smaller compared to the protease or antibodies. This is supported by synthetic peptide studies, which mapped this region as the interaction site for the adhesion of malaria-infected erythrocytes to endothelial cells, as well as a host receptor for the *Plasmodium falciparum* invasion of erythrocytes (Crandall et al., 1993; Goel et al., 2003). As this region is rich in proline and charged amino acids, it would be interesting to locate the sidedness of this loop precisely. The exposure of this loop on the surface of erythrocytes caused by malaria-infections has offered us a plausible approach to resolve the location of this loop. We could infect erythrocytes with malaria parasite to induce the exposure of this loop, and then probe the

loop extracellularly by specific antibodies, such as BRIC 132 (Wainwright et al., 1990a). This would definitively resolve the intriguing location of this region.

In **chapter 4**, we have identified the anion transport site in the AE1 protein, which opens a door for many opportunities to study the questions that are related to AE1 transport mechanism. As the ping-pong kinetic model suggests a transport site in the protein may alternately face outward and inward transiently to transport the substrates. Thus, it would be interesting to test how this region undergoes such conformational changes. In addition, although we have identified the substrate selectivity filter in the protein, it seems unlikely that these residues are sufficient to form a pore structure. AE1 contains 13 TMs; efforts are still needed to identify all the residues that are involved in forming the substrate translocation pore.

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