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Title of Thesis — Titre de la thèse

Spectroscopic and Interaction Studies on the Subunits of Bovine Cardiac Troponin

University — Université

UNIVERSITY of ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Ph.d.

Year this degree conferred — Année d'obtention de ce grade

1981

Name of Supervisor — Nom du directeur de thèse

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SPECTROSCOPIC AND INTERACTION STUDIES ON THE SUBUNITS OF BOVINE CARDIAC TROPONIN

by

© MAXWELL THOMAS CARL HINCKE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SPECTROSCOPIC AND INTERACTION STUDIES ON THE SUBUNITS OF BOVINE CARDIAC TROPONIN submitted by MAXWELL THOMAS CARL HINCKE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor

External Examiner

Date December 8, 1980

DEDICATION

This thesis is dedicated to Barbara, who was a continuous source of inspiration. I will always be grateful for her faith in me.

ABSTRACT

Thin filament regulation of muscle contraction in cardiac tissue, as compared to that in skeletal tissue, was investigated in several studies. Circular dichroism (CD) and biological activity criteria revealed the functional interchangeability of the troponin subunits from bovine cardiac and rabbit skeletal muscle in reconstituted hybrid complexes. The inhibitory activities of cardiac and skeletal troponin-I (Tn-I) were different, each being most effective with its parent actomyosin system.

The interaction between cardiac Tn-I and troponin-T (Tn-T) was demonstrated clearly by gel filtration, near and far UV CD and UV difference spectroscopy. Modification of both cysteine residues in Tn-I prevented complex formation with Tn-T, although ATPase inhibitory activity and interaction with troponin-C (Tn-C) were unchanged. This observation suggests the sequence region near cysteine 75 and/or 92 in Tn-I may be important for the interaction with Tn-T.

Ca^{2+}-binding to cardiac and skeletal Tn-C was investigated by CD, UV difference and UV absorption spectroscopy. The pH dependence of the Ca^{2+} affinity of the high affinity sites, as monitored by CD, was shown to be due to the protonation of two carboxyl groups with pKa's near 6.6 for each protein, in contrast to the single carboxyl group ($pKa \approx 6$) which controls the enhancement of tyrosine fluorescence observed when Ca^{2+} is bound. Tyrosine 150 and/or 5 in cTn-C is sensitive to these carboxylates ($pKa \approx 6.6$) as it possesses a pH dependent Ca^{2+} induced tyrosine difference spectrum with an apparent pKa of 6.6. No pH dependence was observed for the Ca^{2+} induced tyrosine difference spectrum in sTn-C. Complete nitration of the tyrosyl residues of cTn-C, sTn-C and calmodulin allowed

the nitrotyrosyl chromophore to serve as an optical probe for high affinity Ca^{2+}-binding, as evidenced by CD titrations with Ca^{2+}, monitored at 222 nm and 275 nm.

The effect of Ca^{2+}-binding on the 270 MHz proton nuclear magnetic resonance spectrum of bovine cardiac troponin-C (cTn-C) was examined. Resonances in the aromatic spectral region were assigned to tyrosine residues 5, 111 and 150 for apo- and calcium-bound cTn-C, based on decoupling experiments, pH titrations, temperature induced changes and gadolinium broadening experiments. High affinity calcium binding (up to 2 moles/cTn-C) causes large alterations in the environments of tyrosines 5 and 150, indicating that the N-terminus is probably buried in the protein interior. The evidence suggests that the environment of tyrosine 150 in calcium-saturated cTn-C must closely resemble that of tyrosine 138 in calmodulin, in that it experiences the hydrophobic core of the protein. However, there is no similarity between these environments in the apo-proteins. Dramatic alterations in phenylalanine resonances occur during the binding of the third mole of calcium, corresponding to filling the sole low affinity site. Comparison of the spectral features of cTn-C with those previously reported for sTn-C and calmodulin reveals many structural similarities which stem from their high degree of primary sequence homology.

Laser photo CIDNP ^{1}H NMR experiments were performed with rabbit skeletal Tn-C, bovine cardiac Tn-C and bovine brain calmodulin to study the exposure of histidine and tyrosine residues. In cTn-C, tyrosine residues 5, 111 and 150 were exposed in the apo-protein, becoming buried as Ca^{2+} was bound. A similar phenomenon was observed for tyrosyl residues 10 and 109 of sTn-C. In calmodulin only tyrosine 99 was accessible in

the apo-protein. The lack of exposure of tyrosine 138 observed with this technique correlates with the buried nature of this residue implied by other criteria.

In 6 M urea each of the apo-proteins was observed to be unfolded from the standpoint of the tyrosine environments. A large tyrosyl CIDNP effect was obtained for each protein which decreased as Ca^{2+} was bound, with a stoichiometry of one metal ion per protein. This was correlated for cTn-C with the appearance of 'native' resonances representing tyrosine residues 111 and 150 in Ca^{2+}-saturated cTn-C, also with a stoichiometry of one. Analysis suggests the sole high affinity Ca^{2+} binding site of cTn-C and sTn-C remaining in 6 M urea is site IV.

ACKNOWLEDGEMENTS

My thesis is in part due to the enthusiatic and helpful atmosphere of this Biochemistry department, derived from all its members. To them I shall always be grateful. I wish to express particular thanks to my supervisor, Cyril Kay, for his consideration and thoughtful approach at all levels. To Bill McCubbin, a collaborator in some of this work, I am grateful for numerous discussions and for introducing me to the vexations of the internal combustion engine. I thank the other members of the lab, Dave Byers, Sonia Herasymowich, Toni Keri, Vic Ledsham, Raj Mani and Kim Oikawa, for their input and contribution to all aspects of my graduate career. I express thanks to Ron Reid for many discussions on the nature of Ca^{2+}-binding proteins. I am grateful to Mike Natriss for performing the amino acid analyses and Dr. Peter Krahn of Dr. S. Hanson and Associates for Ca^{2+} determinations. Special thanks to the members of the labs of Larry Smillie and Bob Hodges for sharing their expertise, and to Brian Sykes and the other resonators for initiating me into the mysteries of nuclear magnetic resonance.

The present form of this thesis is due to the excellent typing of Darleen Wilson and the graphic skills of Vic Ledsham.

I acknowledge finanacial support in the form of studentships from the National Research Council and the Medical Research Council.

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ABBREVIATIONS AND SYMBOLS

The units for mass, length, volume and time have been abbreviated according to standard procedure. Terms appearing in a mathmatical relation are defined in the text where that equation is introduced.

ASX	asparagine & aspartate, as determined by amino acid analysis
ATP	adenosine triphosphate
cAMP	adenosine 3',5' monophosphate
C_c, C_s	cardiac, skeletal Troponin-C
CD	circular dichroism
CMC	carboxamidomethyl cysteine
DEAE-	diethylaminoethyl-
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid
DTT	dithiothrietol
$E^{1\%}_{1\ cm,\ \lambda}$	absorbance of a 1% protein solution in a 1 cm pathlength cell at the wavelength, λ
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycolbis(β-aminoethylether)N,N'-tetraacetic acid
f	fraction titrated
FID	free induction decay
FMN	flavin mononucleotide
I_c, I_s	cardiac, skeletal Troponin-I
IAANS	2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid
MNP	2-chloromercurinitrophenol
MOPS	morpholinopropanesulfonic acid
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PAGE	polyacrylamide gel electrophoresis

pCa	negative logarithm of the free Ca^{2+} concentration
pH	negative logarithm of the hydrogen ion activity
photo CIDNP	photochemically induced dynamic nuclear polarization
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
SAM	synthetic actomyosin
SDS	sodium dodecyl sulfate
T_c, T_s	cardiac, skeletal Troponin-T
Tm	tropomyosin
Tn	troponin
cTn-C	bovine cardiac troponin-C, the calcium binding subunit of the troponin complex
sTn-C	rabbit skeletal troponin-C
cTn-I	bovine cardiac troponin-I, the inhibitory subunit of the troponin complex
cTn-T	bovine cardiac troponin-T, the tropomyosin binding component of the troponin complex
Tn-T*, Tn-I*	proteins modified by reaction with iodoacetate or iodoacetamide
TNM	tetranitromethane
UV	ultraviolet
λ	wavelength
$[\theta]_\lambda$	mean residue ellipticity at the wavelength λ
$\simeq$	approximately equal to
$\sim$	approximately
$\geq$	greater than or equal to
$\leq$	less than or equal to

CHAPTER I

INTRODUCTION

A. VERTEBRATE STRIATED MUSCLE

The molecular nature of contraction in vertebrate skeletal muscle has been recently treated in several excellent reviews (Harrington, 1979; Lymn, 1979). Skeletal and cardiac muscle are cross-striated and, although there are large similarities between these tissues, distinct differences also exist, which have also been reviewed extensively (Katz, 1970; Ebashi et al., 1974; Léger et al., 1975). Therefore only a brief general review of muscle structure will be presented. A starting point for this discussion is the schematic description of a striated muscle in Figure 1, the main features of which are found in both cardiac and striated muscle.

A muscle fiber is composed of a longitudinally arranged, in register, array of myofilaments which exhibit a periodic band pattern when observed under the light microscope. The cross-striations represent alternating transverse regions along the myofibril which differ in optical density. When viewed under polarized light, it is seen that the optically dense or A bands are strongly birefringent (anisotropic), indicating that the molecular components of this region are asymmetric and oriented in a specific direction. Conversely, the optically light or I bands are relatively non-birefringent (isotropic).

Each A band is bisected by a dark line, the M line. Immediately adjacent to each side of the M line is a relatively less dense region. This narrow zone of relatively low optical density in the center of the dense A band and containing the M line is known as the H zone. The Z lines bisect the I bands and can be seen as a set of dark lines. The space be-

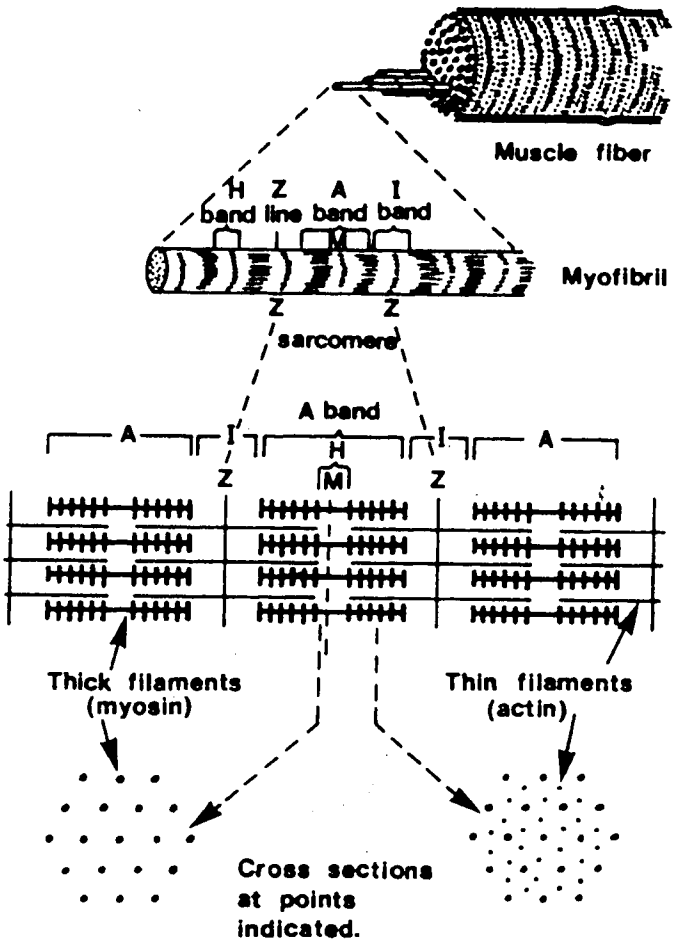


Figure 1. Schematic representation of myofibrillar structure [from Lehninger, 1970].

tween two adjacent Z lines represents the functional unit of muscle contraction, the sarcomere. Sarcomere length ranges maximally from 1.5 to 2.5 μ and is a function of total muscle length.

The band pattern of the sarcomere and its alterations during the contractile cycle is now known to reflect both the disposition of the contractile proteins in the myofibril and their mode of action. Electron micrographs reveal thick filaments, about 150 Å in diameter and 1.6 μ long, which extend the length of the A bands. There are also thin filaments, about 60 Å in diameter, running from the Z line at the sarcomere edge, completely through the I band and into the A band up to the edge of the H zone. The thick and thin filaments overlap solely within the A band. In this overlap region interfilamentous crossbridges between these two types of filaments may be visualized by electron microscopy. A cross sectional view of this arrangement is also presented in Figure 1. The myofilaments are arranged in a hexagonal lattice. The thick and thin filaments are easily discernable and their interdigitation is apparent in the overlap region of the A band.

Upon contraction, the sarcomere shortens. However this is brought about by the relative movement of the thick and thin filaments. Electron micrographs demonstrate that the length of the A band and the distance from the Z line to the edge of the H zone does not change. This may be seen schematically in Figure 2, which represents, in a simplified version, the sliding filament theory of muscle contraction. There are three important features of this model.

1. The proteins of the sarcomere are arranged in two separate kinds of filaments which can cross-connect;
2. Each type of filament is discontinuous along the length of the myo-

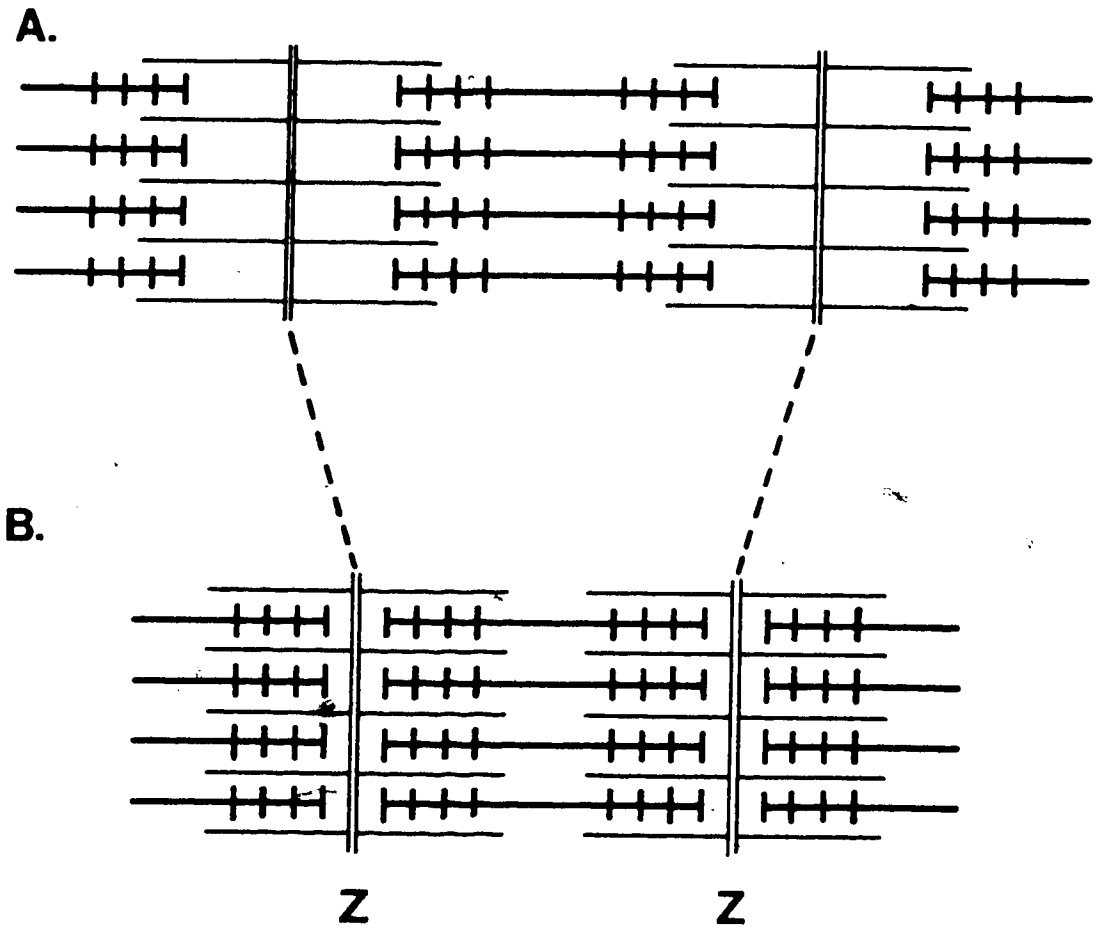


Figure 2. Model for sarcomere contraction.
A. Resting muscle.
B. Contracted muscle.
This representation of the sliding filament hypothesis demonstrates the relative sliding of the thick and thin filaments in one complete sarcomere [after Burtnick, 1977].

fibril;

3. Muscle shortening is due to a relative motion of one type of filament with respect to the other. There is no appreciable change in the lengths of the filaments themselves. The relative sliding motion occurs during the formation and disruption of the crossbridges between the two myofilament classes.

B. REGULATION OF STRIATED MUSCLE CONTRACTION IN VERTEBRATES

The most important functional proteins in muscle are myosin and actin, the main constituents, respectively, of the thick and thin filaments. These proteins play a major structural role but are also important in a regulatory sense. The region of the myosin molecule which forms the crossbridge to the thin filament contains an ATPase enzymatic site. The ATP hydrolysis provides the energy to drive contraction. The actin thin filament is found associated with two important regulatory proteins which confer calcium sensitivity upon the interaction between actin and myosin. The ability of these proteins to switch muscle contraction on and off represents the excitation-contraction coupling in which the calcium ion plays the role of a second messenger.

The manner in which the troponin-tropomyosin complex influences the ability of actin and myosin to interact is thought to be qualitatively similar in cardiac and skeletal muscle. The most extensive research has been carried out on the rabbit skeletal muscle system and it is believed to be fairly well understood (for reviews see Perry, 1979; McCubbin and Kay, 1980).

Skeletal troponin is made up of three proteins which interact strongly with each other: troponin-I (T[illegible]-I), the inhibitory subunit,

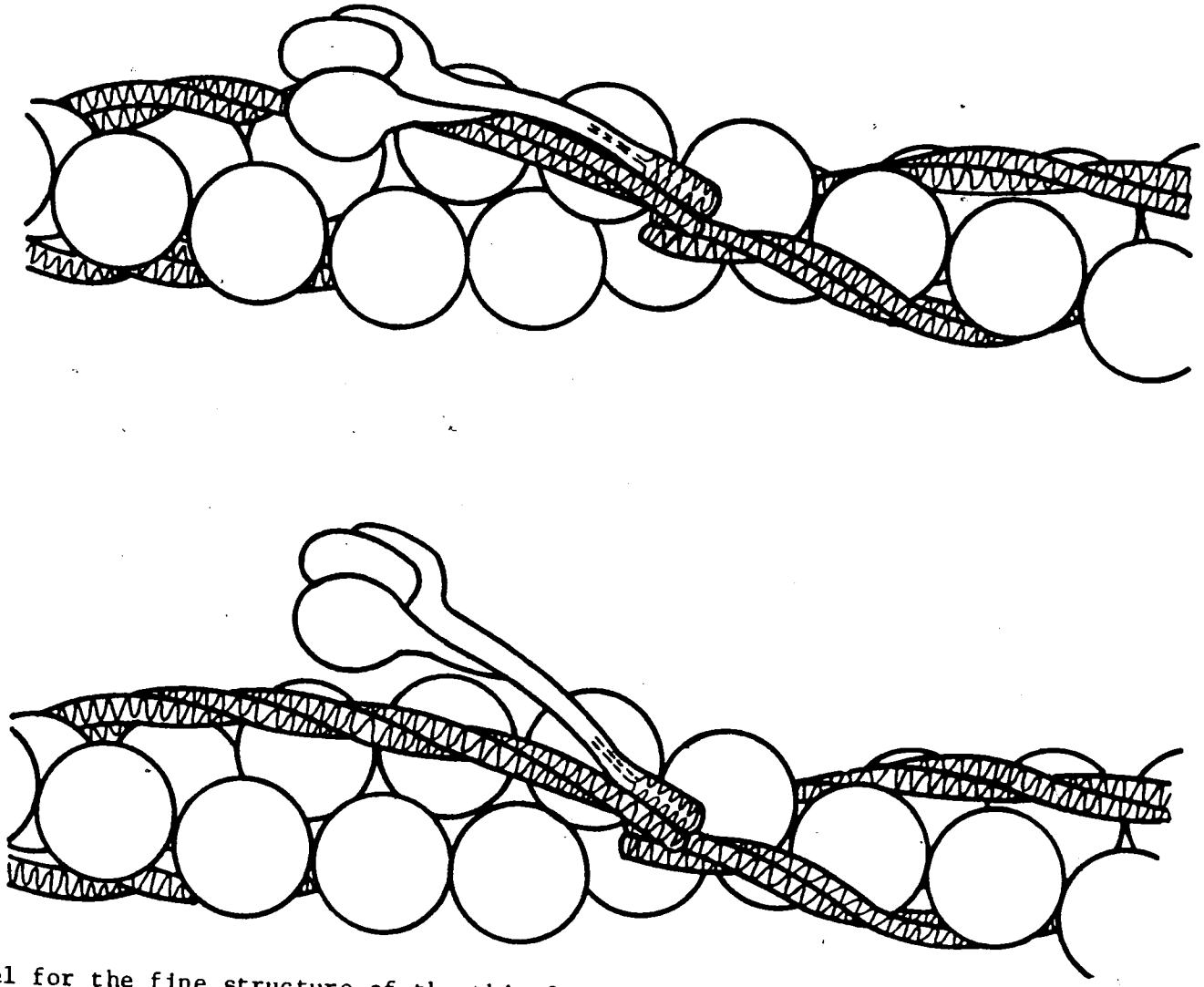


Figure 3. Model for the fine structure of the thin fi ment of muscle [from Mak and Smillie, (1980); after Ebashi et al., (1969)].

inhibits the Mg^{2+}-activated ATPase of actomyosin; troponin-C (Tn-C) is the calcium-binding subunit which reverses the inhibition of Tn-I, and troponin-T (Tn-T), which interacts strongly with tropomyosin. Each subunit has been characterized extensively. Studies of troponin and tropomyosin and their interactions have been incorporated into a model which seeks to explain their role in the regulatory process. This concept can be discussed in relation to the schematic representation of the thin filament seen in Figure 3. F-actin is depicted as two strands of pearls or beads wound around each other. Tropomyosin molecules are represented as rods, lying end to end in the grooves of the actin strands and each spanning the length of 7 actin monomers. Associated with each tropomyosin, at a specific site, is one unit of troponin. The known interaction properties of the troponin subunits between each other and with tropomyosin have been incorporated into this diagram. Tn-C interacts with Tn-I and Tn-T. Tn-I binds to Tn-T and to a site comprised of both actin and tropomyosin. Tn-T is shown binding to tropomyosin at two sites. A Ca^{2+} insensitive interaction between tropomyosin and Tn-T is found near the C-terminal head-to-tail overlap region of tropomyosin, (Mak and Smillie, 1980), while the bulk of the troponin complex has been localized about 90 Å closer to the N-terminus of the molecule. In this model, the Ca^{2+}-sensitive step is associated with the release of the troponin complex from this site.

A cross-sectional representation of the thin filament is depicted in Figure 4a. In resting muscle (pCa ≈ 8), troponin binds to the actin-tropomyosin filament at two sites. In this configuration, the troponin-tropomyosin attachment to F-actin sterically blocks the site on each actin monomer which would interact with myosin.

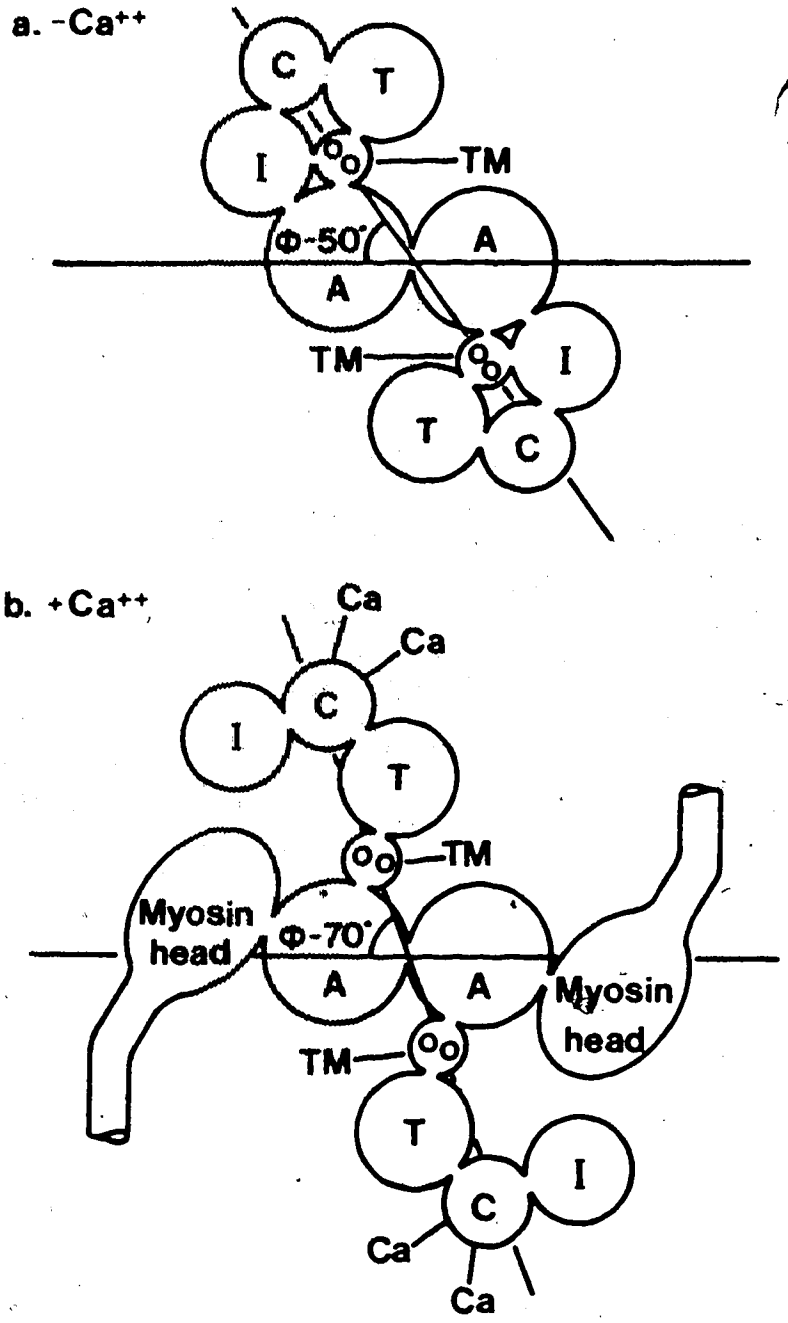


Figure 4. Model for the regulation of muscle contraction. The proteins are labelled as follows: actin, A; tropomyosin, Tm; troponin-I, I; troponin-C, C; troponin-T, T. Open areas between components indicate intermolecular interactions.

A. Positions of the proteins in relaxed muscle (pCa ≈ 8).

B. Positions of the proteins in contracting muscle (pCa ≈ 5) [from McCubbin and Kay, (1980) after Potter and Gergely, (1974)].

A nervous signal triggers the release of Ca^{2+} ions into the sarcoplasm bathing the myofilaments (pCa ≈ 5), saturating the calcium-binding sites on Tn-C. The resulting conformational change in Tn-C is transmitted in some manner to the other regulatory components, causing the tropomyosin molecules to shift deeper into the grooves between the F-actin strands. This situation is presented in Figure 4b. The rotation of the tropomyosin exposes the previously blocked interaction sites on the actin monomers, allowing myosin to form crossbridges. ATP hydrolysis occurs with concomittant muscle contraction.

C. CARDIAC TROPONIN SUBUNITS

The rabbit skeletal troponin subunits have been most widely studied and are better characterized than the corresponding proteins from any other tissue or source. These will be introduced and discussed briefly, but emphasis will be placed on the similarities and differences with the corresponding cardiac subunits. Some details have not been extensively discussed because of their thorough treatment later in the text.

1. Troponin-C

Tn-C molecules from all vertebrate striated muscle sources are acidic proteins, with high ratios of phenylalanine to tyrosine residues. This composition results in a distinctive UV absorption spectrum which is also observed for other Ca^{2+} binding proteins such as parvalbumin and calmodulin. These proteins all demonstrate great homology in Ca^{2+}-binding properties and amino acid sequence, leading to the hypothesis that they have all evolved from a common ancestor, a Ca^{2+}-binding protein, by gene duplication events (Collins et al., 1973). The crystal structure of a carp parvalbumin (carp MCBP 4.25) has been determined (Kretsinger

and Nockolds, 1973), revealing that the Ca^{2+}-binding sites consist of two of the three highly sequence homologous regions of the protein. The arrangement of each site can be generalized as a helix-loop-helix structure of the protein backbone, 33-37 residues in length. The calcium ion is bound to the 12 residue loop (Reid and Hodges, 1980). Parvalbumin is the only Ca^{2+}-binding protein for which the X-ray structure is known, and this only corresponds to the Ca^{2+}-bound form.

The highly sequence homologous regions within parvalbumin which bind Ca^{2+} have been compared to the primary sequences of many other Ca^{2+}-binding proteins. Four such regions have been located in the rabbit skeletal Tn-C sequence (Collins, 1976), three in that of bovine cardiac Tn-C (van Eerd and Takahashi, 1975) and four in the sequence of bovine brain calmodulin (Vanaman et al., 1977). These are presented in Figure 5, as aligned for maximum homology. The Ca^{2+}-binding regions are indicated, as are the tyrosine residues which have served as such useful spectroscopic probes. The predicted Ca^{2+}-binding sites agree with the stoichiometries that have been experimentally determined for these proteins. The Ca^{2+}-binding sites are labelled by designating the one closest to the N-terminus as site I and the others as II, III and IV.

It should be noted that the large similarities in primary sequence between these proteins does not indicate similarities in biological function or biological activity. Calmodulin is a ubiquitous Ca^{2+} binding protein found in non-muscle tissue. After binding Ca^{2+} it is able to interact with many different enzyme systems, causing a large enhancement of their enzymatic activity (see Cheung, 1980; Klee et al., 1980 for reviews). It differs from Tn-C in this aspect as Tn-C will bind to Tn-I and Tn-T in the absence of Ca^{2+}. This interaction is strengthened by

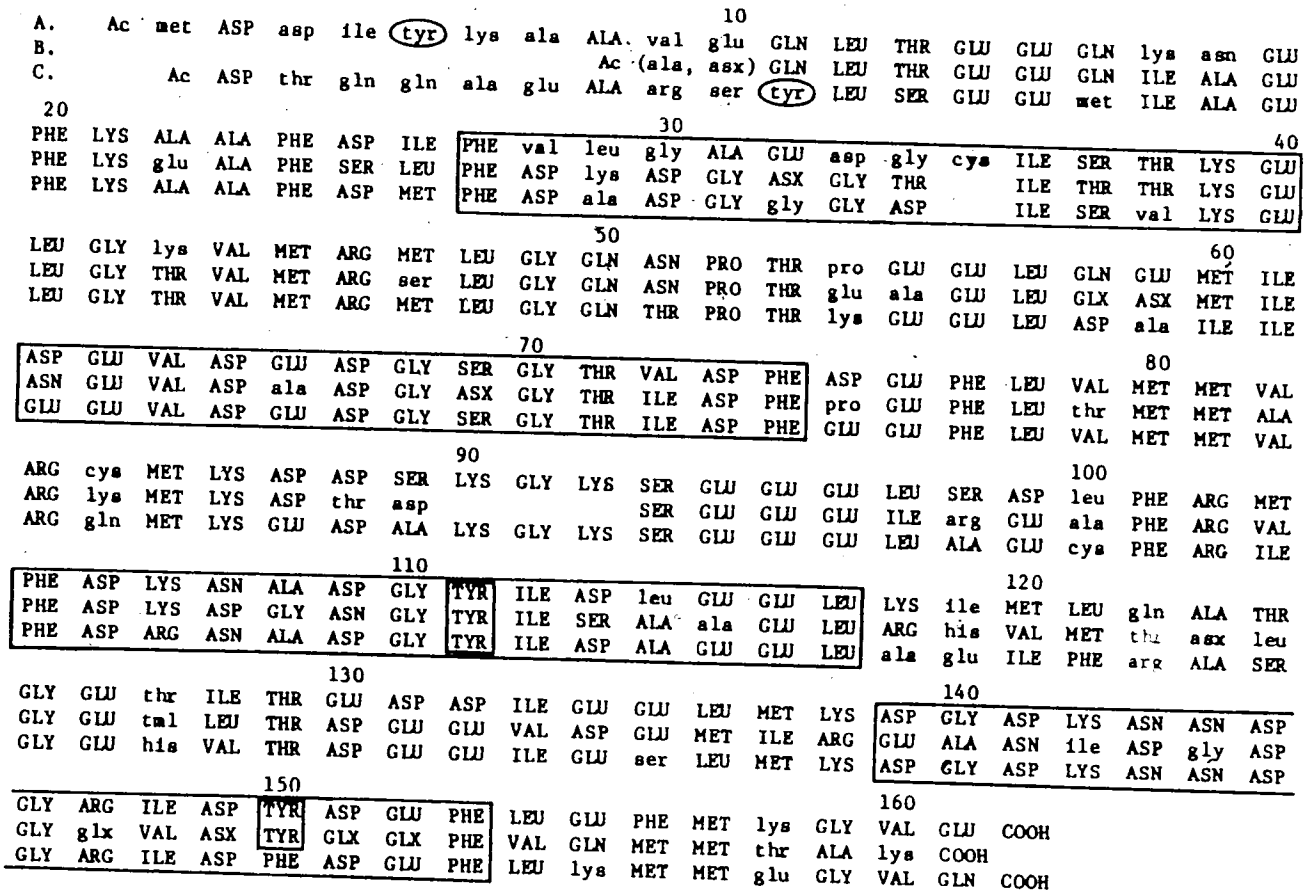


Figure 5. Amino acid sequences of muscle Tn-C's and calmodulin.
A. Bovine cTn-C (van Eerd and Takahashi, 1975),
B. Bovine brain calmodulin (Watterson et al., 1980),
C. Rabbit sTn-C (Collins, 1974).
Residues shown in capital letters are identical or functionally conservative replacements; those in lower case are assumed to be nonconserved. The numbers refer to positions in the cTn-C sequence. Trimethyllysine is abbreviated as tml. The presumed Ca^{2+}-binding sites are enclosed in boxes.

Ca^{2+}, the Tn-C - Tn-I complex becoming stable to 6 M urea (Head and Perry, 1974). Calmodulin displays similar binding properties with Tn-I, but only in the presence of Ca^{2+}. In the absence of Ca^{2+} no complex is formed (Amphlett et al., 1976). Although calmodulin can replace Tn-C in restoring the Ca^{2+} sensitivity of the actomyosin ATPase (Amphlett et al., 1976), sTn-C will not substitute for calmodulin in the activation of cyclic nucleotide phosphodiesterase except at 600-fold higher levels (Dedman et al., 1977). This effect, of uncertain physiological importance, is also seen with phosphorylase kinase, which sTn-C is 100-200-fold less effective in activating, as compared with calmodulin (Cohen et al., 1979).

Comparison of the primary sequences of Tn-C's from skeletal and cardiac sources reveals the major difference between the protein from these two tissues. The Ca^{2+}-binding region corresponding to site I in the skeletal sequence has been extensively substituted in the cardiac proteins, indicating it would not function as a Ca^{2+}-binding site. sTn-C binds 4 Ca^{2+} ions, two with high affinity (2×10^7 M^{-1}) and two with lower affinity (3×10^5 M^{-1}) (Potter and Gergely, 1975). The high affinity sites also bind Mg^{2+} (3×10^3 M^{-1}) and competition can be observed between Mg^{2+} and Ca^{2+} binding when Mg^{2+} is present at millimolar levels (approximately physiological). This reduces the apparent affinity for Ca^{2+} by about an order of magnitude. Similar Ca^{2+}-binding properties are observed for the cardiac protein. There are two high affinity Ca^{2+}-binding sites (1×10^7 M^{-1}) which also bind Mg^{2+} with low affinity (3×10^3 M^{-1}), and one low affinity binding site (5×10^4 M^{-1}) (Leavis and Kraft, 1978). The Ca^{2+} affinity of each class of binding sites is increased by one order of magnitude in the cardiac and skeletal Tn complex.

Comparison of these data implies the defunct site I in cTn-C corresponds to a low affinity site in sTn-C. This has been verified by two different studies. A chemical modification study (Sin et al., 1978) indicated that the two sites found closest to the N-terminal in sTn-C are the low affinity sites (I and II), and the high affinity sites are located in the C-terminal half of the molecule (sites III and IV). Further confirmation has come from Ca^{2+}-binding studies with fragments prepared from sTn-C (Leavis et al., 1978).

Although initially it was reported that the affinity of the sole low affinity site of cTn-C was about an order of magnitude lower than those of sTn-C, a recent report indicates it is of equivalent affinity (2×10^5 M^{-1}) (Holroyde et al., 1980).

Ca^{2+}-binding to cardiac and skeletal Tn-C has been monitored by a number of spectroscopic methods. For cTn-C, these have included increases in secondary structure by CD (Hincke et al., 1978), changes in intrinsic tyrosine fluorescence (Leavis and Kraft, 1978) and changes in fluorescence of the sulfhydryl label IAANS (Johnson et al., 1978b). Generally it has been observed that most of these techniques only probe the high affinity binding. The low affinity transition has been most readily monitored by incorporating reporter groups on the N-terminal half of the molecule. Thus IAANS covalently bound at cysteine residues 35 and 84 in cTn-C demonstrated a 6 to 7% decrease in fluorescence when the high affinity sites were occupied, but a 1.7-fold increase when the low affinity site bound Ca^{2+} (Johnson et al, 1978b). This phenomenon is also observed with the dansylaziridine fluorescent probe on sTn-C at methionine-25 (Johnson et al., 1978a).

Stopped-flow fluorescence studies utilizing the above mentioned

fluorescent analogs of cTn-C and sTn-C have suggested that it is the lower affinity site(s) in each protein which regulate muscle contraction (Johnson et al., 1978b; Johnson et al.,1979). Apparently the time scale of Ca^{2+}-exchange from these sites, which is related to their affinities for Ca^{2+}, and the associated changes in the protein structure, are fast enough to occur during one contraction cycle. Ca^{2+}-exchange at the high affinity sites in each protein, on the other hand, is too slow to regulate contraction.

Studies of the isometric twitch contraction times in fast mammalian skeletal muscle indicate that in the rat extensor digitorum longus (hind limb) maximum tension occurs in 10 to 13 ms after excitation (Close, 1965). This tension is observed to decay in 40 to 50 ms after excitation. Ca^{2+} transients associated with the development of tension in muscle have been measured utilizing the Ca^{2+} sensitive dye murexide (Jobsis and O'Conner, 1966) and the photoprotein Aequorin (Ashley and Ridgeway, 1968). $[Ca^{2+}]$ reaches a maximal level in the myofibrillar space just as 25 to 30% of the maximal tension is developed and then decays exponentially. The maximum height of the Ca^{2+} transient corresponded temporally to the maximum rate of rise of tension. This has also been observed in sartorus muscle fibers from frog and toad utilizing the Aequorin technique to detect Ca^{2+} transients (Blinks et al., 1978).

Similar data is not as abundant for cardiac tissue, apparently because of the difficulty in working with these myofibrils. However the Aequorin technique has been adapted and utilized by microinjection into the cells of frog atrial trabeculae (Allen and Blinks, 1978). As observed in the muscles so far discussed, the peak of the luminescent response (ie. Ca^{2+} transient) occurred at about the time of maximum rate of in-

crease in tension. They noted that the time course of the Ca^{2+}-transient was much slower in cardiac tissue than observed in skeletal muscle preparations (Blinks et al., 1978). Their data indicates that in the frog sartorus muscle about 30 ms is required from stimulation to maximal Ca^{2+} levels, but in the frog cardiac tissue, this time period is about 130 ms. This difference can also be inferred from data presented on the rate of left ventricular pressure development in dog hearts (Levine, 1967). The maximal rate of pressure development, corresponding to the point of maximal Ca^{2+} release, was reached about 60 ms after stimulation. This is obviously slower than similar data for vertebrate skeletal muscle (Close, 1965).

These considerations indicate that in fast skeletal muscle, Ca^{2}— must be released from the sarcoplasmic reticulum, bind to the regulatory sites of Tn-C, and produce the structural changes in the thin filament proteins which allow activation of the actomyosin ATPase activity, all within 10 to 13 ms after excitation. It should be noted that this figure is probably the fastest which can be found in the literature for excitation-contraction coupling; however other skeletal muscle systems should at least in principle be capable of achieving this speed. In vertebrate cardiac tissue these events probably occur within 60 ms. Relaxation of skeletal muscle, following removal of Ca^{2+} from Tn-C and reversal of the Ca^{2+}-induced structural changes would occur within 40 to 50 ms after excitation. Similar data for cardiac tissue is not available, but longer time periods would be involved, perhaps 4 to 5 times longer.

Stopped flow fluorescent experiments with dansylaziridine labelled sTn-C indicate the Ca^{2+} off-rates from the Ca^{2+}-Mg^{2+} high affinity sites and the lower affinity Ca^{2+}-specific sites are 4.8 s^{-1} and 313 s^{-1},

respectively (Johnson et al, 1979). In the functional troponin complex the Ca^{2+} affinity of each class of sites is increased. From the increased binding constants, and assuming a diffusion controlled 'on-rate' of $1 \times 10^{8}\ M^{-1}\ S^{-1}$, the calculated off-rates from these classes of sites in troponin would be $0.2\ S^{-1}$ and $20\ S^{-1}$, for the Ca^{2+}-Mg^{2+} and Ca^{2+}-specific sites respectively. Since Ca^{2+}-exchange with the Ca^{2+}-Mg^{2+} sites is much too slow to regulate contraction, while Ca^{2+}-exchange with the Ca^{2+}-specific sites is sufficiently rapid, the Ca^{2+}-specific sites are anticipated to be those involved in the regulation of contraction.

Similar phenomena have been reported for cTn-C (Johnson et al., 1978b). A Ca^{2+} off-rate of $277\ S^{-1}$ was determined for the sole Ca^{2+}-specific site in this protein. Again, there is a much slower rate of exchange from the high affinity sites. Calculations based on the affinities of the cTn sites predict rates of Ca^{2+}-exchange of $0.3\ S^{-1}$ and $50\ S^{-1}$ for the high and low affinity sites, respectively. Although the contraction processes in cardiac muscle occur on a slower time scale than in skeletal muscle, Ca^{2+}-exchange at the level of the high affinity sites is apparently too slow to fill this role.

A study of Ca^{2+}-binding and tension development in dog papillary strips found that between 10^{-8} and 10^{-6} M free Ca^{2+} an appreciable amount of Ca^{2+} was bound to myofibrils, but little activation of isometric tension occurred (Solaro et al., 1974). The Ca^{2+} requirement for half-maximal tension development was similar to that required for activation of myofibrillar ATPase ($K_{app} = 5 \times 10^{-5}$ M). These data suggests contraction was only associated with binding to the low affinity site. An interesting point is that their data indicates the Ca^{2+} requirement for maximal activation of cardiac contraction was about one-third of that for maxi-

mal activation of skeletal muscle contraction. This may be related to cTn possessing one regulatory site while sTn has two.

2. Troponin-I

Troponin-I is the inhibitory subunit of the troponin complex. It will inhibit the actomyosin ATPase on its own but this inhibition is greatly enhanced in the presence of tropomyosin (Wilkinson et al., 1972). The inhibition is neutralized by troponin-C, while the restoration of full Ca^{2+} sensitivity requires troponin-T (Greaser and Gergely, 1973). Although the general features of this inhibition appear to be similar in cardiac and skeletal systems, some unique aspects exist in each muscle type. Four Tn-I molecules from different sources have been sequenced (Wilkinson and Grand, 1978) which will form the basis for these general structural comparisons. These are fast and slow skeletal Tn-I and cardiac Tn-I from the rabbit, and the protein from chicken fast skeletal muscle. In this discussion I will assume the unique sequence features of rabbit cardiac Tn-I are present in the bovine cardiac protein as well. The amino acid composition and molecular weights of rabbit and bovine cardiac Tn-I are very similar (Burtnick, 1977).

The fast muscle Tn-I's have a smaller molecular weight, the main basis for which appears to be an additional 26 residues at the N-terminus of rabbit cardiac Tn-I, once the sequences have been aligned for maximum homology. This part of the molecule contains a serine residue at position 20, whose phosphorylation appears to be under hormonal control. Purified cTn-I can be phosphorylated at two sites by cAMP-dependent protein kinase, with a much more rapid rate of incorporation into serine-20 than serine-146 (Moir and Perry, 1977). However in the troponin complex phosphorylation at serine-146 is almost completely

inhibited (Stull and Buss, 1977). A similar phenomenon is observed for the corresponding residue, serine-117, in rabbit skeletal Tn-I. Studies with perfused intact hearts have clearly demonstrated that stimulation of the β-adrenergic receptors with catecholamines (adrenaline or isoprenaline) causes the incorporation of one mole of phosphate per Tn-I molecule, in hearts from a number of different species (Stull et al., 1979). This site has been shown to be serine-20 in perfused rabbit hearts (Moir et al., 1980). Other manipulations which increase the force of contractions in perfused hearts without increasing the cAMP content of the cardiac tissue have no effect upon the phosphate content of cTn-I (Ezrailson et al., 1977). The phosphorylation of serine-20 has been correlated with a decrease in the calcium sensitivity of cardiac actomyosin ATPase activity (Ray and England, 1976; Solaro et al., 1976).

The increased myocardial contractility observed with β-adrenergic agents probably does not stem from Tn-I phosphorylation. Force increase precedes phosphorylation (Perry, 1979), indicating the decrease in Ca^{2+} sensitivity may represent a negative feedback response associated with adrenaline action (Solaro et al., 1976). The decrease in Ca^{2+} sensitivity resulting from phosphorylation could explain the increased rate of relaxation that is characteristic of the inotropic effect of adrenaline. The fall in ATPase activity that accompanies the reduction of intracellular Ca^{2+} concentrations to normal levels after adrenaline intervention would be more rapid as a consequence of the decrease in Ca^{2+} sensitivity resulting from phosphorylation.

3. Troponin-T

Troponin-T is the tropomyosin binding subunit of the troponin complex. Little is known regarding structural or sequence differences

between cardiac and skeletal Tn-T. Their amino acid compositions are fairly different (Burtnick, 1977) and the molecular weight of the cardiac protein (36,000) is higher than the sequence molecular weight of sTn-C (30,500) (Pearlstone et al., 1977). Some differences in the interactions of cardiac and skeletal Tn-T with tropomyosin have been reported, based on different paracrystal patterns obtained with each system (Yamaguchi and Greaser, 1979).

4. Tropomyosin

Bovine cardiac tropomyosin has been thoroughally studied by physicochemical techniques and shown to be extremely similar to the rabbit skeletal protein (McCubbin et al., 1967). Both proteins form a dimer under physiological conditions, resulting from a non-staggered, parallel coiled-coil arrangement of α-helical subunits. The protein aggregates readily, in a head-to-tail fashion, involving an overlap region of 8 to 9 residues at the carboxy and amino terminals (Smillie, 1976). Rabbit skeletal tropomyosin is made up of two isomers, the α and β chains, in a molar ratio of about 3.5:1 (Cummins and Perry, 1973). These forms can be differentiated by gel electrophoresis, where the α chain has a faster mobility. Comparison of the sequences of the α and β forms of the rabbit skeletal protein indicated a large degree of similarity (Mak et al., 1980). The two forms have the same number of residues per chain (284) and only 39 amino acid substitutions, most of which are conservative replacements. Rabbit cardiac tropomyosin is found in only the α form, which is identical to the rabbit skeletal α protein (Lewis and Smillie, 1980). Other evidence indicates that β tropomyosin is found as about 15-20% of the total tropomyosin in large and slowly beating hearts (sheep, pig, human) and is absent in the hearts of smaller animals

(rabbit, guinea pig, rat, dog) (Léger et al., 1976). On this basis bovine cardiac tissue probably contains some β form as well.

Analysis of the rabbit skeletal α tropomyosin sequence indicates there is a pattern of 14 pseudo-repeat regions along the length of the tropomyosin molecule, involving both acidic and nonpolar residues (Parry, 1975; McLachlan and Stewart, 1976). These are postulated to contain the actin interaction sites, as each tropomyosin molecule is believed to overlap 7 actin monomers, in agreement with the stoichiometries of these proteins in the thin filament (Potter, 1974).

5. Interactions Between Skeletal Tn Subunits

Sequence studies of the skeletal troponin subunits have yielded well characterized portions of the molecules by proteolytic enzyme or chemical fragmentation techniques. Therefore it has been possible to define regions in the primary sequences which are involved in specific interactions with other thin filament components. The limitations of this technique are evident, in that interaction sites can involve distant segments of the primary sequence, brought together by the tertiary folding. However these studies have proved very successful with the skeletal troponin subunits, suggesting at least some of the interaction regions consist of highly localized stretches in the primary sequence. Such studies have not been performed with the cardiac proteins. However, as these probably represent the most fundamental interactions between the proteins, it is probable that similar interactions also occur between the cardiac components.

a. Troponin-C

The Ca^{2+}-binding domains can be localized onto individual fragments, in good agreement with the predictions based on homology with

parvalbumin (Leavis et al., 1978). The reduced Ca^{2+}-affinity of these fragments indicates the affinities of the native molecule probably result from interactions between the Ca^{2+}-binding sites as well as local effects at each site. A CNBr fragment of sTn-C, residues 84-135 (CB-9), can neutralize the inhibitory activity of Tn-I and form a complex with Tn-I in a Ca^{2+}-dependent manner (Weeks and Perry, 1978). This peptide corresponds to site III, a high affinity Ca^{2+}-binding site. The part of the molecule which is on the N-terminal side of the Ca^{2+}-binding loop is considered to be the region that binds to Tn-I (Perry, 1979). This part of the fragment undergoes a large increase in α-helix when Ca^{2+} is bound (Nagy and Gergely, 1978).

b. Tn-I

Interaction studies have indicated the N-terminal region of Tn-I (residues 1-47) and residues 96-117 form complexes with Tn-C (Syska et al., 1976). The peptide consisting of residues 96-117 also acted as a specific inhibitor of the Mg^{2+}-ATPase of desensitized actomyosin. As is the case with native Tn-I, this inhibitory activity was increased in the presence of tropomyosin (Syska et al., 1976). Since the CB-9 peptide of Tn-C can neutralize the inhibitory activity of Tn-I, it is this region of Tn-C which is believed to interact with the inhibitory peptide of Tn-I.

sTn-I also interacts with sTn-T (Horwitz et al., 1978) but no studies have been performed to localize this phenomenon in Tn-I. This phenomenon will be discussed in more depth at a later point.

c. Tn-T

Fragmentation studies on sTn-T demonstrate that the region which binds to Tn-C resides within residues 159-259, the highly basic C-terminal portion of the molecule (Pearlstone and Smillie, 1978). This inter-

action is strengthened by Ca^{2+}-binding to Tn-C.

Most of the α-helix of native sTn-T appears to be found at the level of one peptide, residues 71-151, which binds to tropomyosin near the C-terminus of that molecule (Mak and Smillie, 1980). Other evidence, such as immuno-electron microscopy, has indicated Tn-C and Tn-I are found about one third the length of the tropomyosin molecule from the C-terminal end. However, antibodies raised against two fragments of sTn-T indicate part of the Tn-T molecule is found at the same position as Tn-I and Tn-C, and part is located near the C-terminus of the tropomyosin molecule (Ohtsuki, 1979). Taken together, this evidence suggests Tn-T may interact with tropomyosin over a considerable length of the molecule, or at two distinct and well separated sites.

D. AIMS OF THE PROJECT

Most of the data available on striated muscle troponin has been obtained using protein prepared from rabbit skeletal muscle. Previous work in this laboratory has centered on the isolation and characterization of the individual troponin subunits from beef cardiac tissue (Burtnick, 1977). These results, as well as those of others, have demonstrated basic similarities between the troponin subunits in the cardiac and skeletal muscle systems. Important differences also exist, which presumably reflect subtle variations in the regulation of muscle contraction in each tissue. The beating of the heart, unlike skeletal muscle contraction, is not under direct conscious control. Skeletal muscle is capable of being switched on and off rapidly and completely, while cardiac muscle beats rhythmically but never appears to exist in a completely relaxed state. It displays a high resting tension with respect to skeletal muscle. It

is possible that differences between the troponin subunits are responsible for some of the functional differences between these tissues.

With these factors in mind, the present study was undertaken to more thoroughly investigate the properties of the cardiac troponin subunits and compare these to the skeletal counterparts. This was pursued at several levels. A direct comparison of the biological activity of the cardiac and skeletal subunits was undertaken in which the reconstitution of 'hybrid' complexes allowed a functional test of their interchangeability. A study of the interaction between cTn-I and cTn-T demonstrated similarities between this complex and its skeletal counterpart. As the Ca^{2+}-binding subunit, Tn-C, is the initial receptor molecule, the conformational alterations it undergoes after responding are of great interest. These were investigated by several optical techniques in a comparison study with skeletal Tn-C. Due to interesting results obtained by other workers from proton NMR studies of sTn-C and calmodulin, an NMR investigation of cTn-C was undertaken, allowing direct comparison of the solution conformations of these proteins. These were also studied using a closely allied technique, laser photo CIDNP, to probe the surface exposure of certain aromatic residues in each of these proteins.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. PREPARATION OF PROTEINS

The bovine cardiac troponin subunits were prepared according to methodology developed in this laboratory (Burtnick et al., 1975a; 1975b; 1976) for Tn-C, Tn-I and Tn-T, respectively. Trace Tn-T contamination in Tn-C preparations was removed by further chromatography in the absence of urea. Tn-C was dissolved in 50 mM TRIS-HCl, pH 7.5 and dialysed overnight at 4° C. If necessary this solution was clarified by centrifugation (Beckman Model L Ultracentrifuge). The protein was applied to an equilibrated DEAE-Sephadex-A25 column and eluted with a linear NaCl gradient from 0 to 0.5 M. Tn-C eluted as a sharp peak (conductivity ≈ 14 mmhos). The procedure was performed with either 2 mM EDTA or 2 mM $CaCl_2$ included, with identical results. This step also removed some unidentified contaminant which necessitated redetermination of the extinction coefficient. $E^{1\%}_{277\ nm}$ was determined to be 2.3 for this material.

The cTn-I and cTn-T preparative techniques were not altered, except that N_2 (Linde) was bubbled through all solutions used in their preparation for 15 minutes prior to use to minimize oxidation of DTT. Rabbit skeletal Tn-C was prepared by similar technique to cTn-C, from frozen rabbit muscle tissue (Pelfreeze). sTn-I and sTn-T were a kind gift of Dr. W.D. McCubbin and were prepared by standard methodology (Mani et al., 1973; 1974b).

Cardiac and skeletal actin were prepared from acetone powders of the tissue (Spudich and Watt, 1971). Myosin from beef cardiac muscle and rabbit skeletal muscle was prepared by standard procedures (Tonomura

et al., 1966). Bovine cardiac tropomyosin was purified from an ammonium sulfate fraction obtained during the preparation of crude Tn (Burtnick, 1977), by either repeated isoelectric precipitation (McCubbin et al., 1967) or hydroxyapatite chromatography (Eisenberg and Kielley, 1974). Calmodulin was prepared from bovine brain tissue (Walsh, 1978). Protein purity was verified by SDS-PAGE (Weber and Osborn, 1969).

B. pH MEASUREMENTS

pH measurement and adjustment of solvents and protein samples were routinely performed using a Radiometer model PHM62 pH meter equipped with a variable temperature compensator and combination glass electrode. pH measurements within a 5 mm or 10 mm NMR tube were accomplished with a 6030-04 Ingold combination electrode. These instruments were calibrated using standard pH buffers (Fisher Scientific Co.). No attempt was made to correct for the deuterium isotope effect with NMR samples dissolved in D_2O.

1. Spectrophotometric Titrations

Spectrophotometric titrations were performed on bovine cardiac and rabbit skeletal Tn-C in 0.1 M KCl, 5 mM acetate with either 1 mM EGTA or 0.1 mM $CaCl_2$ included. Samples (2.0 ml) were titrated manually with KOH in a constantly stirred vessel maintained at 20° C. After addition of base an aliquot was removed and the absorbance measured at 295 nm. Nitrated proteins were titrated similarly, but the absorbance change at 425 nm was monitored. A Gilford 240 spectrophotometer was utilized for these measurements. The pH of the solution was determined with a Radiometer 64 pH meter after the aliquot was replaced in the titration vessel.

A computer program was used to perform a nonlinear curve fitting

iterative procedure which fitted the data to an equation of the form:

$$\Delta A = \sum_{i=1,2} \frac{\eta_i K_{OH,i} [OH^-]}{1 + K_{OH,i}[OH^-]} \quad [1]$$

where $pH = 14 - pOH$ [2]

and $pK_{a,i} = 14 - pK_{OH,i}$ [3]

derived from the Henderson-Hasselbach equation. In this case η_i refers to the relative contribution of each class of tyrosyl residues to the total absorbance change. Thus

$$\text{Total } \Delta A = \eta_1 + \eta_2 \quad [4]$$

C. AMINO ACID ANALYSIS

Amino acid analyses (Moore and Stein, 1963) were performed utilizing a Beckman 121 or Durram D-500 automated amino acid analyser. Samples were hydrolysed in constant boiling 6 N HCl for 24 hours. Phenol (1%) was included to minimize degradation of tyrosine residues.

Amino acid analysis was performed to measure the 3-nitrotyrosine content of nitrated cTn-C, sTn-C and calmodulin. This was determined using the peak size of nitrotyrosine at its unique Rf value or from the decreased peak size of tyrosine.

The CMC content of carboxyamidomethylated Tn-I or Tn-T was determined by amino acid analysis, assuming a color value which was 0.905 of the Asx value (Hodges, 1971).

1. Extinction Coefficient Determination

Cardiac Tn-C samples were dialysed versus 0.1 M KCl, 20 mM MOPS, pH 7.2 and their absorption spectra measured. Aliquots of these solutions were placed in tubes for analysis and an aliquot of a standard solution of norleucine added to each. Following lyophilization, 6 N HCl was

added to each sample and amino acid analysis performed as usual. The results for each sample were normalized by the norleucine content and the concentrations determined using the known amino acid content of cTn-C. Correlation of the protein concentration determined in this manner with its absorbance allowed an extinction coefficient ($E^{1\%}_{1\ cm,\ 277\ nm}$) to be calculated.

D. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Standard methodology was used (Shapiro et al.,1967). Samples were prepared in 2% or 10% SDS containing 1 mM DTT by heating in a boiling water bath for 5 to 10 minutes. Electrophoresis was performed on 10% or 15% polyacrylamide gels for 3 to 4 hours at 6 mamps/gel. The dye marker bromphenol blue was used. Gels were stained with Coomassie Brilliant Blue for 20 to 40 minutes and destained in several changes of 7% acetic acid, 7.5% methanol (V/V).

Molecular weight estimations were made by comparing the migration distances of the unknown proteins with those of proteins of known molecular weight (Weber and Osborn, 1969).

E. OPTICAL METHODS

1. Absorption Spectroscopy

Routine absorption measurements were performed using a Gilford 240 spectrophotometer. UV absorbance, difference spectra and first derivative spectra were recorded employing a Cary 118C recording spectrophotometer. Protein samples utilized for spectroscopic studies were routinely clarified by centrifugation in a Beckman Model L instrument (Rotor 50). Dialysates used to establish baselines were filtered (millipore

5.0 μ). Difference spectra were obtained using either Helma Quartz cells (1.00 cm path length) or split compartment tandem cells (0.874 cm path length). The instrument was operated in the 'auto-slit' mode with a full scale absorbance range of 0.05 at a scan speed of 0.1 nm/s. Spectra were obtained at ambient temperature. The stability of the instrument was enhanced by constant purging of the optical track and cell compartment with dry N_2.

Two different methodologies were utilized to generate difference spectra. When the effect of a perturbant upon the protein sample was being studied a baseline was first generated with both cells containing equal volumes of the protein solution. Then concentrated perturbant (ie. HCl or $CaCl_2$) was added to the sample cell while an equivalent volume of solvent was added to the reference cell.

Tandem double cells were used to study protein interactions. A baseline was first generated with separated protein solutions in both reference and sample compartments of the spectrometer. The contents of the sample cell were then mixed to generate a protein complex and the absorption difference spectrum recorded.

Derivative spectra were recorded with the Cary 118C instrument operating in the first derivative mode.

2. Circular Dichroism

Circular dichroism measurements were performed using a Cary 6001 CD attatchment to a Cary 60 recording spectropolarimeter (Oikawa et al., 1968). The instrument was calibrated with an aqueous solution of recrystallized d-10- camphor sulfonic acid. Constant nigrogen flushing was employed.

The mean residue ellipticity, $[\theta]_\lambda$, at a particular wavelength λ, was calculated from the equation:

$$[\theta]_\lambda = \frac{\theta_{obs} \cdot MRW}{100 \cdot \ell \cdot c} \qquad [5]$$

where:

MRW is the mean residue weight (taken as 115 in these studies),

θ_{obs} is the observed ellipticity value at the wavelength of interest,

ℓ is the cell pathlength in cm, and

c is the protein concentration in gcm^{-3}.

The units of $[\theta]_\lambda$ are degree . cm^2 . $dmole^{-1}$.

When protein solutions were titrated with calcium, the ellipticity at the wavelength of interest was determined following careful addition of an aliquot of $CaCl_2$. The process was continued until no further change in the spectrum could be discerned.

F. ULTRACENTRIFUGAL METHODS

Ultracentrifugal studies (Chervenka, 1969) utilized a Beckman Spinco Model E analytical ultracentrifuge. This instrument was equipped with a photoelectric scanner, multiplex accessory and high intensity light source. A Rayleigh interference optical system was also used.

1. Protein Extinction Coefficient

The concentrations of a number of a number of protein solutions were determined following absorbance measurements using ultracentrifugal synthetic boundary runs with Rayleigh interference optics (Babul and Stellwagen, 1969). A measurement of the number of fringes crossed on each photographic plate, using a Nikon model 6C microcomparater, was rela-

ted to the protein concentration using the average refractive increment of 4.1 fringes/mg/ml determined for proteins by Babul and Stellwagen. This allowed correlation of protein concentration with absorbance for calculation of the extinction coefficient ($E^{1\%}_{1\ cm,\ 277\ nm}$).

2. Sedimentation Equilibrium Experiments

The apparent weight average molecular weight Mw of a homogeneous protein is given by the equation:

$$Mw = \frac{2RT}{(1-\bar{V}\rho)\omega^2} \cdot \frac{d \ln c}{dr^2} \qquad [6]$$

where:

R is the universal gas constant,

T is the experimental temperature (°K),

$\bar{V}$ is the partial specific volume of the protein,

ρ is the solvent density,

ω is the angular velocity, and

c is the protein concentration at a distance r from the axis of rotation.

When Rayleigh interference optics were employed the protein concentration at the meniscus C_m was calculated according to:

$$C_m = C_o - \frac{r_b^2 (C_b - C_m) - \int_{cm}^{cb} r^2\, dc}{r_b^2 - r_m^2} \qquad [7]$$

where:

C_m is the protein concentration at the meniscus,

C_o is the initial concentration of the protein,

r_m is the distance from the meniscus to the axis of rotation,

C_b is the protein concentration at the cell bottom, and

c is the protein concentration at any distance r from the axis

of rotation.

A direct measure of protein concentration was determined by adding the fringe displacement at point r to the concentration at the meniscus in terms of fringes.

In using the photoelectric scanner the absorption value A at any point along the cell gave a direct measure of protein concentration.

A plot of the natural logarithm of the protein concentration (expressed as either Y, the number of fringes, or by A, absorbance units) versus r^2 yielded a straight line whoe slope, d ln c/dr^2, was used to determine the weight average molecular weight at any position along the cell by application of equation [6].

G. BIOLOGICAL ACTIVITY ASSAYS

Synthetic cardiac and skeletal actomyosin systems were reconstituted to investigate the differences in regulation by hybrid troponin complexes.

The assay system contained 750 µg of synthetic actomyosin (SAM) and 50 µg of tropomyosin in 10 mM TRIS-HCl, pH 7.6, 2.5 mM $MgCl_2$, 1 mM EGTA and 2.5 mM ATP at 20° C (Shaub and Perry, 1969). SAM was prepared by mixing myosin and actin in a 4:1 ratio by weight (Shi [illegible] Tonomura, 1972). Total volume of this reaction mixture was 3.0 ml and also contained troponin components in equimolar amounts of Tn-I. Addition of SAM initiated the reaction, which was monitored by measuring inorganic phosphate ions liberated on hydrolysis of ATP (Fiske and Subbarow, 1925).

H. CALCIUM BINDING STUDIES

In studying the properties of a molecule such as Tn-C, as a

function of $[Ca^{2+}]$, it is necessary to control the concentration of free Ca^{2+} very precisely. This may be accomplished by the use of metal-ion buffers which provide a controlled source of free metal ions in a manner similar to the regulation of hydrogen ion concentration by pH buffers.

In a solution containing a metal ion, M, and a chelating agent, L, metal complexes are formed

$$M + nL \rightleftharpoons MLn$$

and the equilibrium can be expressed quantitatively by the appropriate stability constant:

$$Kn = \frac{(MLn)}{(M)(L)^n} \qquad [8]$$

This may be solved for [M] and expressed as pM, the negative logarithm of the free metal ion activity which is analogous to pH. When the chelating agent is present in excess and almost all of the metal ion is bound in a complex, the free metal ion is buffered.

Very frequently the chelating agent can undergo protonation

$$H^+ + L \rightleftharpoons HL, \text{ etc}$$

to form species that have little or no complexing ability. Thus protons will compete with metal ions for the chelator and the extent of this competition depends upon the pH.

Calculation of pM for metal ion buffers containing an excess of complexing agent is straightforward (Raaflaub, 1956; Schwarzenbach, 1957) and has been discussed extensively (Burtnick, 1977; Perrin and Dempsey, 1974). The calculations in this work utilized EGTA and the following logarithmic association constants for Ca^{2+} and H^+ binding to EGTA: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^-, 2.0; Ca^{2+} to $EGTA^{4-}$, 10.97 (Sillén and Martell, 1964).

Only plasticware was used for sample preparation and handling and this was previously washed with 3 N HCl/25% ethanol solution, followed by doubly distilled water to eliminate Ca^{2+} contamination. Quartz CD and UV absorbance cells were treated in the same manner. A given Tn-C sample was dissolved in, and dialysed versus, 100 mM buffer at a pH near the pKa, 50 mM KCl and 1 mM EGTA for about 18 hours. This high buffering capacity was necessary to prevent pH changes during the Ca^{2+} titration, which would result from the displacement of protons from EGTA upon metal binding.

CD titration data was normalized using the following expression:

$$f = \frac{[\theta]^{obs}_{222\ nm} - [\theta]^{i}_{222\ nm}}{[\theta]^{f}_{222\ nm} - [\theta]^{i}_{222\ nm}} \qquad [9]$$

where:

$[\theta]^{obs}_{222\ nm}$ is the ellipticity value at a given Ca^{2+} concentration,

$[\theta]^{i}_{222\ nm}$ is the initial ellipticity value before the addition of any Ca^{2+},

$[\theta]^{f}_{222\ nm}$ is the final ellipticity value after saturation of the protein with Ca^{2+}.

The fractional conformational change (f) was plotted versus pCa to generate a titration curve. Since the concentration of the protein (typically ca. 1mg/ml) is small compared to that of EGTA, Ca^{2+} binding to Tn-C can be neglected in calculating the free $[Ca^{2+}]$. Apparent binding constants were extracted from the data by computer fitting using a non-linear least-squares iterative procedure, which fit the data to an equation of the form:

$$f = \Sigma \frac{\eta_i K_i [Ca^{2+}]}{1 + K_i [Ca^{2+}]} \quad i = 1 \text{ or } 2 \text{ or } 3 \ldots . \qquad [10]$$

and yielded the values of η_i and K_i.

Here K_i is the apparent association constant of the i^{th} class of binding sites,

η_i is the proportion of the total conformational change due to the i^{th} class of Ca^{2+}-dependent transitions, and

$[Ca^{2+}]$ is the concentration of free Ca^{2+} calculated from the EGTA parameters.

This equation describes the binding of small molecules to proteins and is based upon the simplifying assumption that the binding sites in any class are equivalent, independent and noninteracting (Edsall and Wyman, 1958). If there is more than one class of binding sites with different affinities for Ca^{2+}, i is increased to equal the number of different classes.

In practice it was an easy matter to decide from the shape of the Ca^{2+}-titration curve which value of i was most appropriate. The extent to which a given fit would minimize the mean standard error was also used in this assessment.

I. ^1H NUCLEAR MAGNETIC RESONANCE

1. Instrumental Settings and Pulse Sequences

All ^1H NMR spectra were obtained on a Bruker HXS 270 spectrometer equipped with quadrature detection operating in the fourier transform mode. Typical instrumental settings for the 5 mm probe were: sweep width = $\pm$ 2000 Hz, 4096 points, pulse length 9.8 μs (~90°), line broadening =

1.0 Hz. The HDO resonance was suppressed with homonuclear decoupling. All chemical shifts are reported relative to the methyl resonance of DSS, and, except where noted otherwise, were taken at 301° K.

Several different pulse sequences were used:

a. In order to completely suppress the nuclear Overhauser effect (NOE) during decoupling of aromatic resonances, the decoupler was only turned on during the FID aquisition and a delay of 1.5 seconds was incorporated between scans.

b. When decoupling experiments retaining the NOE were desired, the decoupler was left on throughout the pulse sequence.

The effects of decoupling were most easily seen in difference spectra, where the blank was obtained by decoupling at a frequency where there were no resonances. Because of the overall correlation time of cTn-C, decoupling resulted in a negative NOE which decreased the intensity of the collapsed peak. This is represented in Figure 6b, where subtraction of the smaller singlet peak from the doublet yields an inverted doublet in the difference spectrum.

2. Gadolinium Broadening Experiments.

In these experiments equal amounts of $GdCl_3$ and $LaCl_3$ solutions (in 10 mM PIPES, pH 6.5) were added to two identical samples. The specific paramagnetic effects of the Gadolinium were seen by subtracting the Gd^{3+} spectrum from the La^{3+} one. The broadening of the resonances of the protons close to the bound Gd^{3+} ion was easily detected in difference spectra. This is represented in Figure 6a, where subtraction of the broadened peak from the normal resonance (equal areas) yields a sharpened peak in the difference spectrum.

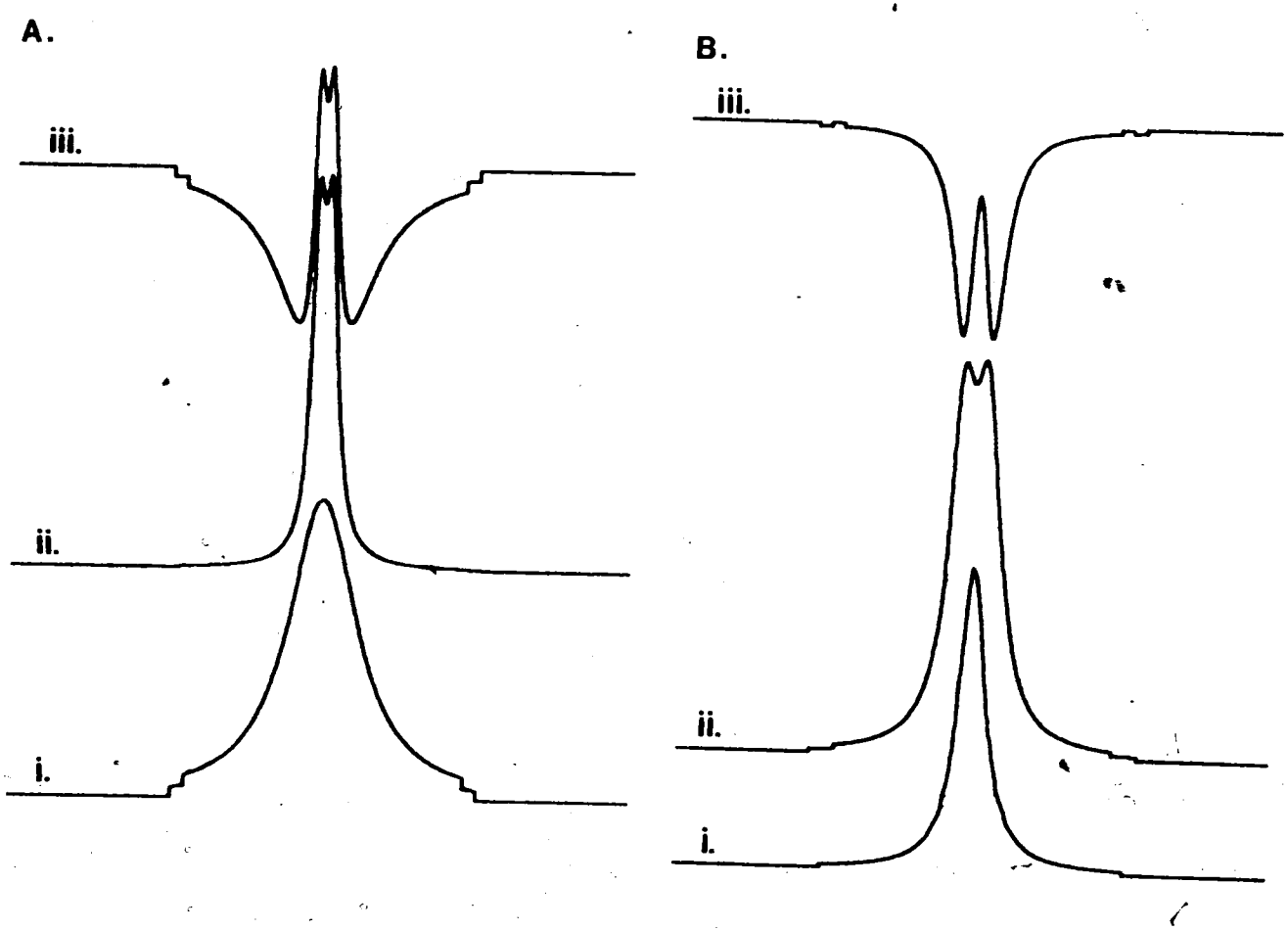


Figure 6. Idealized difference spectra for different NMR situations.

A. Subtraction of a broadened tyrosine doublet(i) from one which has not been broadened(ii) to produce a sharpened doublet(iii).

B. Subtraction of a doublet which has been collapsed to a singlet in a decoupling experiment, with concomitant loss of intensity due to the negative NOE(i) from the normal doublet(ii) to produce a negative doublet(iii).

3. Metal Ion Solutions

Metal ion solutions ($LaCl_3$, $GdCl_3$ and $CaCl_2$) were prepared gravimetrically and standardized by titration with an EDTA standard solution. The lanthanide chlorides were dissolved in 10 mM PIPES, pH 6.5 to prevent hydroxide formation and precipitation. $CaCl_2$ was dissolved in D_2O. A syringe microburet (Micro-Metric Instrument Co.) calibrated with H_2O gravimetrically was used for titrations. The indicator eriochrome black T was used for calcium standardization (Skoog and West, 1969) and xylenol orange for lanthanide solutions (Korbl and Přibil, 1956).

4. Protein Solutions

Calcium-free Tn-C and calmodulin was generated by exhaustive dialysis versus doubly distilled water in plasticware following protein dissolution in 0.1 M EDTA, pH 8.0. The protein was then dialysed for 24 hours against 0.15 M KCl, 25 mM MOPS, pH 6.8, which had been passed through a column of chelex 100, and lyophilized. The protein was redissolved in D_2O to the original volume for NMR measurements. The Ca^{2+}-free status of this protein was confirmed by atomic absorption measurements (<0.1 mole Ca^{2+}/mole protein) and also by comparison of spectra of these samples with those obtained with samples containing EDTA. Ca^{2+} was removed from some samples by passing them slowly through a column of chelex 100 (Blinks et al., 1978).

In other experimental situations, in order to generate the Ca^{2+}-saturated or Ca^{2+}-free proteins, samples were dialysed versus the solvent containing 2 mM $CaCl_2$ or 2 mM EDTA for 24 hours. After lyophilization, the protein samples were redissolved in D_2O.

Protein concentrations were determined on each sample by quantitative dilution of a small aliquot into a similar solvent (but H_2O, not

D_2O). Typically this was 50 µl protein sample plus 1.0 ml solvent. UV absorption measurements, utilizing extinction coefficients ($E^{1\%}_{1\ cm,277\ nm}$) of 2.3 for cTn-C, 1.9 for sTn-C (unpublished observations from this laboratory) and 2.0 for calmodulin (Walsh, 1978) allowed calculation of the protein concentration.

pH's were adjusted with 0.5 N solution of NaOD or DCl.

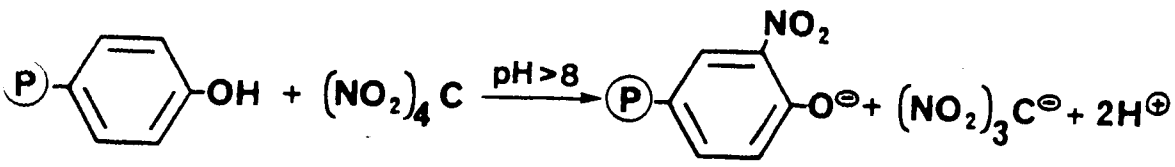
5. Laser Photo CIDNP

Laser photo CIDNP experiments (Kaptein, 1978) were performed utilizing a Spectra Physics model 164 Argon ion laser, operating at 3.5 Watts in the multiline mode. 10 mM flat bottom NMR tubes were used for these experiments, at an ambient temperature of 301° K. A 14.0 µsec pulse (~90°) was used for the 10 mM Probe. In the experimental routine, alternating light and dark FID's were collected, in which the sample was irradiated for 1.0 seconds prior to recording the light spectrum, with a 30 second delay between scans. Subtraction of the dark from the light spectrum allowed the specific effects of irradiation to be seen.

J. PROTEIN MODIFICATION TECHNIQUES

1. Nitration of Tyrosyl Residues

Nitration of proteins was achieved by treatment with TNM (0.84 M solution in 95% ethanol). This reaction takes the form:



The reaction with skeletal Tn-C resulted in complete nitration of both tyrosine residues (McCubbin and Kay, 1975). For cardiac Tn-C two sets of conditions were chosen. Complete nitration of all three tyrosine resi-

dues could be obtained by treatment of the protein, at a concentration of 1 mg/ml, with an 8-fold molar excess of TNM at room temperature, for six hours, in a solvent consisting of 50 mM TRIS-HCl, 2 mM EGTA pH 8.0, followed by another addition of an 8-fold molar excess of TNM and overnight incubation. After reaction the modified protein was separated from the nitroformate ion side-product by desalting with Biogel P-2 equilibrated with 50 mM NH_4HCO_3, 1 mM EGTA. The product was lyophilized from this mixture. Partial nitration could be achieved by treatment with a 10-fold molar excess of TNM for 1 hour at room temperature in 0.1 M NH_4HCO_3, 2 mM EGTA pH 8.5, followed by desalting and lyophilization. A Ca^{2+}-free system was chosen for these reactions since preliminary experiments revealed that the rate of nitration in 50 mM TRIS-HCl, 1 mM $CaCl_2$ pH 8.0 was approximately one half the rate for the protein in the minus Ca^{2+} state although the same degree of nitration could be obtained under both conditions. Chromatography on a 125 x 1.5 cm column of Sephadex G-100 in 50 mM NH_4HCO_3, 1 mM EGTA pH 8.5 yielded monomeric nitrated protein. Complete nitration of calmodulin was achieved utilizing the methodology of Walsh and Stevens (1977). This sample of calmodulin was a kind gift of Drs. F.C. Stevens and M. Walsh. The concentration of nitrotyrosine was estimated from the absorption at 381 nm using 2200 M^{-1} cm^{-1} as the extinction coefficient (Riordan and Vallee, 1972). The concentrations of nitrated protein were determined by interference fringe counts in the ultracentrifuge assuming 41 fringes equivalent to 10 mg/ml (Babul and Stellwagen, 1969). The extent of nitration was also determined by amino acid analysis, in which the color value for nitrotyrosine was assumed to be equal to that of tyrosine.

2. Carboxamidomethylation of Troponin I and T

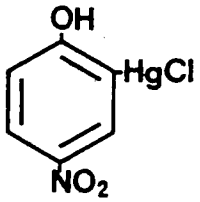
This reaction can be represented:

$$\text{(P)}\text{-}S^{\ominus} + ICH_2CONH_2 \xrightarrow{pH \geq 7} \text{(P)}\text{-}S\text{-}CH_2CONH_2 + I^{\ominus}$$

Proteins to be modified were dissolved in 6 M urea, pH 4.0 and dialyzed overnight at room temperature versus 0.5 M NaCl, 50 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT with N_2 bubbling throughout. Samples were clarified by centrifugation (Beckman Model L, 50 rotor) and concentrations determined. To these solutions a volume of 12 mM iodoacetamide (aqueous, freshly prepared) was added to yield a 2 mM solution. A volume of 4 M NaCl, adequate to maintain the ionic strength, was first added to the Tn-T solution to prevent precipitation. These were incubated in the dark for 20 minutes at room temperature and then solid DTT was added to quench the reaction. The samples were desalted in 5% formic acid and lyophilized. A similar procedure was used to modify Tn-I with iodoacetate. Concentrations of solutions of the modified proteins were determined using the extinction coefficients established for the native proteins. This was possible because the modification does not alter the aromatic chromophores present, nor introduce any new ones.

3. 2-chloromercurinitrophenol (MNP) Titration of Cysteine Residues

MNP, shown below, reacts quantitatively and rapidly with cysteine residues.



Tn-I and Tn-T, dissolved in 10^{-3} N HCl, were dialyzed overnight at room temperature versus 0.5 M NaCl, 50 mM TRIS-HCl, pH 8.0, 1 mM DTT with N_2 bubbling continuously through the solution. After clarification

by ultracentrifugation (Beckman Model L, 50 rotor), the proteins were desalted (BioGel P-2) into 0.5 M NaCl, 50 mM TRIS-HCl, pH 8.0. The spectrophotometric titrations were performed by adding small aliquots of 5 mM MNP (Eastman) in 100 mM NaOH to a sample cell containing the protein solution (Quiocho and Thomson, 1973). Identical aliquots of reagent were added to the reference cell which contained solvent but no protein. The reaction as manifested by a decrease in absorbance at 395 nm was measured in a 118C Cary spectrophotometer. The decrease in absorption at 395 nm was plotted as a function of the molar ratio of MNP:protein to obtain the titration curve.

CHAPTER III

STUDY ON HYBRID SPECIES FORMED FROM CARDIAC SKELETAL TROPONIN SUBUNITS

This study was initiated to investigate one aspect of the physiological differences in the action of skeletal and cardiac muscle. Although each type of muscle is regulated by similar mechanisms, involving molecular interactions at the level of the troponin subunits and tropomyosin, it is not clear to what extent the differences between the troponin subunits in skeletal and cardiac tissues contribute to the observed physiological differences between these tissues. The material in this chapter is based upon published data (Hincke et al., 1977) in which hybrid combinations of the components of the regulatory systems of bovine cardiac and rabbit skeletal muscles were examined by CD and bioassay techniques.

A. CIRCULAR DICHROISM

The maximum Calcium-induced change in ellipticity, ($\Delta[\theta]$), occurred at 220 nm and this wavelength was therefore monitored for comparison purposes.

Comparison of the observed $[\theta]_{220\ nm}$ values with the theoretical results in the absence of Ca^{2+} (Tables 1 and 2) reveals that interaction can be detected by this technique for most of the C-T, C-I and C-I-T complexes. Interaction for a specific complex is indicated when the calculated and observed $[\theta]_{220\ nm}$ differ by more than $\pm$ 500°, which is considered experimental error (Burtnick and Kay, 1976). The only exceptions occur for the C_cI_c, C_cT_c and C_sT_c complexes, where the difference values, given in the final column of both tables, are borderline in

terms of significance. However, both the C_cI_c and C_cT_c complexes have been demonstrated by migration patterns of samples on polyacrylamide gels in the absence of SDS (Burtnick et al., 1975b; Burtnick et al., 1976).

Representative spectra for the C_sI_c complex are presented in Figure 7 where 7a reveals the theoretical and observed patterns for the minus Ca^{2+} state, and 7b the corresponding spectra for the plus Ca^{2+} situation. The theoretical spectra have been calculated from those of the individual troponin subunits, assuming no interprotein interaction. Therefore, the difference between the theoretical spectra in the presence and absence of Ca^{2+} is due solely to the $\Delta[\theta]$ induced in sTn-C by Ca^{2+}. These values were then compared with the extent of the experimentally observed Ca^{2+} induced conformational change for the corresponding complexes.

Data collected in this fashion for the TnC-TnI and TnC-TnT complexes are presented in Table 1. The C_cI_c complex shows an almost identical change in ellipticity at 220 nm in the observed and theoretical spectra upon Ca^{2+} addition (-1300° versus -1350°). This suggests that the Ca^{2+}-induced conformational change occurs almost entirely at the level of Tn-C, even when it is complexed with Tn-I.

A different phenomenon is observed for the C_sI_s complex. Here the observed $\Delta[\theta]_{220\ nm}$ value is -1451°, as opposed to a calculated difference of -2728°. This implies that sTn-I has a damping effect upon the magnitude of the Ca^{2+}-induced change in the conformation of sTn-C. On the other hand, sTn-I has a potentiating effect upon the $\Delta[\theta]^{Ca^{2+}}_{220\ nm}$ of cTn-C ($\Delta[\theta]^{Ca^{2+}}_{220\ nm}$ values of -2058° versus -1434° for observed and calculated spectra, respectively), while cTn-I does not

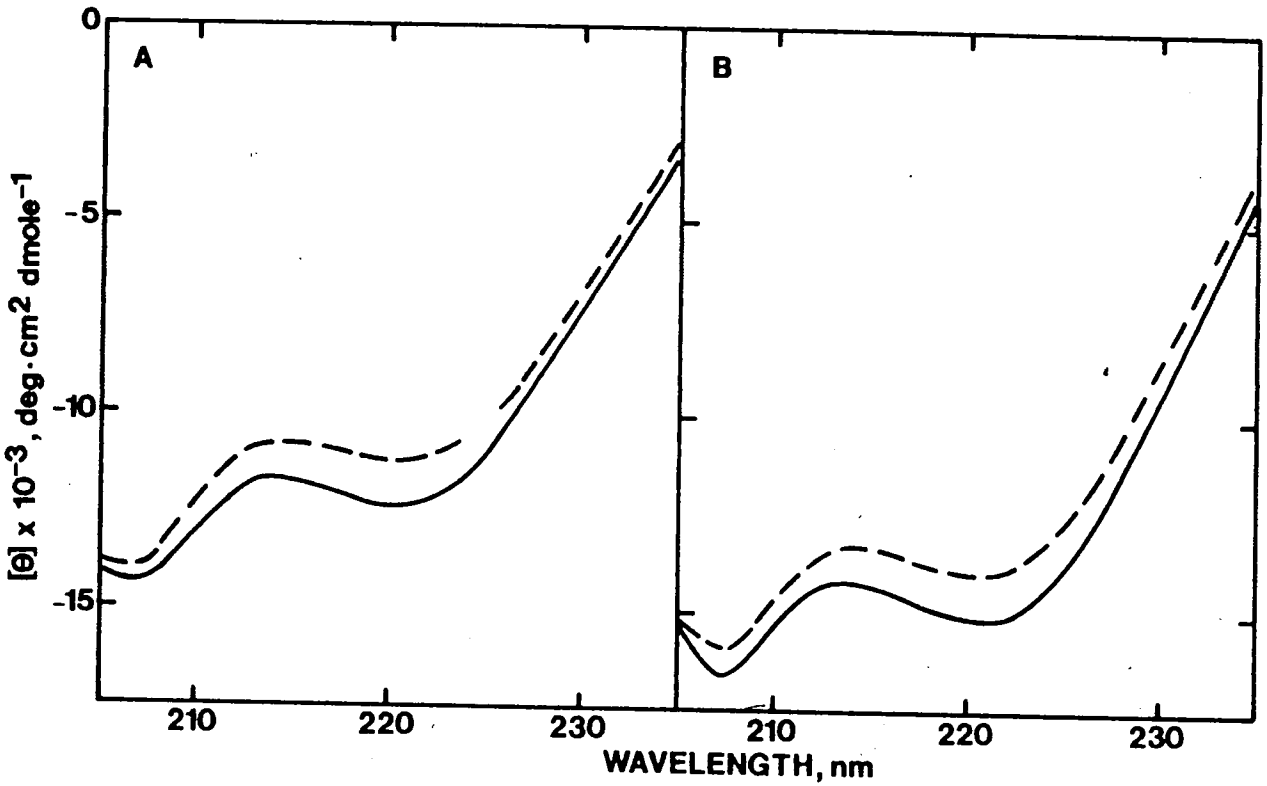


Figure 7. Theoretical (- - -) and observed (———) CD spectra for the hybrid complex skeletal Tn-C and cardiac Tn-I.
A. Minus Ca^{2+} state (1 mM EGTA).
B. Plus Ca^{2+} state (2 mM $CaCl_2$).

TABLE I

CD Parameters for Binary Hybrid Complexes

	Observed			Calculated (theoretical)			
	$[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$%	$[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$%	$[\theta]^{obs.}_{220\ nm}-[\theta]^{calc.}_{220\ nm}$
C_cI_c	-11450			-10950			-500
$+Ca^{2+}$	-12750	-1300	11.4	-12300	-1350	12.3	-450
C_cI_s	-14046			-12914			-1132
$+Ca^{2+}$	-16104	-2058	14.6	-14348	-1434	11.1	-1756
C_sI_c	-12327			-11174			-1153
$+Ca^{2+}$	-15050	-2723	22.1	-13936	-2762	24.7	-1114
C_sI_s	-18784			-15677			-3107
$+Ca^{2+}$	-20235	-1451	7.7	-18405	-2728	17.4	-1830
C_cT_c	-12150			-12900			+750
$+Ca^{2+}$	-12950	-800	6.6	-13500	-600	4.6	+550
C_cT_s	-13515			-11847			-1668
$+Ca^{2+}$	-15061	-1546	11.4	-12849	-1002	8.4	-2212
C_sT_c	-14128			-13503			-625
$+Ca^{2+}$	-15454	-1326	9.4	-15026	-1523	11.3	-428
C_sT_s	-12019			-12797			+778
$+Ca^{2+}$	-13535	-1516	12.6	-14483	-1686	13.2	+948

TABLE II

CD Parameters for Ternary Hybrid Complexes

	Observed			Calculated (theoretical)			
	$[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$%	$[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$	$\Delta[\theta]_{220\ nm}$%	$[\theta]^{obs.}_{220\ nm} - [\theta]^{calc.}_{220\ nm}$
$C_sI_sT_s$	-14556			-11442			-3114
$+Ca^{2+}$	-15924	-1368	9.4	-12783	-1341	11.7	-3141
$C_sI_sT_c$	-15940			-13415			-2525
$+Ca^{2+}$	-17413	-1473	9.2	-14512	-1097	8.2	-2901
$C_sI_cT_s$	-11200			- 9707			-1493
$+Ca^{2+}$	-12433	-1233	11.0	-11117	-1410	14.5	-1316
$C_cI_sT_s$	-11274			-10354			- 920
$+Ca^{2+}$	-12880	-1606	14.2	-11039	- 685	6.6	-1841
$C_sI_cT_c$	-12492			-11225			-1267
$+Ca^{2+}$	-13973	-1481	11.8	-12529	-1304	11.6	-1444
$C_cI_cT_s$	-10996			- 9924			-1072
$+Ca^{2+}$	-12003	-1007	9.2	-10987	-1063	10.7	-1016
$C_cI_sT_c$	-15968			-12656			-3312
$+Ca^{2+}$	-17820	-1852	11.6	-13349	- 693	5.5	-4471
$C_cI_cT_c$	-12200			-11500			- 700
$+Ca^{2+}$	-13430	-1230	10.1	-12200	- 700	6.1	-1230

appear to affect the magnitude of this change in sTn-C (ca. -2700° in both cases).

These effects are not carried over very dramatically to the TnC-TnT complexes, where only a small potentiating effect is observed for those com[redacted]lving cardiac Tn-C. The C_cT_c complex has a larger Ca^{2+} chang[redacted]mpared to the theoretical spectrum, and the same trend [redacted]rved for the C_cT_s complex (-1546° versus -1002°). In contrast, with the complexes involving sTn-C the observed change is slightly less than the theoretical one.

Examination of the data for the ternary complexes (Table 2) reveals several important features at the level of the Tn-C component. Those complexes containing sTn-C do not show any potentiation, when the $\Delta[\theta]_{220\ nm}$ for the observed and theoretical spectra are compared. Although large changes in $[\theta]_{220\ nm}$ are noted upon Ca^{2+} addition (about -1500°), they are predictable and suggest that the reconstituted skeletal troponin is interacting with calcium in a similar manner to the uncomplexed sTn-C. In contrast, those complexes containing cTn-C all show large potentiation effects, a phenomenon previously observed for the exclusively cardiac ternary complex (Burtnick and Kay, 1976). Typically the observed $\Delta[\theta]_{220\ nm}$ is about double that predicted by the theoretical calculations. For example, with the $C_cI_sT_c$ complex the observed $\Delta[\theta]_{220\ nm}$ is -1852° as opposed to a calculated value of -693°.

B. BIOASSAY

As shown in figures 8 and 9 both skeletal and cardiac Tn-I have inhibitory activity in the skeletal and cardiac SAM systems. An interesting feature is that while each Tn-I component is operative in both

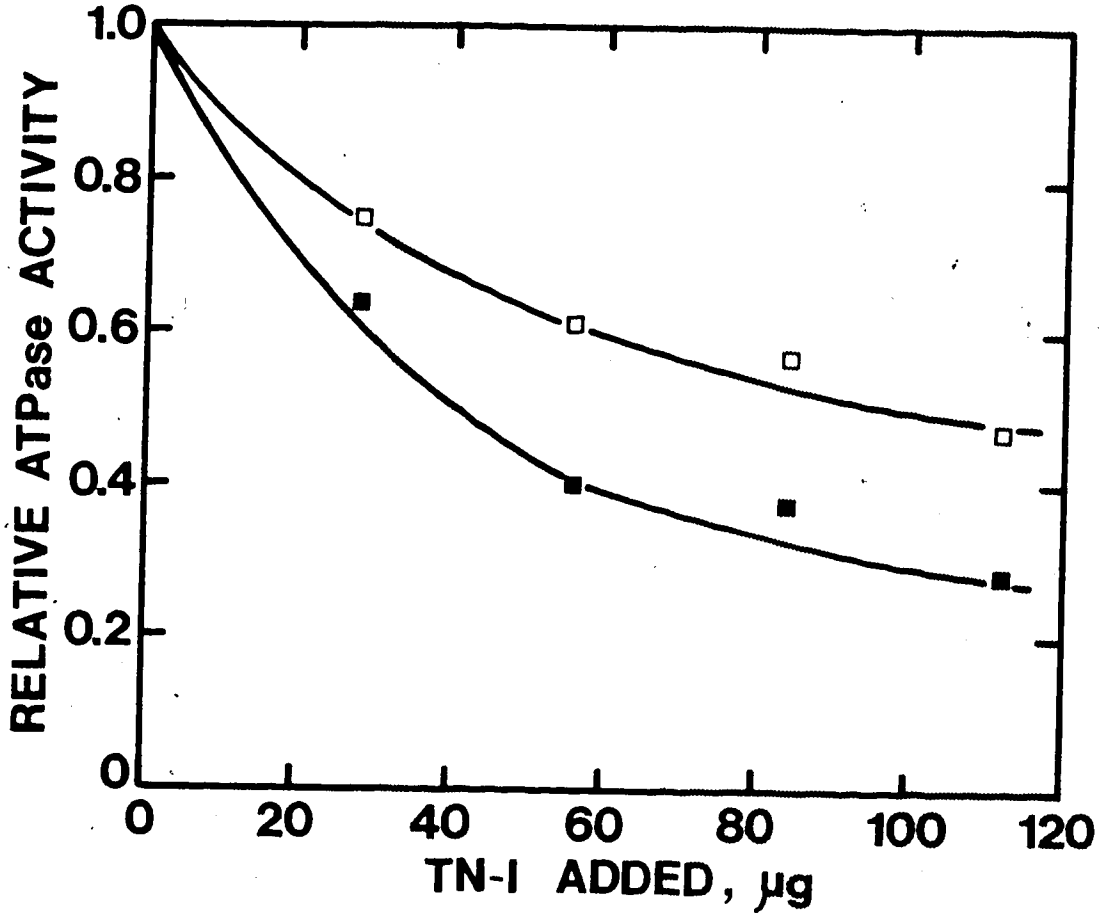


Figure 8. Relative ATPase activity of cardiac SAM as a function of amount of Tn-I added to the assay system for cardiac Tn-I (■-■) and skeletal Tn-I (□-□).

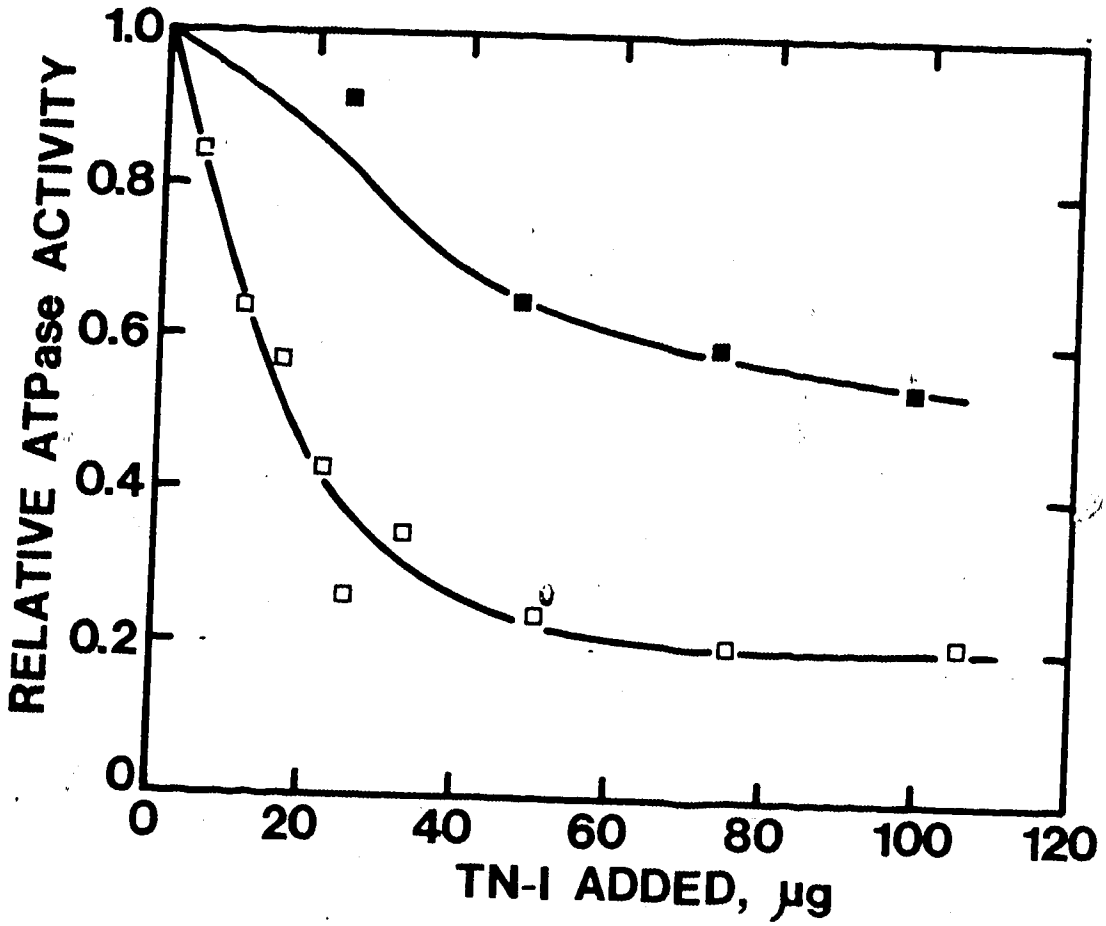


Figure 9. Relative ATPase activity of skeletal SAM as a function of amount of Tn-I added to the assay system for cardiac Tn-I (■-■) and skeletal Tn-I (□-□).

SAM systems, a given Tn-I is most active with its parent actomyosin. This phenomenon was also observed by other workers, comparing rabbit cardiac and skeletal muscle Tn-I's in a desensitized skeletal actomyosin system (Syska et al., 1974). The present results confirm that observation but extend it to comparative studies with both the skeletal and cardiac actomyosin systems.

It is also noteworthy that cardiac Tn-I is a less effective inhibitor against cardiac SAM than is skeletal Tn-I versus its parent actomyosin system. In this connection, to produce 50% inhibition in the cardiac SAM system requires 50 µg cTn-I whereas only 20 µg skeletal Tn-I will produce the same degree of inhibition in the skeletal actomyosin system. This finding of a smaller degree of inhibition of cardiac actomyosin ATPase activity by cardiac Tn-I may have physiological relevance, since cardiac muscle must function continuously, without an opportunity for rest and recovery.

Addition of the Tn-C components reversed the inhibitory effects of the Tn-I subunits (Table 3), while incorporation of Tn-T into the assay systems with Tn-I and Tn-C conferred Ca^{2+} sensitivity on the regulatory process (Table 4). In the presence of EGTA, the ATPase activities were inhibited, while in the presence of Ca^{2+}, reversal of these inhibitions was noted.

C. SUMMARY

The biological activity of the hybrid complexes studied is in good agreement with the CD data, which indicates that interaction can occur among the troponin subunits of the two tissues. This implies that the regulatory proteins in each system are structurally similar

TABLE III

Restoration of ATPase Activity by TN-C

to Skeletal SAM System Partially Inhibited by TN-I

	Skeletal TN-I (6:1)	Cardiac TN-I (2:1)	Skeletal TN-C	Cardiac TN-C	Relative Activity
1.	-	-	-	-	1.0
2.	25 µg	-	0	-	0.27
	25 µg	-	15 µg	-	0.7
	25 µg	-	45 µg	-	0.81
	25 µg	-	5 µg	-	0.93
3.	25 µg	-	-	0	0.27
	25 µg	-	-	15 µg	0.73
	25 µg	-	-	45 µg	0.76
	25 µg	-	-	75 µg	0.86
4.	-	71 µg	0	-	0.60
	-	71 µg	45 µg	-	0.81
	-	71 µg	75 µg	-	0.90
	-	71 µg	112 µg	-	0.98
5.	-	71 µg	-	0	0.60
	-	71 µg	-	45 µg	0.78
	-	71 µg	-	75 µg	0.79
	-	71 µg	-	112 µg	0.81

Note: The control contained 750 µg synthetic actomyosin and 50 µg tropomyosin in 10 mM TRIS-CHL, pH 7.6, 2.5 mM $MgCl_2$, 1 mM EGTA and 2.5 mM ATP at 20°C. The ratio of actin to Tn-I is given in brackets.

TABLE IV

Ca^{2+} Activation of ATPase Activity of Skeletal and Cardiac SAM with Reconsituted Hybrid Tropo in

A- Skeletal SAM, B- Cardiac SAM

	Skeletal Troponins			Cardiac Troponins			2 mM $CaCl_2$	Relative Activity
	TN-C	TN-I	TN-T	TN-C	TN-I	TN-T		
	(6:1)			(2:1)				
A.	30 µg	25 µg	40 µg					0.69
	30 µg	25 µg	40 µg				✓	0.93
				75 µg	71 µg	120 µg		0.74
				75 µg	71 µg	120 µg	✓	1.00
	30 µg	25 µg	-			40 µg		0.70
	30 µg	25 µg	-			40 µg	✓	0.98
	(3:1)							
B.				68 µg	56 µg	84 µg		0.47
				68 µg	56 µg	84 µg	✓	0.69
	62 µg	56 µg	75 µg					0.53
	62 µg	56 µg	75 µg				✓	0.77
			75 µg	68 µg	56 µg	-		0.60
			75 µg	68 µg	56 µg	-	✓	0.76
	62 µg	56 µg	-			84 µg		0.32
	62 µg	56 µg	-			84 µg	✓	0.41

Conditions are given in the legend to Table III.

enough to form functional hybrid complexes. However, there are known differences between the same proteins within the two tissues.

The potentiation of the Ca^{2+}-induced conformational change in those ternary complexes containing cTn-C and its absence in those complexes constructed with sTn-C could be a reflection of the different number of calcium-binding sites the Tn-C molecules contain, as well as the smaller CD conformational change which cTn-C undergoes when Ca^{2+} is bound.

There may also be different types of interactions between these Tn-C molecules and the other subunits when comparing the two tissue sources. A recent study utilizing tropomyosin paracrystals indicated the cardiac Tn-C - Tn-T interaction was stronger in the absence of calcium than that of the skeletal counterparts (Yamaguchi and Greaser, 1979). They also suggested a different type of interaction occurred between Tn-T and tropomyosin in these two systems, as evidenced by the type of paracrystals which could be obtained.

There are important structural differences between skeletal and cardiac Tn-I which could be responsible for the different inhibitory activity these proteins exhibit. One significant difference in primary sequence occurs in a region corresponding to a peptide fragment of skeletal Tn-I which is 40-70% inhibitory on a molar basis as the parent molecule (Syska et al., 1976). A leucine-for-arginine substitution at residue 141 in the cardiac sequence has been shown by work with synthetic peptide analogs of this active fragment to result in a large decrease in inhibitory activity, similar to that seen for whole cTn-I compared to sTn-I (J.A. Talbot and R.S. Hodges, priviledged communication).

The specific sequence difference of this peptide cannot completely account for the different inhibitory activity of cTn-I and sTn-I.

Rabbit slow muscle Tn-I, possessing an intermediate inhibitory activity between that of cardiac and fast muscle Tn-I in the skeletal actomyosin system (Syska et al., 1976), has an identical sequence at this site to the fast muscle Tn-I (Wilkinson and Grand, 1978). As well, the observation that cardiac and skeletal Tn-I is each most active in its parent actomyosin system indicates other factors are involved.

Both the potentiation of the Ca^{2+}-induced conformational change in those ternary complexes containing cTn-C, and the smaller degree of inhibition elicited by cTn-I in its parent actomyosin system are expressions of unique structural differences between the two muscle regulatory systems.

CHAPTER IV

THE DEMONSTRATION OF AN INTERACTION BETWEEN CARDIAC Tn-I AND Tn-T

As discussed earlier, the interaction of Tn-C with Tn-T and Tn-I has been well documented. We have also shown this to be true for hybrid complexes prepared between proteins from the two tissues. Previously a specific interaction between Tn-T and Tn-I had not been considered likely, based mainly on the failure of these proteins to comigrate in standard disc gel electrophoresis (van Eerd and Kawasaki, 1973). However, this technique is not completely satisfactory for detecting complex formation when one or both of these proteins are highly insoluble at the low ionic strengths employed, as is the case for Tn-I and Tn-T. Indeed, it is difficult to satisfactorily demonstrate an interaction between cTn-C and cTn-T with this method (Burtnick et al., 1976).

There is some indirect evidence in the literature, however, which suggests such a complex does occur in the rabbit skeletal system. The addition of Tn-I to a tropomyosin-Tn-T complex markedly increases the solubility of the mixture. As well, a unique form of paracrystal results from magnesium precipitation of this mixture (Margossian and Cohen, 1973). Tn-I and Tn-T copurify under certain conditions (Mani et al., 1973; Hartshorne and Mueller, 1969) and furthermore, cosediment in the analytical ultracentrifuge (Hartshorne and Mueller, 1969). Copurification of the cardiac proteins has also been observed in our laboratory.

Hitchcock (1975) has demonstrated, in a crosslinking study of skeletal troponin, that crosslinked Tn-T - Tn-I is a major product of the reaction utilizing dimethyl imidoesters. She suggested that Tn-T and Tn-I must lie within 0.6 nm or less of each other.

As an impetus to this study, evidence to suggest the formation of a 1:1 skeletal Tn-T - Tn-I complex was recently presented (Horwitz et al, 1978; Horwitz et al., 1979). Near UV CD spectra and gel filtration chromatography on Sephacryl S-200 were used to demonstrate this interaction which they argued was also present in the native troponin complex. Because of the presumed as well as demonstrated parallel nature of the regulation of muscle contraction in the rabbit skeletal and beef cardiac systems, an investigation of a possible Tn-T - Tn-I interaction in the cardiac system was undertaken (Hincke et al., 1979).

A. CIRCULAR DICHROISM

Initial CD experiments in which 0.5 mM or 1 mM DTT were included in the solvent could demonstrate no interaction between Tn-T and Tn-I in the near or far UV regions. However, when N_2 was bubbled continuously through these solvents during dialysis to exclude oxygen, complex formation could be demonstrated, a phenomenon in agreement with other results for the rabbit skeletal proteins (Horwitz et al., 1979). They found that complete reduction of the 3 Tn-I sulfhydryls was necessary for Tn-I - Tn-T interaction and also for reconstitution of a troponin complex which was active in the synthetic actomyosin bioassay (skeletal Tn-T has no cysteine residues (Pearlstone et al., 1977)).

Cardiac Tn-I possesses two cysteine residues (Burtnick et al., 1975b; Grand et al., 1976) and Tn-T one (Burtnick et al., 1976). This was verified by spectrophotometric titration of the proteins with 2-chloromercurinitrophenol (Figure 10), and amino acid analysis of the carboxamidomethylated proteins (see Table 5). Presumably, if the sulfhydryls were not maintained in a reduced state or protected, intermole-

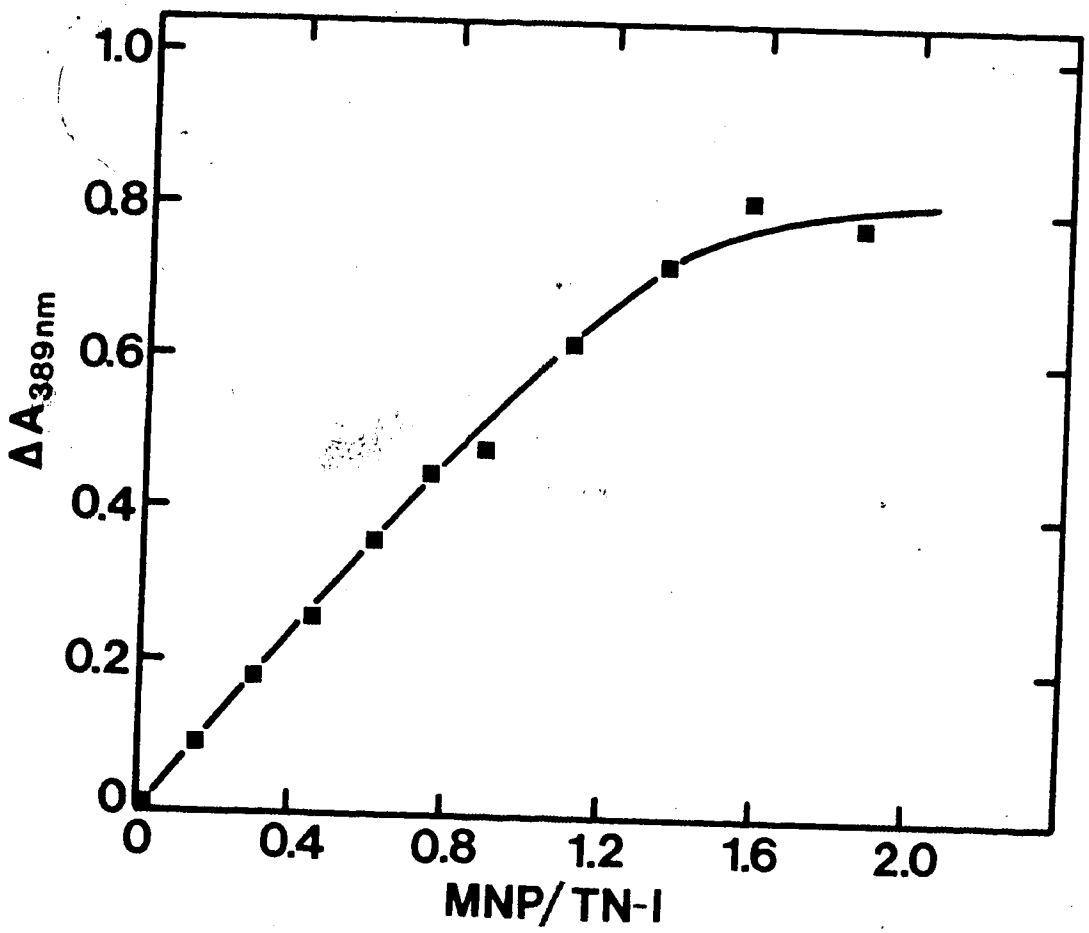


Figure 10. MNP titration of the cysteine residues in cTn-I. The conditions are described in the experimental section.

TABLE V

THE AVERAGE ELLIPTICITY VALUES AT SELECTED WAVELENGTHS NEAR THE 220 nm MINIMUM FOR THE MODIFIED AND NATIVE PROTEINS

Protein (No. of spectra averaged)	[θ] $deg.cm^2.dmole^{-1}$			
	225 nm	222.5 nm	220 nm	217.5 nm
Tn-T (3)	-12,080	-12,910	-12,910	-12,540
Tn-T* (4)	-11,880	-12,670	-12,660	-12,310
Tn-I (4)	- 5,260	- 5,770	- 5,860	- 5,870
Tn-I* (6)	- 6,260	- 6,770	- 6,840	- 6,690

* indicates carboxamidomethylated protein; by amino acid analysis, the Tn-I had 2.03 moles of CMC incorporated per molecule, and the Tn-T 0.99 moles CMC per molecule (relative to alanine).

cular disulfide formation in Tn-T and inter- and intra- molecular disulfide formation in Tn-I could alter some necessary conformation associated with the 'native' state.

Figure 11 shows a far UV CD spectrum in which Tn-T and Tn-I have been mixed in a 1:1 molar ratio, and compares this spectrum to a theoretical one generated from those of the individual proteins. Differences between the two spectra greater than 500° are taken as evidence for interaction, in which formation of a complex has caused secondary structure alterations in either or both of the proteins (Bürtnick and Kay, 1976; Hincke et al., 1977). In this case, the difference 1000° at 220 nm, providing definite evidence for a Tn-T - Tn-I interaction.

As the status of the sulfhydryl residues in these proteins seemed important in the complexation event, the properties and interaction of the carboxamidomethylated proteins were studied. Typically 90% of the SH residue(s) in each protein could be modified without resorting to denaturing conditions. The far UV CD spectrum of the modified Tn-T seemed unaltered compared to that of the native Tn-T (see Table 5), indicating that carboxamidomethylation of the sole cysteine residue has no gross effects upon the secondary structure of this molecule.

On the other hand, modification of the two cysteine residues of Tn-I caused an increase in negative ellipticity of almost 1000° (Table 5). This gain in secondary structure with modification is unusual since chemical modification of most proteins usually leads to a loss of structure, if there are structural alterations. These results must indicate that one or both cysteine residues are located in a key structural region of the molecule, which can be readily altered by even the mild modifications which have been employed here, with a fairly dramatic conformational

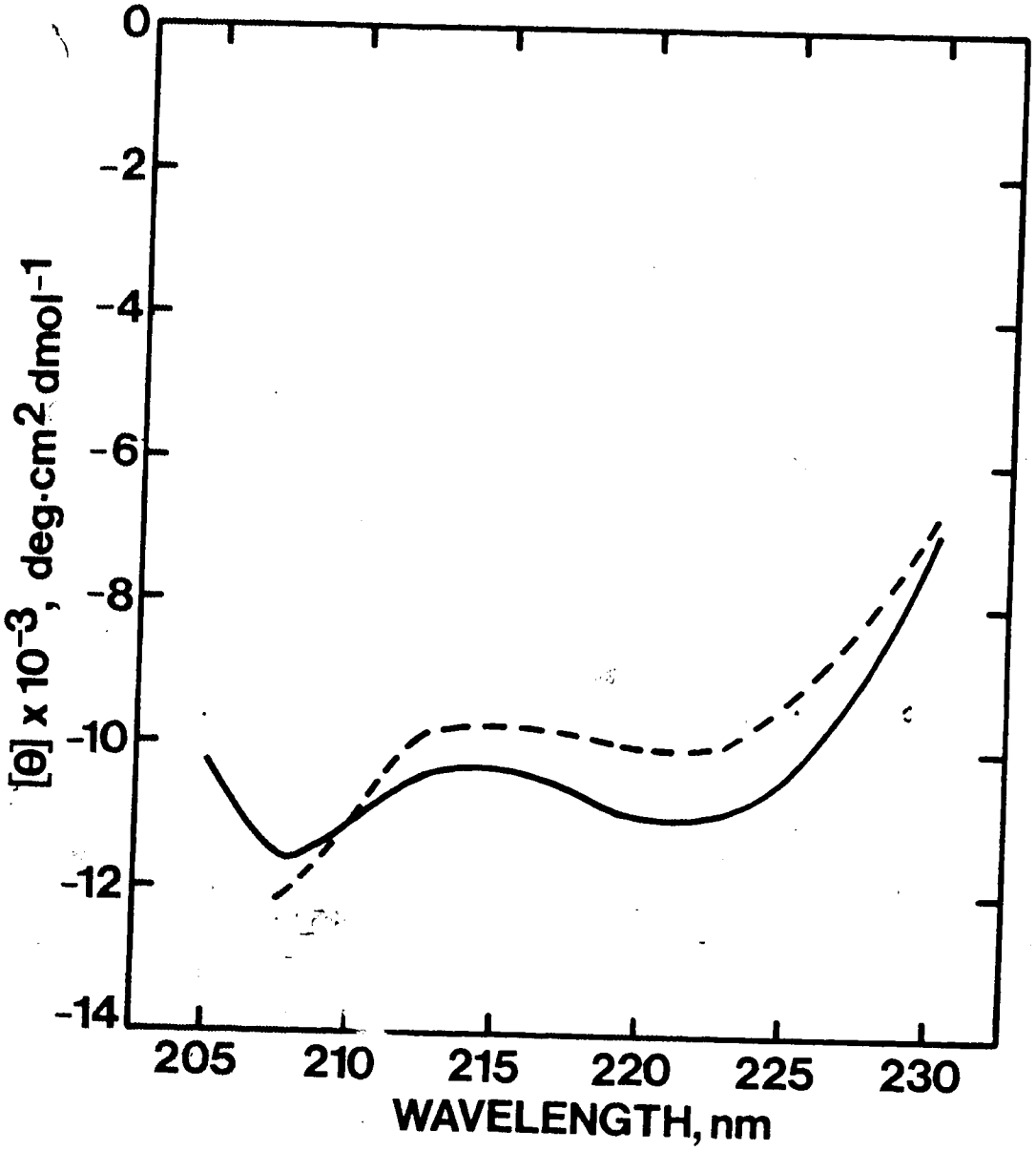


Figure 11. Far UV CD spectrum of a 1:1 mixture of Tn-T and Tn-I (———) and a theoretical spectrum generated from those of the individual proteins (- - -). The solvent was 0.5 M NaCl, 50 mM $NaPO_4$, pH 7.0, 2 mM EDTA, 0.5 mM DTT (N_2).

change. In this context, it is of interest to note the results of studies with calmodulin (Walsh et al., 1978). They found that the mild oxidation of 3 or 4 surface methionine residues to methionine sulfoxide resulted in a drastic loss of biological activity and a major alteration in secondary structure. These methionine residues appear to play an important role in determining the secondary structure of this protein.

It was of interest to attempt to identify the mechanism for such a large change in secondary structure in Tn-I, upon the modification of its cysteine residues to S-carboxamidomethyl cysteines. α-helix, β-sheet and β-turn conformational potentials have been determined for all 20 amino acids (Chou and Fasman, 1974; 1977). This analysis has been used to predict secondary structural domains in proteins whose primary sequence is known. Rabbit cardiac Tn-I has been sequenced (Grand et al., 1976) and this sequence is expected to be largely homologous to that of the beef cardiac protein, since the sequences of Tn-I's from different sources appear to be tissue-specific and not as homologous when compared within a species for different tissues (Grand and Wilkinson, 1977). The cysteine residues are found at positions 75 and 92.

In using the conformational parameters, it was assumed that S-carboxamidomethyl cysteine could be approximated by glutamine or methionine both of which have similar α-helix or β-sheet parameters. Cysteine is an indifferent α-former, while both glutamine and methionine are strong α-formers. Thus the modification could be enough to convert a region of indifferent α-helix-forming amino acids into a section of α-helix. Indeed, this type of sequence is found about cysteine residue 75. A second possibility involves the proline residue at position 77. In this case we investigated the β-turn probability for the sequence in

this region to see if conversion of cysteine (a residue with high β-turn probability) to a methionine or glutamine analog could affect this type of secondary structure. In no case was the probability high enough to suggest a β-turn in this region, in spite of the proximity of the proline residue. Thus one might expect the α-helix parameters to dominate and further, that the conversion of an indifferent helix former to a strong one might alter the conformation about cysteine-75 into an α-helix, thereby accounting for some, at least, of the increased secondary structure in the modified protein.

The interaction of the modified proteins with each other and with their native counterparts was also studied by CD. The general finding was that while an interaction could be demonstrated for the Tn-T* - Tn-I pair by the far UV CD criterion, this was not possible for the Tn-T - Tn-I* or Tn-T* - Tn-I* pairs. This suggests that the large change in secondary structure which occurs in the modified Tn-I is taking place in a region which is important for the interaction with Tn-T.

In the study on the skeletal Tn-T - Tn-I interaction, the near UV CD region was examined almost exclusively (Horwitz et al., 1979). The CD signals of these proteins in this region are very small, but this problem was overcome by using electronic signal averaging techniques. Alterations produced in the microenvironments of various aromatic residues by the complex were utilized to good effect, titrating Tn-I with Tn-T to show a 1:1 complex of these proteins was being formed.

Although hampered by our lack of this technology, it was found that under the rigorous reducing conditions employed, the aromatic spectrum for Tn-T was significantly sharpened with a minimum centered at 275 nm, compared to that reported for the absence of DTT (Burtnick et al,

1976). An identical spectrum was recorded for the modified Tn-T. The Tn-I and Tn-I* spectra were essentially identical and featureless, as reported in the literature (Burtnick et al 1975b).

The 1:1 mixtures of Tn-T and Tn-I were made and their near UV CD spectra recorded. A typical Tn-T + Tn-I spectrum is presented in Figure 12. The theoretical calculated spectrum is also shown. A difference spectrum generated by subtracting the theoretical spectrum from that observed is presented as an insert. The resulting minimum trough, centered at 280 nm, seems to be clear evidence for an environmental alteration of some tyrosine residue(s) in the complex, relative to the uncomplexed proteins.

A similar difference spectrum could be generated from the Tn-T* + Tn-I mixture, again indicating interaction. However, both mixtures of Tn-T + Tn-I* and Tn-T* + Tn-I* produced difference spectra which were essentially random throughout the near UV region, correlating with the far UV results, where no detectable protein interaction was observed.

B. UV DIFFERENCE SPECTROSCOPY

Ultraviolet difference spectroscopy was performed to detect any perturbation of aromatic residues in Tn-T or Tn-I which could occur when a complex was formed. The results obtained are shown in Figure 13. This difference spectrum demonstrates perturbation of tryptophan (293 nm), tyrosine (283 and 276 nm) and phenylalanine (268 nm) residues (Donovan, 1969). It is not possible to determine whether these residues are in Tn-I, Tn-T or perhaps both proteins. The positive peaks of this difference spectrum at these wavelengths, indicative of red shifts in their absorption spectra, suggest that complex formation causes these aromatic

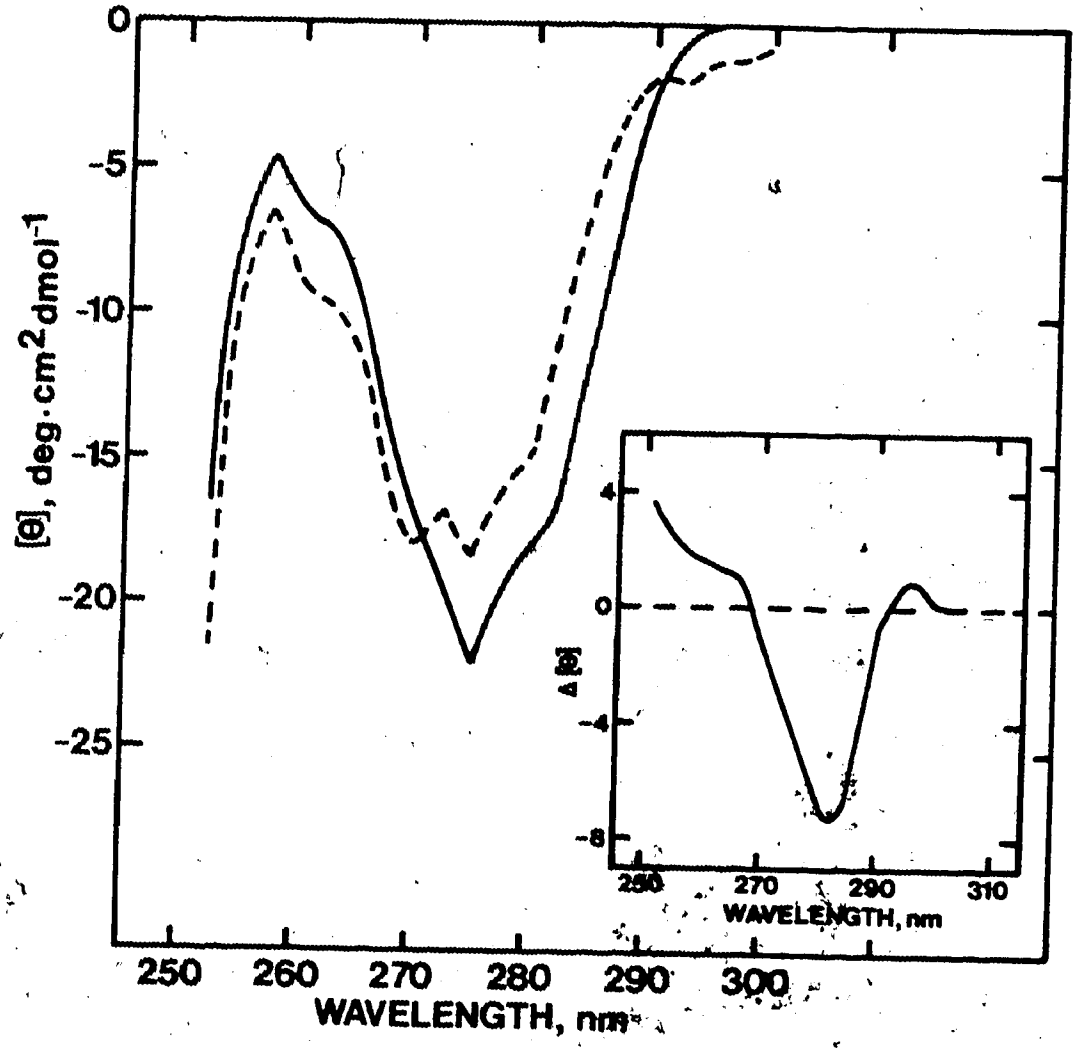


Figure 12. Near UV CD spectrum of a 1:1 mixture of Tn-T and Tn-I (———) and a theoretical spectrum generated from those of the individual proteins (- - -). The insert shows a difference spectrum generated by subtracting the theoretical spectrum from that observed. The solvent was 0.5 M NaCl, 50 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT (N_2).

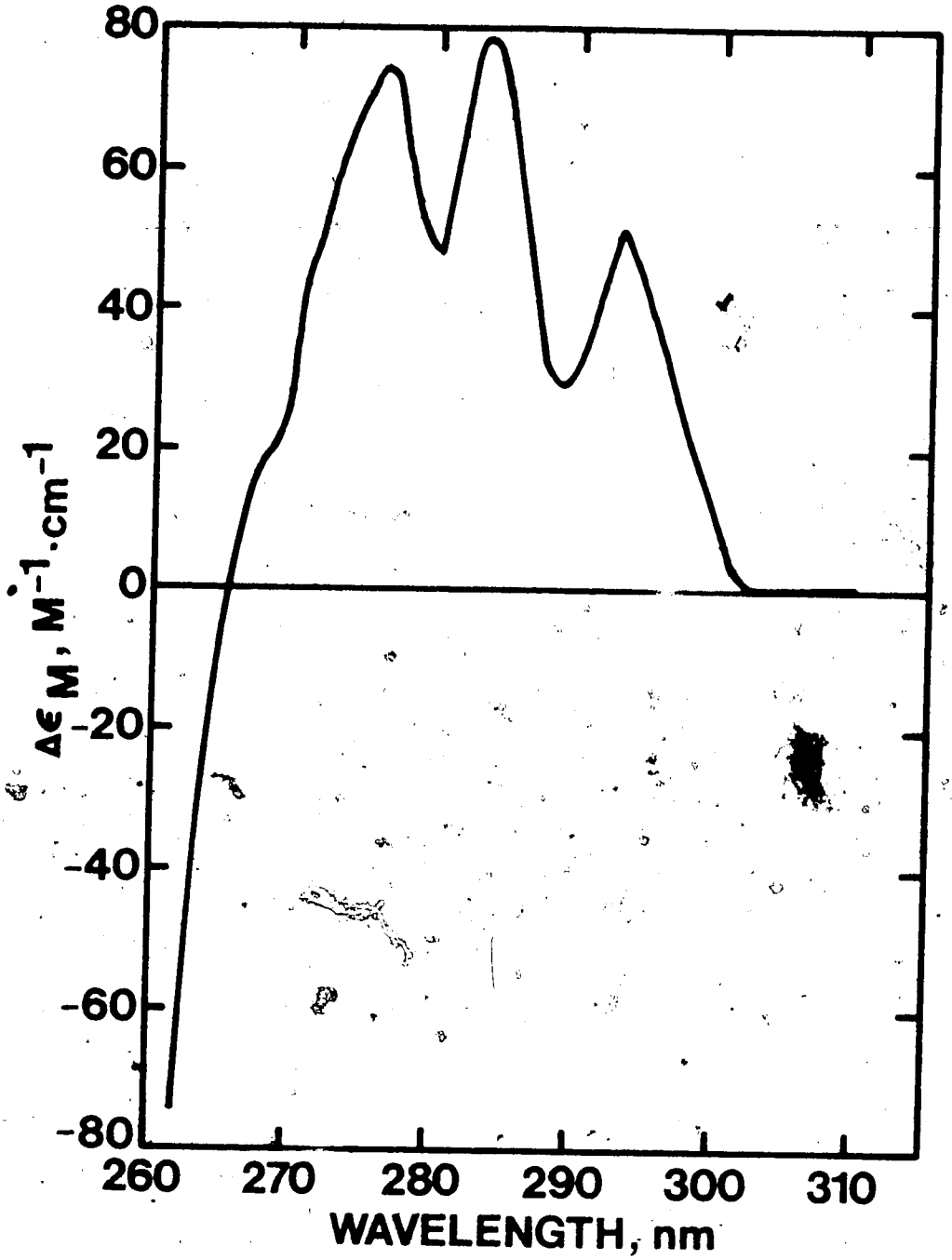


Figure 13. UV difference-absorption spectrum of a 1:1 mixture of Tn-T and Tn-I. The solvent was 0.5 M NaCl, 50 mM NH_4HCO_3, pH 8.2, 2 mM EDTA, 2 mM DTT.

amino acids to experience more hydrophobic environments.

C. GEL FILTRATION CHROMATOGRAPHY

In order to confirm and extend the CD findings, gel filtration was performed utilizing Sephacryl S-200 resin as with this material high flow rates are possible, without sacrificing resolution. Because of the protein dilution which occurred during passage through the column, a dye-binding assay system was used which was simple, reproducible and amplified protein absorption 20-30 fold (Bradford, 1976).

Samples were prepared and complexes mixed under the same conditions as for the CD work. When the modified and native Tn-T and Tn-I were mixed at approximately 1:1 molar ratios in the four combinations possible and separately applied to the gel filtration column, the elution profiles presented in Figure 14 were obtained. Note that a very similar pattern is observed for the Tn-T - Tn-I and Tn-T* - Tn-I complexes - a main peak with an elution volume intermediate to those of the Tn-T and Tn-I alone. The slight shoulder present on the higher molecular weight side of the peak in each profile is due to uncomplexed Tn-T and Tn-T* since in each case a slight excess (~10%) was present over Tn-I.

However, very different results were observed for the Tn-T + Tn-I* and Tn-T* + Tn-I* pairs. In each case, two peaks were observed which correspond to free Tn-T and Tn-I, indicating an absence of interaction under these conditions. This substantiated the CD data, suggesting that no complex is formed with modified Tn-I, while the modified Tn-T is able to interact as well as the native protein.

In order to assess the biological activity of these modified proteins, the binary complexes with Tn-C were made and their elution pro-

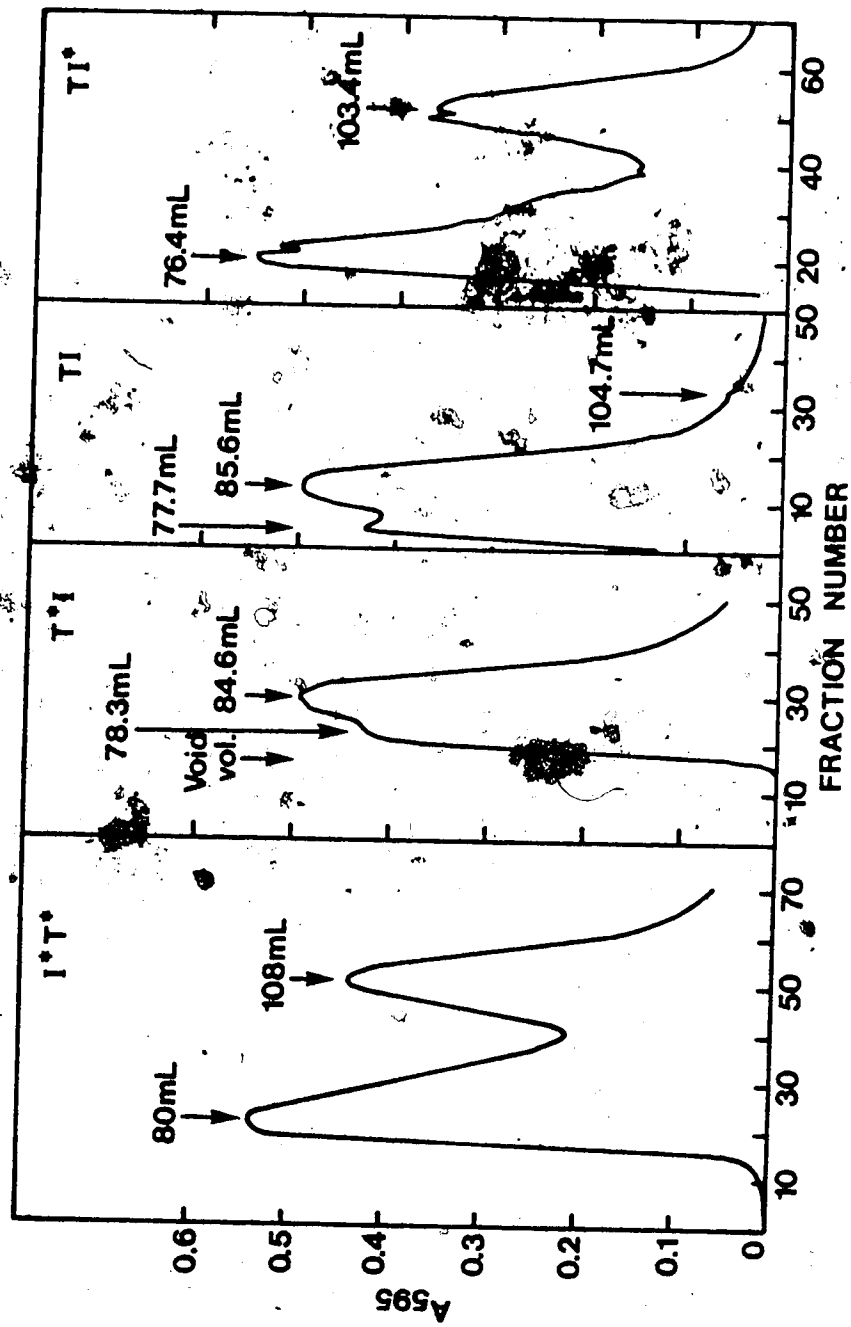


Figure 14. Gel filtration chromatography of 1:1 mixtures of Tn-T and Tn-I (native and modified (*)). The column profiles were determined using a dye-binding assay. In each case sample volumes were 450 λ. The solvent was 0.5 M NaCl, 50 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT (N_2).

files determined. The Tn-C - Tn-I and Tn-C - Tn-I* complexes displayed identical patterns: two peaks poorly resolved from each other. The elution volume of the first peak was about 5 ml less that that for Tn-I alone (higher molecular weight), while the second corresponded to that of Tn-C. This was assumed to be evidence for interaction (weaker than the Tn-T - Tn-I complex), since Tn-C - Tn-I is known to form a complex (Burtnick et al., 1975b; Mani et al., 1974a) under these conditions which can readily be demonstrated by far UV CD techniques. Under the high ionic strength conditions employed, the complex may be weakened, relative to that present under physiological conditions. Since Ca^{2+}-binding by Tn-C enhances its interactions with Tn-I and Tn-T, the Tn-C - Tn-I* mixture was chromatographed in the presence of 1 mM $CaCl_2$. Here, one symmetrical peak was obtained with an elution volume equivalent to the first peak obtained in the absence of $CaCl_2$. No free Tn-C peak observed. This suggests that the interaction of the modified Tn-I with Tn-C is similar to that of the native protein. Therefore, the secondary structure alterations induced by the modification must be specific in nature, affecting the region of the molecule which interacts with Tn-T, but not that which complexes with Tn-C. This is not an unlikely finding. In fast-skeletal muscle Tn-I, the inhibitory activity can be localized in the CNBr peptide containing residues 96 to 117, while Tn-C binds to two regions: residues 1 to 47 and 96 to 117 (Syska et al., 1976). Our results suggest that the region of Tn-I which interacts with Tn-T is found in some other discrete location of the protein and it is altered preferentially when one or both of the cysteine residues is modified.

Studies were also performed at a lower ionic strength than the above (0.3 M NaCl versus 0.5 M NaCl) to see if the lack of interaction

for complexes containing modified Tn-I could be ascribed, at least partially, to the high ionic strength used, since it is possible that the interaction could be ionic in nature. It is noteworthy that when Tn-T - Tn-I, Tn-T* - Tn-I* and Tn-T - Tn-I* mixtures in 0.5 M NaCl, 50 mM MOPS, pH 7.2, 1 mM EDTA and 1 mM DTT were dialyzed versus 0.3 M NaCl, 20 mM Na phosphate, pH 7.0, 1 mM EDTA and 1 mM DTT, no precipitate was observed due to the decrease in ionic strength. However, similar dialysis regimes with only Tn-T or Tn-I resulted in a slight precipitate following overnight dialysis. In the skeletal system, the increased solubility of Tn-T at low ionic strengths following interaction with Tn-I has been demonstrated (Horwitz et al., 1980). This observation suggested that complexes containing modified Tn-I possessed a potential for interaction which was not being expressed at the higher ionic strength.

The gel filtration results at 0.3 M NaCl ionic strength are presented in Figure 15. Again, the profiles from the Tn-T - Tn-I* and Tn-T* - Tn-I* mixtures are different from that of the Tn-T - Tn-I complex. It is important to note, however, that in neither profile have the components been resolved into clearly separated peaks as observed at the higher ionic strength. Indeed, the initial peak elution volume is about 20 ml larger than that for Tn-T while it is about 10 ml less than for Tn-I. It is suggested that this smearing effect, unique to these mixtures, results from a weakly associating complex which undergoes extensive association-dissociation during its passage through the column, resulting in a broad, diffuse profile possessing a unique elution volume (compared to that of the Tn-T - Tn-I complex).

This situation approximates that of frontal anaysis or steady-state gel filtration, a technique used to analyze the gel filtration

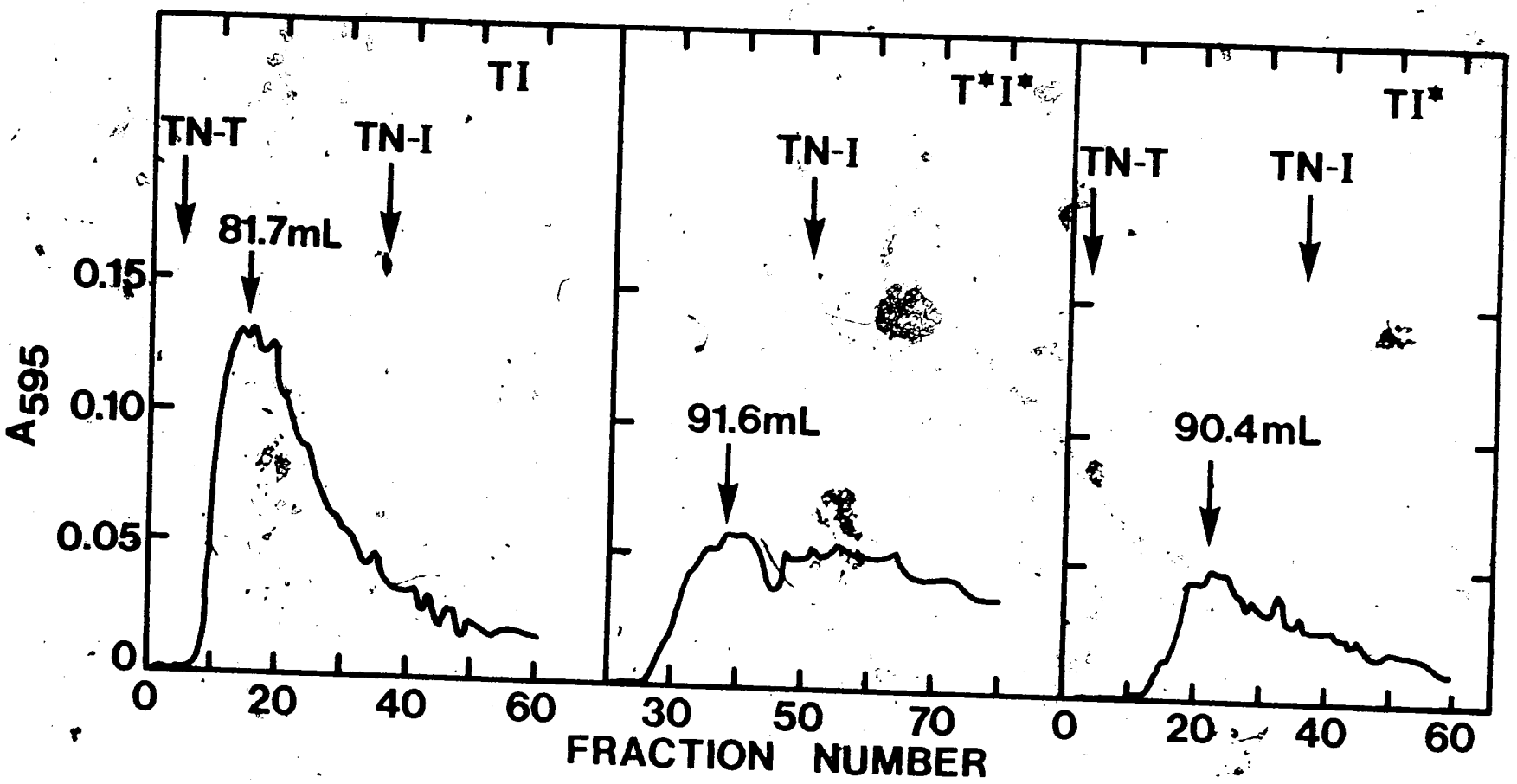


Figure 15. Gel filtration chromatography of 1:1 mixtures of Tn-T and Tn-I (native and modified (*)). The column profiles were determined using a dye-binding assay. The indicated elution volumes for Tn-T and Tn-I were determined for the separate proteins in the same solvent. The solvent was 0.3 M NaCl, 20 mM $NaPO_4$, pH 7.0, 1 mM DTT (N_2).

behavior of soluble adenyla· cyclase from bovine corpus luteum (Young and Stansfield, 1978) in which an equilibrium of the type $AC \rightleftharpoons A + C$ existed. Here AC corresponds to a high activity complex, A is an activatory subunit and C is the free catalytic subunit of low activity. The activity profile that was obtained from application of a large volume of dilute sample to Sepharose 6B showed a large, diffuse, uneven plateau, as predicted by the analysis.

In the system described here, aggregation of the Tn-T and Tn-I and perhaps even of a Tn-T - Tn-I complex will complicate the analysis significantly. However, it is suggested that the profiles obtained for the Tn-T - Tn-I* and Tn-T* - Tn-I* mixtures under these conditions reveal a weakly interacting system in each case. The Tn-T - Tn-I complex is of course much stronger, yielding a single peak with a predictable elution volume.

It is interesting to note the abnormal elution volumes of the proteins studied here, which were also observed for their skeletal counterparts (Horwitz et al., 1979). A linear plot of elution volume versus log molecular weight has been constructed using BSA, ovalbumin and chymotripsinogen, as globular protein markers (data not shown). The corresponding globular protein molecular weight for Tn-T was close to 225,000, for Tn-I 46,000 and that of the Tn-T - Tn-I complex 137,000. These data indicate either extensive aggregation occurs and/or marked deviations from the globular ideal are present, particularly for Tn-T and the Tn-T - Tn-I complex. Recent hydrodynamic evidence indicates that cardiac Tn-T possesses an extended structure in solution (Byers and Kay, 1980).

The aggregation of both cardiac and skeletal Tn-T under these solvent conditions is a well-reported phenomenon (Burtnick et al., 1976;

Mani et al., 1974b). It appears that the interaction of Tn-T with Tn-I prevents or decreases the extent of this aggregation, although the large apparent molecular weight for Tn-T - Tn-I may represent an aggregated species, as well. This is slightly different from the case in the skeletal system, where the elution volume for Tn-T is identical to that of Tn-T - Tn-I complex (Horwitz et al., 1979). Apparently there are qualitative differences between the two systems.

D. BIOLOGICAL ACTIVITY OF MODIFIED Tn-I

The biological activity of modified Tn-I was also investigated. This primarily involved the interaction of Tn-I with actin. Since the modification disrupts the ability of Tn-I to interact with Tn-T but not with Tn-C, it was of interest to see if this affected the ability of the protein to interact with actin. Figure 16 clearly demonstrates that modification of both cysteine residues with iodoacetamide (neutral function) or iodoacetate (negatively charged function) does not affect the inhibitory activity of Tn-I.

E. SUMMARY

Evidently the sulfhydryl modification only causes structural alteration in a region of the Tn-I molecule which interacts with Tn-T. The inhibitory activity of sTn-I (ie. interaction site with actin) has been localized to a 21 residue CNBr fragment which possesses as much as 75% of the activity of native sTn-I (Syska et al., 1976). Chemical synthesis of the corresponding rabbit cardiac sequence reveals a similar inhibition (J.A. Talbot and R.S. Hodges, priviledged communication). Possibly the Tn-T interaction region of Tn-I is similarly highly localized

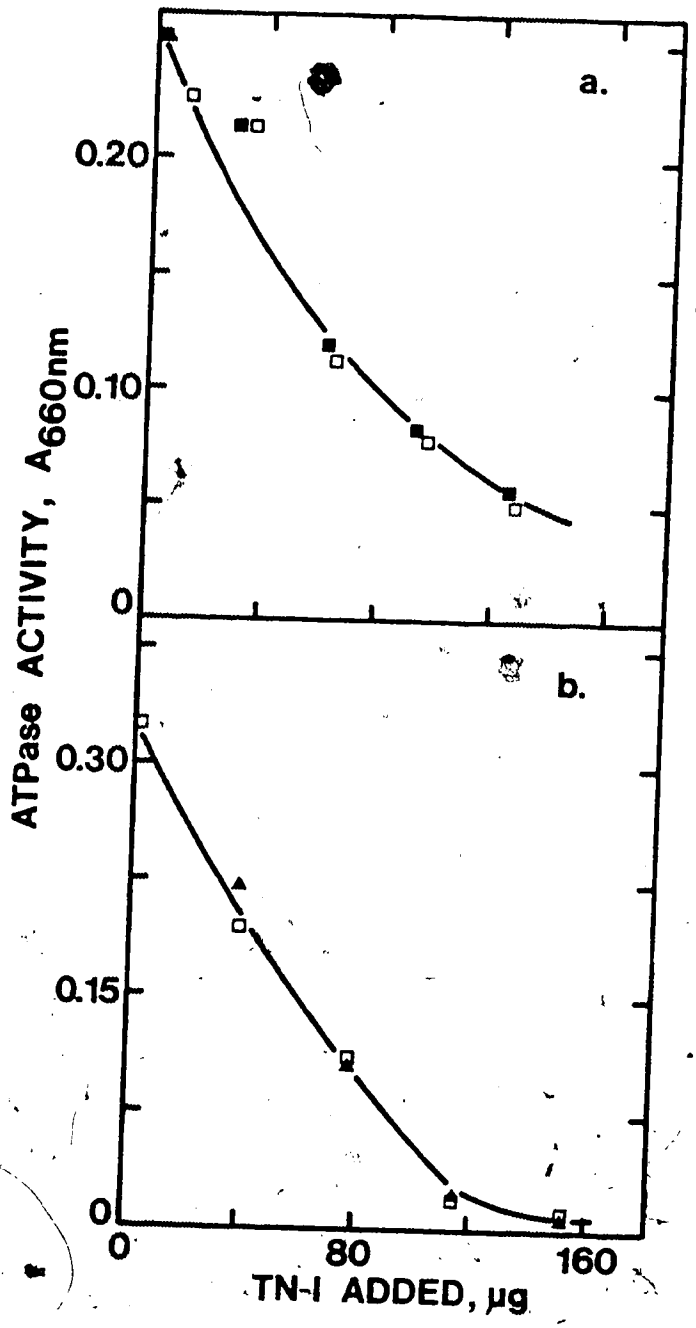


Figure 16. ATPase activity of bovine cardiac SAM as a function of added Tn-I. The solvent system was 2.5 mM $MgCl_2$, 50 mM TRIS-HCl, pH 8.0, 2,5 mM ATP, 1 mM EGTA. Reactions were carried out for 15 minutes at 37° C. Tn-I was dissolved in 3.5% formic acid, 1 mM DTT for addition to the reaction solutions.

A. Native cTn-I (□); cTn-I iodoacetate (■).

B. Native cTn-I (□); cTn-I iodoacetamide (▲).

and close to a cysteine residue. An analysis of the conformational parameters of the primary sequence of rabbit cardiac Tn-I indicates cysteine 75 may be the source of the modification-induced increase in secondary structure. This residue is homologous in the sequences of Tn-I from 3 sources: rabbit fast and cardiac muscle, and chicken fast muscle (Wilkinson and Grand, 1978). The other cysteine residue in cardiac Tn-I, at position 92, is also found in a homologous position in rabbit fast and slow muscle, and chicken fast muscle. It seems reasonable to conclude that the region of Tn-I which interacts with Tn-T is found near or between residues corresponding to 75 and 92 in the cardiac sequence.

It has been previously demonstrated that modification of the SH residues in skeletal troponin with NEM or PCMB or merely their oxidation causes drastic loss of Ca^{2+}-sensitivity in the actomyosin superprecipitation assay system (Yasui et al., 1968; Parker and Kilbert, 1970). In the skeletal system Tn-T has no cysteine residues, Tn-I three and Tn-C one. Thus, in retrospect, this effect appears to originate at the level of the Tn-I subunit. In the current concept of muscle contraction, Tn-I interacts at the level of actin, as well as with Tn-C and Tn-T. This modification may alter the actin interaction as well but the evidence presented here and elsewhere (Horwitz, 1979) suggests that the interaction with Tn-T is necessary to form a fully functional regulatory system.

In this study and others, investigating the interaction between Tn-I and Tn-T, it has become apparent that the status of the sulfhydryl groups in the troponin subunits must be maintained by use of a rigorously reducing environment with DTT and N_2. Unless cysteine residues are protected, it seems apparent that non-physiological disulfide bonds form, involving various members of the troponin complex, when the individual

subunits are reconsituted, or even when dealing with native troponin.

Since this study (Hincke et al, 1978) and the original report on the skeletal system (Horwitz, 1978) have appeared, further information has become known concerning this interaction, but only for the rabbit skeletal proteins. Two studies have investigated the binding of sTn-T fragments to sTn-I in order to localize the interaction region(s) (Katayama, 1979; Pearlstone and Smillie, 1980). The latter study located two regions of Tn-T which interacted with Tn-I, using the technique of gel filtration. Fragments containing the N-terminal region (residues 1 - 70) and residues 159 - 209 of Tn-T displayed specific binding to Tn-I. Although the N-terminal region is acidic at neutral pH and was considered a likely site for interaction with the basic Tn-I molecule, residues 159 - 209 possess a net positive charge under these conditions and must therefore represent a very specific type of binding to Tn-I.

CHAPTER V

SPECTROSCOPIC STUDIES OF CARDIAC AND SKELETAL Tn-C

When this study was initiated it had been demonstrated that Ca^{2+} binding to cardiac and skeletal Tn-C elicited a large conformational change which could be detected by a variety of spectroscopic techniques. This reorganization in protein structure was believed to be the activation step in striated muscle contraction and represented the manner in which Ca^{2+} acted as a second messenger. In order to discern any subtle differences in this process between the cardiac and skeletal 'machinery', a comparison study was undertaken, using the techniques of CD and UV difference spectroscopy. These studies were carried out at a number of pH values between 6 and 8 in order to investigate the effects we discovered were pH dependent in this range. We anticipated this would yield information concerning coordinating residues in these proteins and ionizable groups involved in the process (Hincke et al., 1978).

A. CIRCULAR DICHROISM

Figures 17 and 18 demonstrate the results obtained when the cardiac and skeletal proteins were titrated with calcium. There are two conformational transitions present in each protein. Computer fitting to this data yielded apparent affinity constants of $1.7 \times 10^7\ M^{-1}$ and $4.0 \times 10^3\ M^{-1}$ for skeletal Tn-C and $9.3 \times 10^6\ M^{-1}$ and $2.6 \times 10^3\ M^{-1}$ for the cardiac protein. These compare well with data from other sources, derived by different methods of monitoring the conformational change associated with the high affinity transition. For example, a tyrosine fluorescence and CD titration study of cardiac Tn-C yielded an apparent high affinity transition of $2.5 \times 10^7\ M^{-1}$ at pH 7.5 (Leavis and Kraft, 1978) and

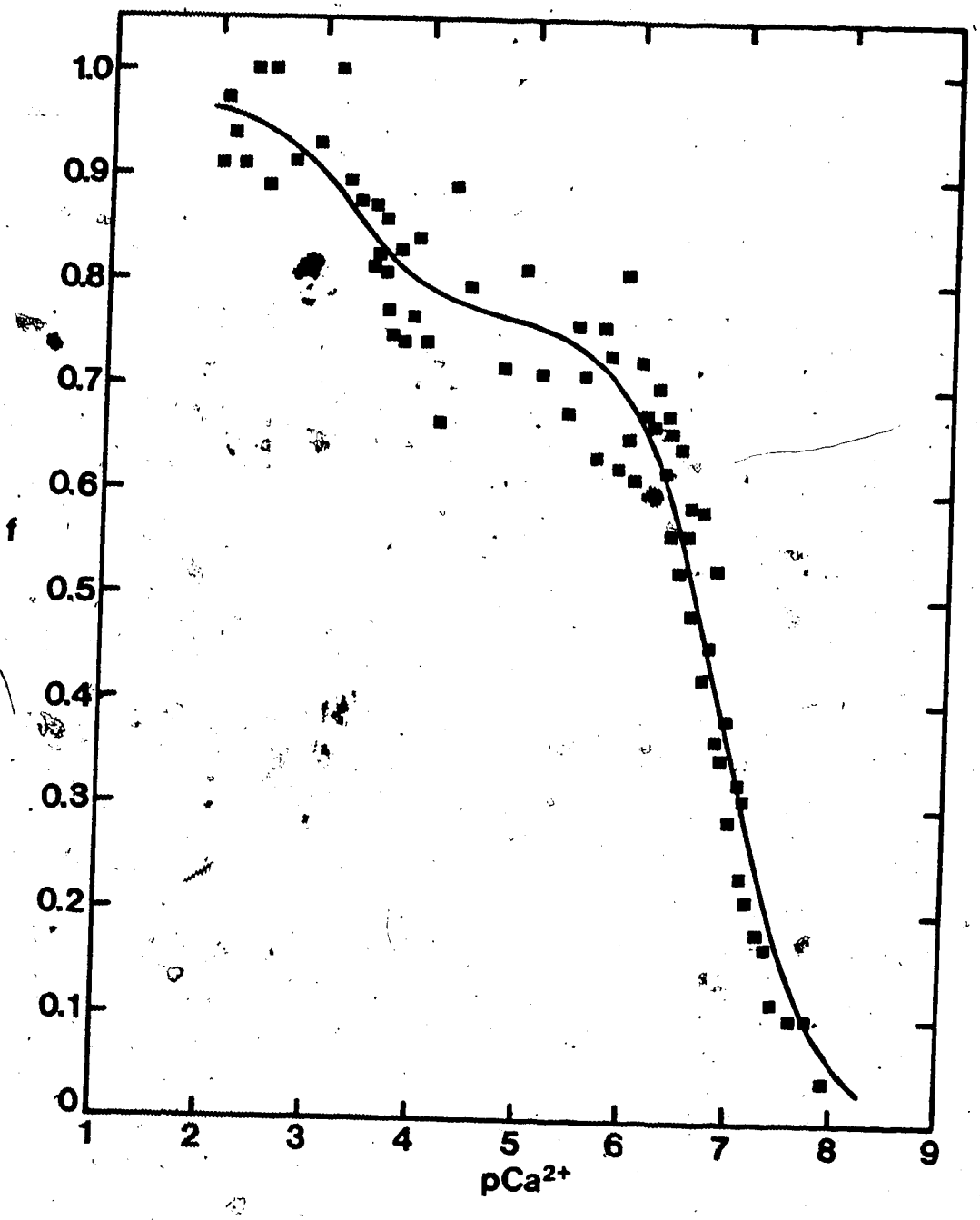


Figure 17. Ca^{2+} titration curve for cTn-C at pH 6.90. The protein was dissolved in 100 mM MOPS, 50 mM KCl, 1 mM EGTA and titrated with $CaCl_2$ while the CD signal at 222 nm was monitored. This figure is a composite of three such runs and the data have been normalized for presentation. The solid line was derived from the computer analysis which extracted the parameters presented in Table VI.

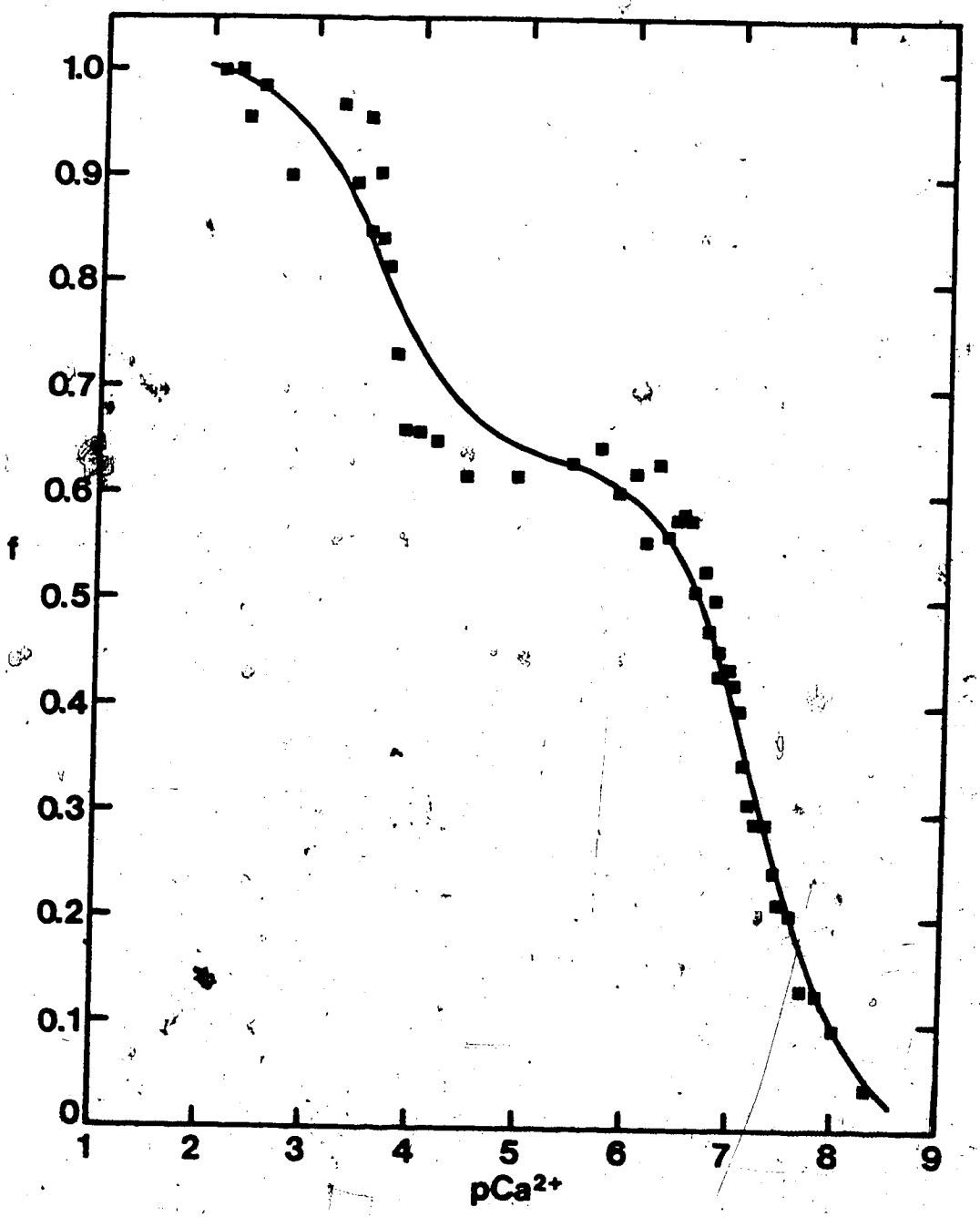


Figure 18. Ca^{2+} titration curve for sTn-C at pH 7.11. The protein was dissolved in 10 mM MOPS, 50 mM KCl, 1 mM EGTA and titrated with $CaCl_2$ while the CD signal at 222 nm was monitored. This figure represents two such runs. The solid line was derived from the computer analysis which extracted the parameters presented in Table VII.

a spin label study of skeletal Tn-C resulted in a measured affinity of $2 \times 10^7\ M^{-1}$ (Potter et al., 1976). These affinities are also in agreement with results from equilibrium dialysis experiments on cardiac Tn-C (Holroyde et al., 1980) and skeletal Tn-C (Potter and Gergely, 1975).

We cannot detect any contribution from the low affinity site(s) in either protein by this technique. Apparently our methodology is not sensitive enough to detect this class of site(s). The very low affinity transition we do detect occurs at an unphysiologically high concentration of calcium, indicating it represents some type of non-specific binding. This is not unlikely in such acidic proteins. For example, the K_{Ca} of citrate is $4 \times 10^3\ M^{-1}$ (Sillén and Martell, 1964) in which the metal ion is bound by two carboxylate groups (Leavis and Lehrer, 1978).

The calcium affinities of these proteins were studied as a function of pH between pH 6.0 and 7.4. The parameters derived from these titrations are presented in Tables 6 and 7. Two parameters change in a pH dependent manner: 1) the apparent affinity of the high affinity transition in each protein decreases as the pH is lowered and 2) the total conformational change which can be elicited by calcium-binding decreases as well.

In Figure 19, plotting the pK'(Ca) versus pH results in a Dixon-Webb plot (Dixon and Webb, 1958), from which the pKa of the ionizing groups controlling this change may be extracted. According to this analysis, extrapolation of the asymptotes to a crossover point yields the pKa of the responsible groups. This suggests ionizing groups with pKa's about 6.6 in each protein are important in controlling the Ca^{2+} affinity of the high affinity sites. The slope at the pH-dependent linear section is ~2.1 for each protein. This equals the change of ionization of the

TABLE VI

Ca^{2+} Binding Parameters For Bovine Cardiac Tn-C as a Function of pH

pH (buffer system)	n_1	K_1 M^{-1}	n_2	K_2 M^{-1}	$\Delta[\theta]_{max,222\ nm}$
8.02 (EPPS)	-	-	-	-	4611 ± 145
7.41 (HEPES)	.605 ± .019	1.10 ± .10 x 10^7	.409 ± .023	5.55 ± 1.02 x 10^3	4584 ± 114
7.11 (MOPS)	.717 ± .025	9.26 ± 1.11 x 10^6	.306 ± .038	2.60 ± 1.15 x 10^3	4587 ± 145
6.90 (MOPS)	.763 ± .015	7.86 ± .69 x 10^6	.209 ± .028	1.84 ± .93 x 10^3	4980 ± 124
6.72 (PIPES)	.728 ± .013	4.87 ± .41 x 10^6	.289 ± .025	2.54 ± .75 x 10^3	3068 ± 145
6.39 (MES)	.793 ± .022	2.94 ± .46 x 10^6	.178 ± .036	4.36 ± 3.16 x 10^2	3547 ± 99
6.01 (MES)	.637 ± .074	5.15 ± 1.51 x 10^5	.339 ± .068	1.44 ± .79 x 10^3	11[illegible] ± 118

TABLE VII

Ca^{2+} Binding Parameters For Rabbit Skeletal Tn-C as a Function of pH

pH (buffer system)	n_1	K_1 M^{-1}	n_2	K_2 M^{-1}	$\Delta[\theta]_{max,222\ nm}$
8.02 (EPPS)	-	-	-	-	7500 ± 140
7.41 (HEPES)	.582 ± .025	1.27 ± .15 x 10^7	.441 ± .031	4.50 ± 1.06 x 10^3	7005 ± 140
7.11 (MOPS)	.634 ± .016	1.73 ± .18 x 10^7	.378 ± .026	3.96 ± .94 x 10^3	7041 ± 153
6.72 (PIPES)	.626 ± .014	6.91 ± .78 x 10^6	.409 ± .026	3.29 ± .72 x 10^3	5253 ± 146
6.39 (MES)	.591 ± .022	4.15 ± .83 x 10^6	.407 ± .026	1.39 ± .39 x 10^4	5356 ± 172
6.01 (MES)	.519 ± .026	7.76 ± 1.50 x 10^5	.511 ± .026	7.59 ± 1.38 x 10^3	3409 ± 146

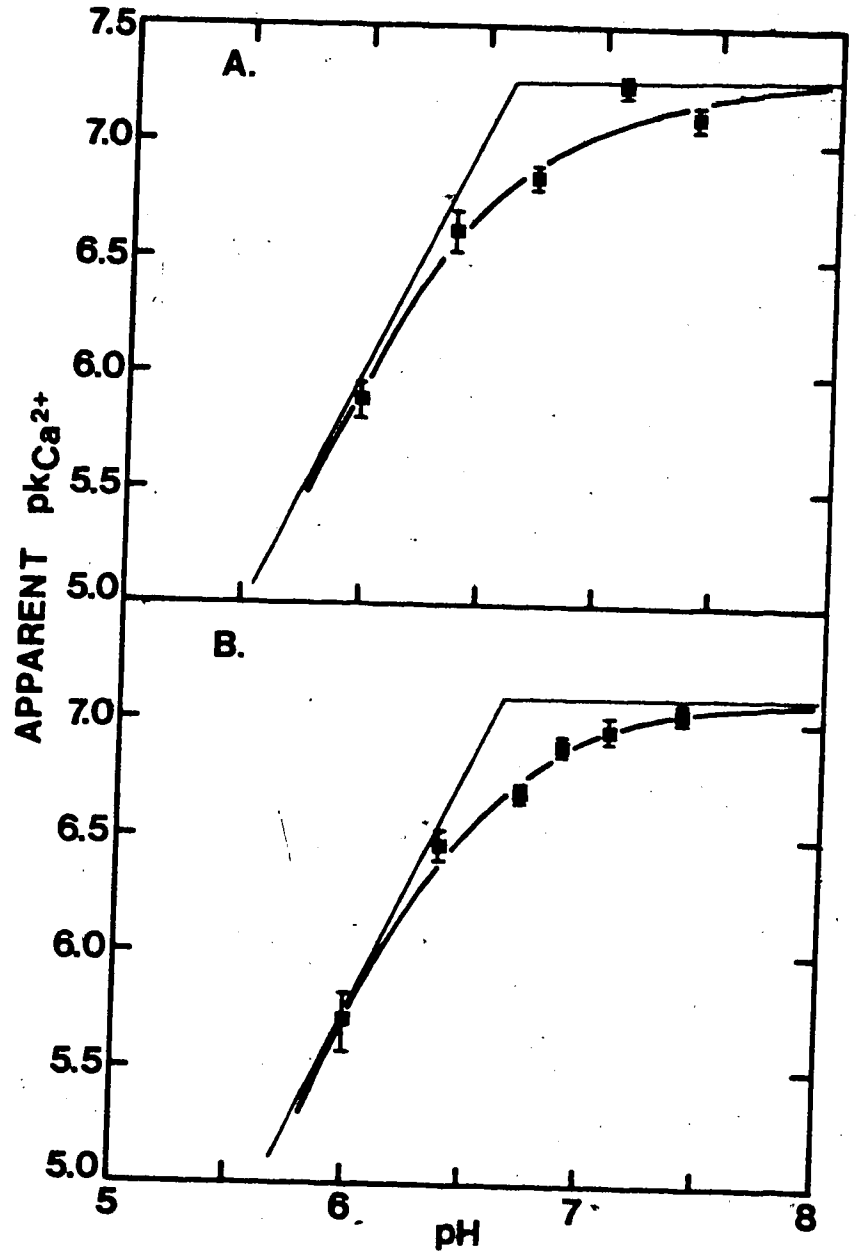


Figure 19. Dixon-Webb plots: The apparent pK'_{Ca} is plotted as a function of pH for A. skeletal Tn-C and B. cardiac Tn-C.

protein that occurs when the complex dissociates to free enzymes and Ca^{2+}.

In Tables 6 and 7 it may be seen that the total conformational change elicited during each conformational transition decreases as a function of pH. This parameter decreases at the level of each class of binding sites, suggesting that protons are able to mimic to a certain extent the ability of Ca^{2+} to evoke a conformational change. Further analysis reveals that this effect is due to an increase in the initial ellipticity. Figure 20, in which the apo-proteins have been titrated with protons, demonstrates this clearly. Here groups with pKa's of 6.2 to 6.3 in each protein are responsible. The final ellipticity after Ca^{2+} addition is the same over this pH range. It can be seen that this effect occurs at the level of both transitions in each protein and must reflect ionizing groups which are able to bind protons in this pH range, ie., they possess pKa's >6. This effect has been demonstrated in sTn-C by others (Leavis and Lehrer, 1978; Lehrer and Leavis, 1974). It was convincingly shown that the conformational change (CD) and increase in tyrosyl fluorescence which occurred as the pH was lowered did not have the same pKa. The pKa for the CD change was determined to be 6.4, that for the fluorescence enhancement was 6.0.

The pKa's of the groups involved in these effects immediately suggest histidine residues are important, However, sTn-C possesses only one histidine, cTn-C has none. Since a similar effect is observed in each protein, carboxyl groups are implicated. A modification study has previously demonstrated their importance in the Ca^{2+}-elicited conformational change (McCubbin and Kay, 1973). Our results indicate some aspartate or glutamate residues are subject to environments which results

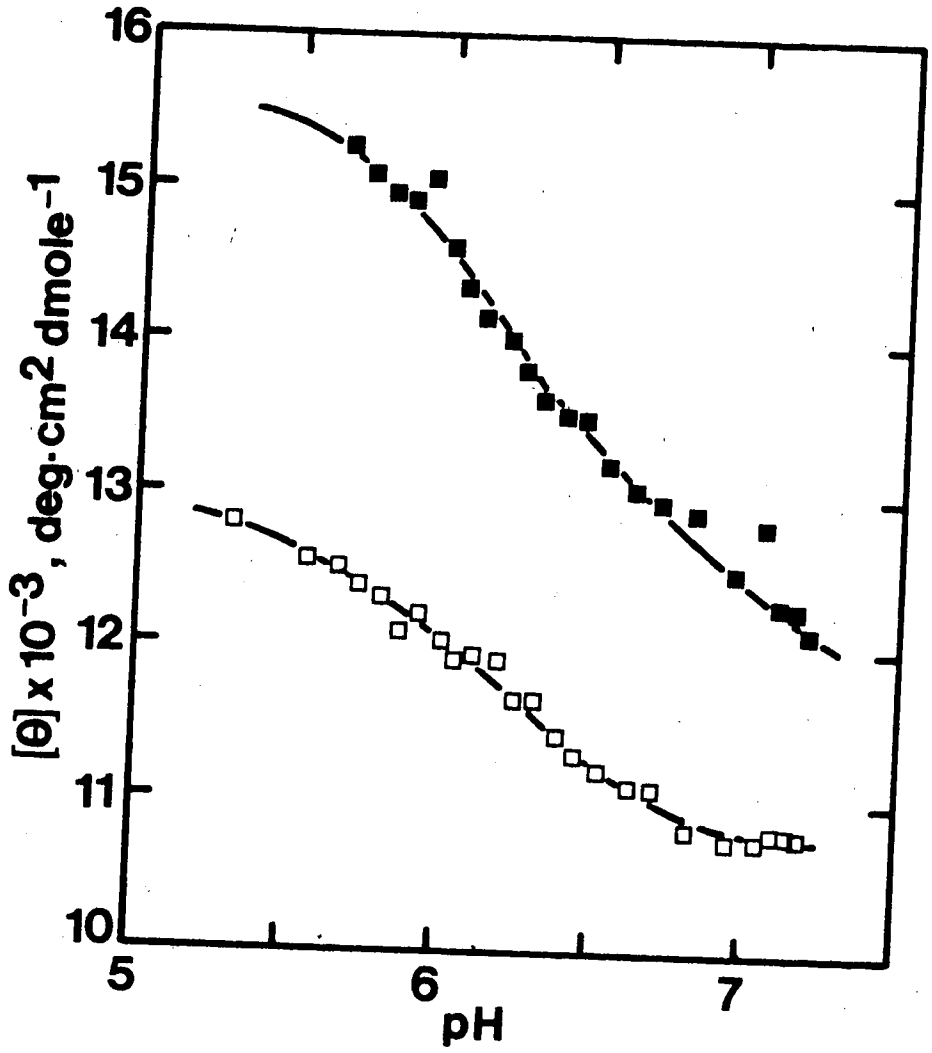


Figure 20. The ellipticity at 220 nm of cTn-C (□) and sTn-C (■) plotted as a function of pH. The solvent system was 0.15 M KCl, 10 mM cacodylate, pH 7.18 and 1 mM EGTA.

in elevated pKa's; the status of these groups is obviously important in determining the protein conformation. The Dixon-Webb plots suggest two such groups may be involved in each protein. The elevation of the pKa of these groups from their free amino acid values (aspartate, 3.9; glutamate, 4.3) is dramatic but not unlikely. This phenomenon has been reported in other protein systems. For example, in lysozyme glutamate 35 shares a proton with aspartate 52 and consequently possesses an elevated pKa of 5.9 - 6.1 (Parsons and Raftery, 1970; Dahlquist and Raftery, 1968).

More appropriately, it has been demonstrated that the affinity of phospholipase A_2 for Ca^{2+} is dependent upon the ionization of a residue whose pKa was determined by Dixon-Webb plots to be 7.2 (Roberts et al., 1977). A carboxyl function was implicated in this effect.

Such an effect is readily obtained in small molecules. In succinic acid the pKa's of the two carboxylic functions are 4.21 and 5.63, which represent a decrease in the first pKa, and an increase of the second, from the value for a monocarboxylic acid such as acetic acid (4.76) (Lehninger, 1970). This phenomenon may be pictured as a sharing of the second proton between the two carboxylate groups, whereby the resulting stabler species possesses an elevated pKa.

It is not unlikely that acidic proteins such as sTn-C and cTn-C would possess some glutamate and aspartate residues with this conformational feature and consequently elevated pKa's. Our evidence clearly implicates these acid residues in Ca^{2+}-binding and the conformational change this evokes.

B. UV DIFFERENCE SPECTROSCOPY

The alterations induced in the protein structure by calcium and proton binding were also studied by monitoring the ultraviolet difference spectra produced by these perturbants.

1. Calcium Difference Spectra

Typical Ca^{2+}-induced difference spectra for cardiac and skeletal Tn-C at pH 7.11 are shown in Figure 21. These spectra were generated by adding a small aliquot of 0.1 M $CaCl_2$ to the sample cell and an equal volume of buffer to the reference cell. The amount of Ca^{2+} added was such that the pCa value was about 3.2 irrespective of pH. This corresponds, approximately, to completion of the calcium induced conformational changes and essential saturation of the high and low affinity sites.

The difference spectra are characterized by maxima near 287 and 277 nm due to perturbations in the environment of tyrosine. These positive peaks imply that the environment of one or more tyrosine residues has become more nonpolar (Donovan, 1969), consistent with the known compacting of the Tn-C structure when binding Ca^{2+}, accompanied by neutralization of negatively charged carboxyl groups. The maxima near 269, 265, 259 and 253 nm can be attributed to contributions from the phenylalanine vibrational fine structure (Donovan, 1969). These peaks are much more difficult to interpret since they represent the averaged environmental effects of each of the 9 or 10 phenylalanine residues in the cardiac or skeletal molecules, respectively.

The magnitude of the tyrosine difference spectra was followed in both systems as a function of pH. These results are presented in Figure 22. The maximum centered near 287 nm in the difference spectrum for skeletal Tn-C is insensitive to pH over the range from 6.1 to 8.0, the

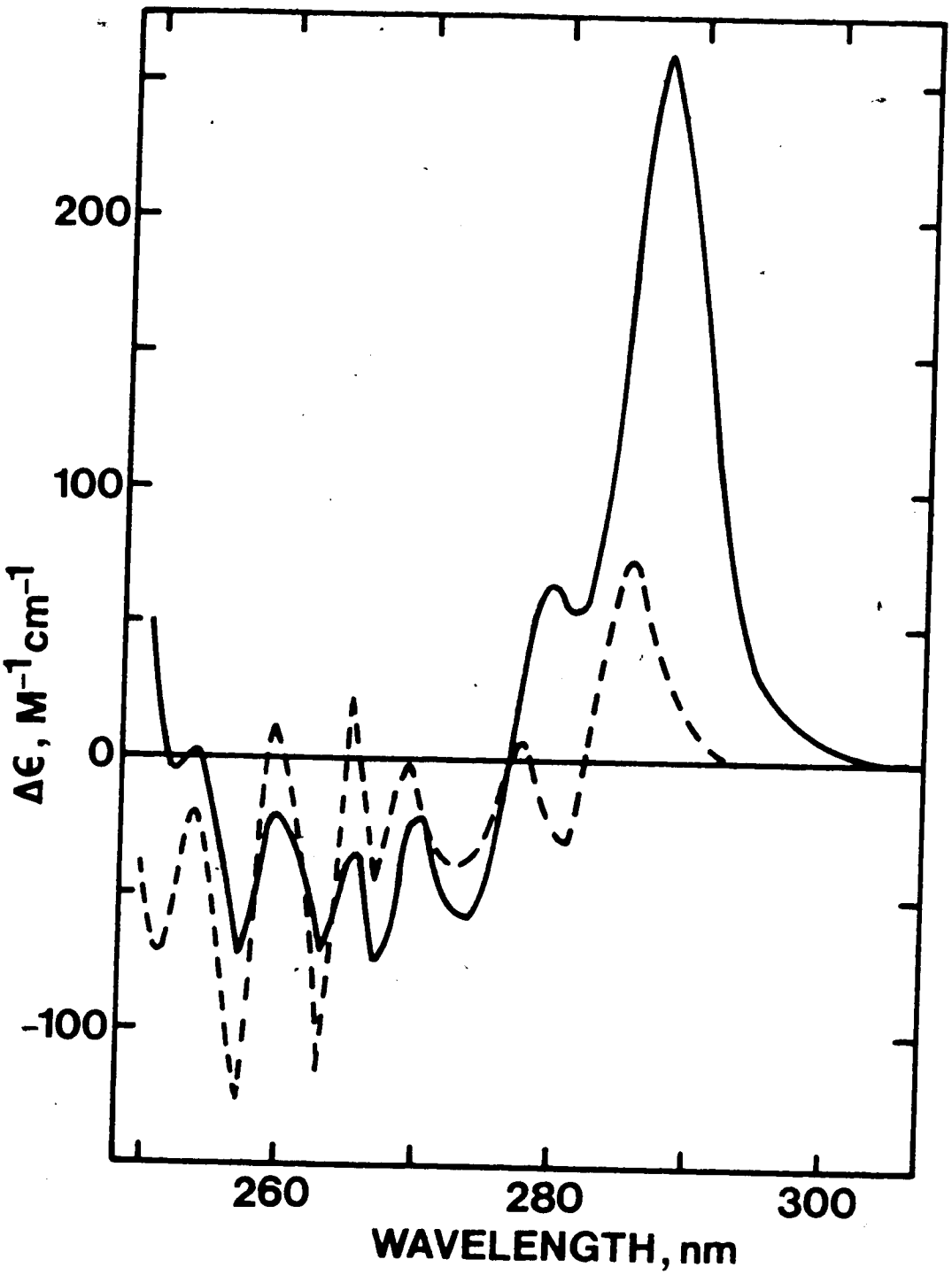


Figure 21. This figure presents typical Ca^{2+}-induced UV difference spectra for cTn-C (———) and sTn-C (- - -). The solvent was 100 mM MOPS, 50 mM KCl, 1 mM EGTA, pH 7.11. These spectra were induced by Ca^{2+} levels of pCa 4.2.

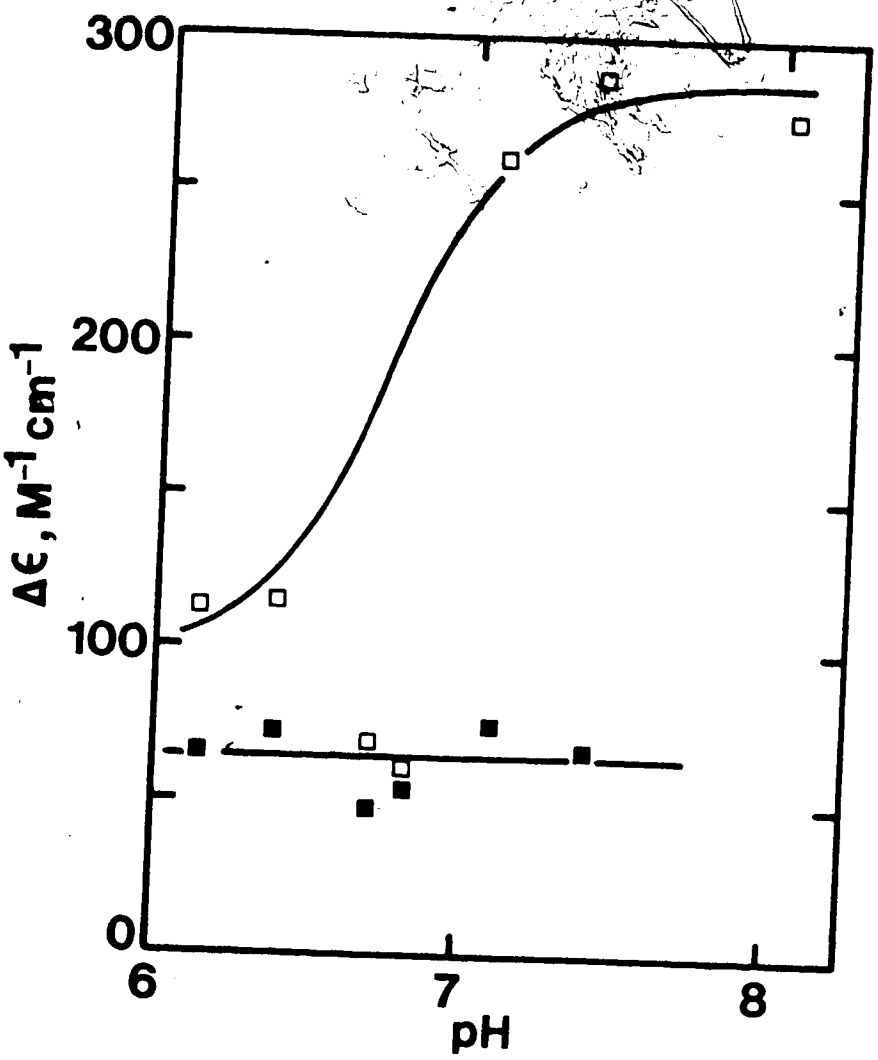


Figure 22. The magnitude of the Ca^{2+}-induced tyrosine difference spectrum at 287 nm is plotted as a function of pH for cTn-C (□) and sTn-C (■). Ca^{2+} levels of pCa 3 to 4 were used to induce these spectra, relative to the Ca^{2+}-free proteins (1 mM EGTA).

value for $\Delta\varepsilon_{M,\ 287\ nm}$ being approximately 68 $M^{-1}\ cm^{-1}$. This is in contrast to the cardiac protein, where $\Delta\varepsilon_{M,\ 287\ nm}$ falls from a value near 285 $M^{-1}\ cm^{-1}$ at pH 8.0 to about 110 $M^{-1}\ cm^{-1}$ at pH 6.1. This appears to be a consequence of protonation of a class of groups with an apparent pKa near 6.6.

The deviation of the values for cTn-C at pH 6.7 and 6.8 from this titration curve is difficult to explain. Examination of the difference spectra throughout this pH range suggests the spectral alterations induced by Ca^{2+} at the different pH values affect more than just the magnitude of this peak. This can be demonstrated by ulilizing a spectral parameter which will be introduced in the section on derivative spectroscopy, the peak resolution parameter (R). This parameter is the ratio of the heights of the two tyrosine bands, measured from the minimum between them. Although developed to interpret first derivative spectra (Brandts and Kaplan, 1973), the similar features of these spectra should allow its application to tyrosine difference spectra, at least for comparison purposes. This parameter was calculated for the cTn-C and sTn-C Ca^{2+}-induced tyrosine difference spectra and is plotted as a function of pH in Figure 23. It is apparent that while the relative heights of the tyrosine bands for sTn-C are invariant with pH, those of the cardiac protein suffer large changes near pH 6.7. The decrease in R value near this pH indicates the smaller band is increasing in height relative to the larger band (or perhaps at its expense). The large change in relative size of the two tyrosyl bands at the midpoint of the titration curve causes the magnitude of the 287 nm band to be much smaller than expected.

The wavelength region 270-250 nm is characterized by phenylalanine transitions for both cardiac and skeletal Tn-C. No trend in peak

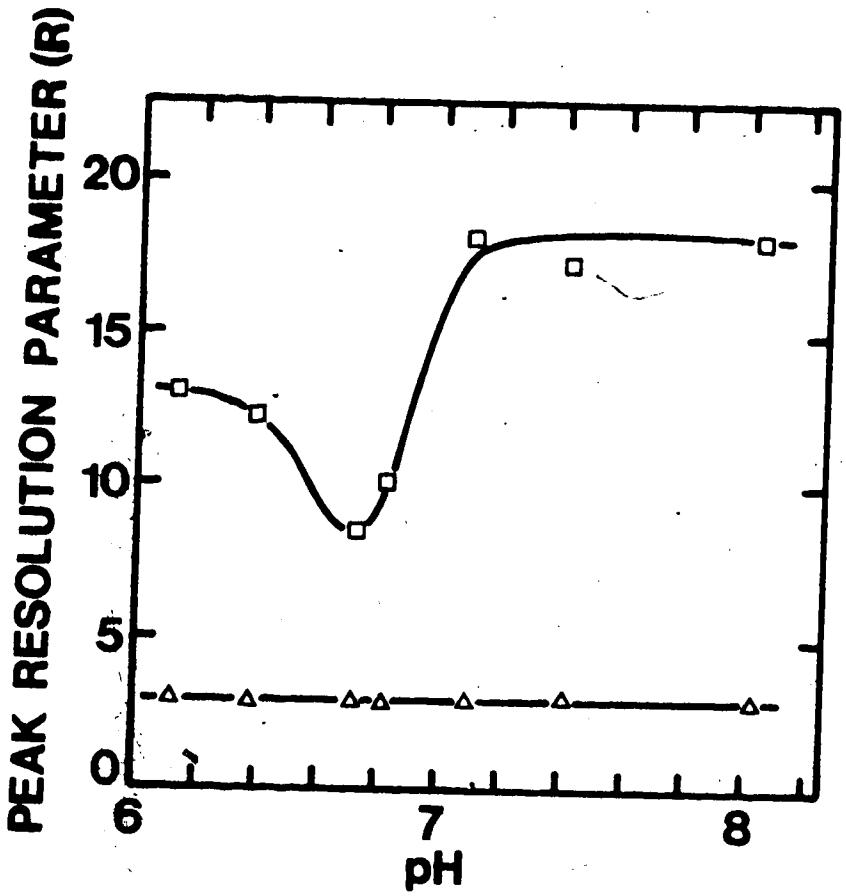


Figure 23. Plot of the spectral resolution parameter (R) as a function of pH for the Ca^{2+}-induced tyrosine difference spectra of cTn-C (□) and sTn-C (△).

height as a function of pH could be discerned.

2. Hydrogen-ion Difference Spectra

The acid difference spectra were obtained in an analogous manner to that for Ca^{2+} ions. The protein sample in the reference cell was maintained at pH 7.0 while the pH of the protein solution in the sample cell was progressively lowered with aliquots of 0.25 M HCl. After addition of the acid, an equivalent volume of buffer was added to the reference cell and the difference spectrum measured. This process was repeated until the onset of turbidity precluded any additional measurements; this occurred at approximately pH 5.0. Typical difference spectra for the two proteins in the absence of Ca^{2+} are presented in Figure 24. These spectra are very similar in shape to those produced by Ca^{2+} and probably have a similar origin. That is, the change in tyrosine environment produced by Ca^{2+} ions is similar to that evoked by H^+ ions.

The $\Delta\varepsilon_M$ values for the major tyrosine peak at 287 nm have been plotted as a function of pH for both systems and are presented in Figure 25. These results seem qualitatively similar to those produced by H^+ titration of the proteins by CD (Figure 20). However the pKa's indicated for this phenomenon (cTn-C, 6.0; sTn-C, 5.8) are lower than those determined from the CD results (6.3 in each case). This suggests that the carboxyl group(s) whose ionization affects the tyrosyl fluorescence in sTn-C may also be detected by this technique. This is not unlikely since the neutralization of an acid group in the tyrosine environment would be expected to decrease the polarity of that environment, causing a red shift in its absorption (Donovan, 1969).

In the presence of Ca^{2+}, the acid difference spectra are drastically reduced. In the cardiac case, $\Delta\varepsilon_{M,\ 287\ nm}$ in the absence of Ca^{2+}

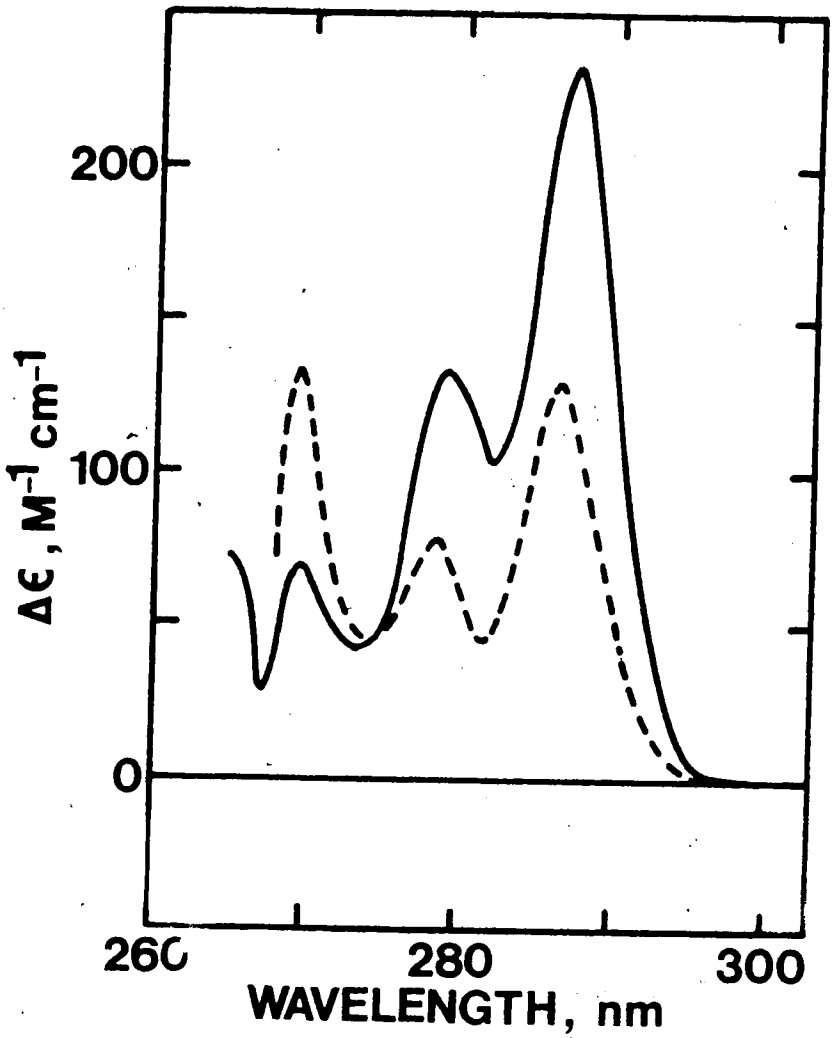


Figure 24. UV difference absorption spectra of cTn-C (———) and sTn-C (- - -) produced by the addition of dilute acid. The proteins were dissolved in 0.15 M KCl, 2 mM EGTA and 10 mM cacodylate, pH 7.00. An aliquot of 0.25 M HCl was added to the sample cell such that the final pH was 5.17 for cTn-C and 5.07 for sTn-C.

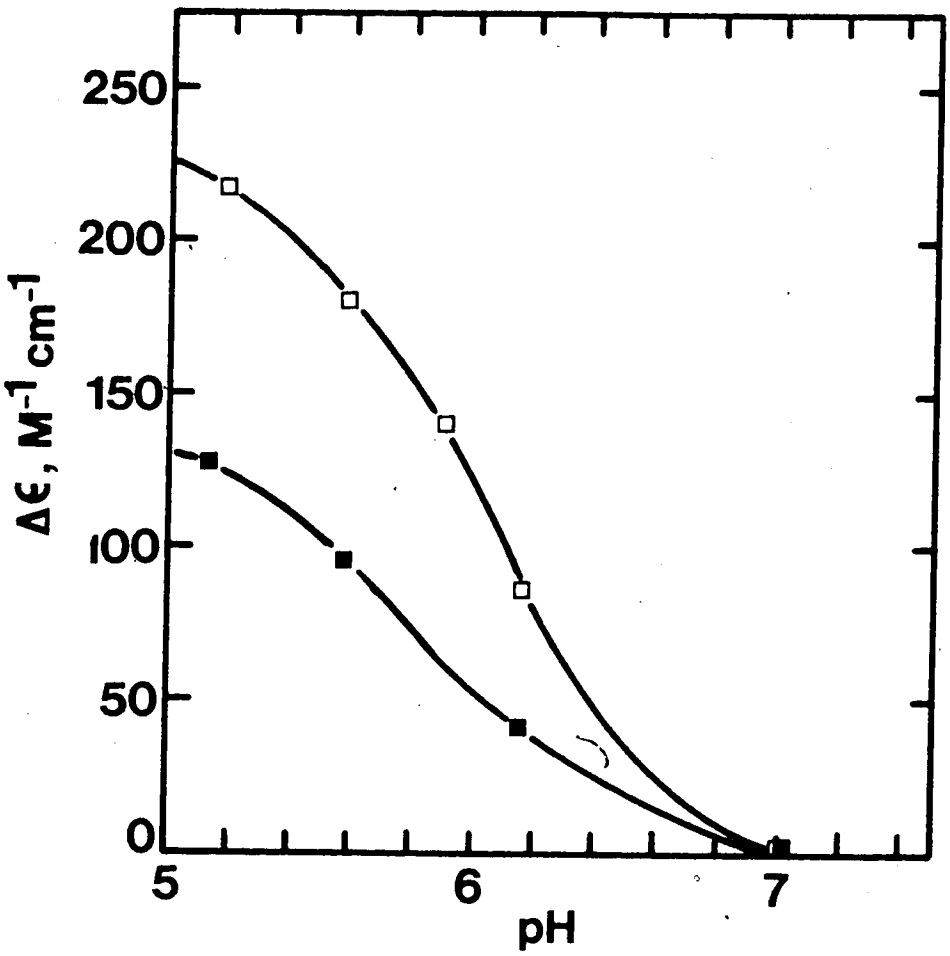


Figure 25. Variation of the magnitude of the acid-produced tyrosine difference peak near 287 nm with pH for cTn-C (□) and sTn-C (■). The solvent was 0.15 M KCl, 2 mM EGTA, 10 mM cacodylate, pH 7.00.

at pH 5.5 is about 189 M^{-1} cm^{-1}, dropping to 20 M^{-1} cm^{-1} in the presence of Ca^{2+}. For skeletal Tn-C, the reduction is not nearly so great. At pH 5.2, $\Delta\varepsilon_{M,\ 287\ nm}$ in the minus Ca^{2+} state is 128 M^{-1} cm^{-1} which becomes 74 M^{-1} cm^{-1} with Ca^{2+} present.

The near UV CD spectrum in the presence and absence of Ca^{2+} was investigated for each protein at a number of pH values to determine if these dramatic environmental alterations would also be expressed as a change in the chirality of the tyrosine or phenylalanine environment. No large differences or trends were detected in either case. Thus the changes in the polarizability of the tyrosine environments produced by Ca^{2+} or H^{+} ions do not reflect large changes in the optical activity of these residues.

C. FIRST DERIVATIVE SPECTROSCOPY

The parameters measured and discussed in this part of the work are those defined and reported earlier (Brandts and Kaplan, 1973). $(\Delta\varepsilon/\Delta\lambda)_{max}$ is the value of the peak height of the major tyrosine band situated near 287 nm. $\Delta\lambda_{1/2}$ is the half band width of this major peak. The degree of resolution of the minor and major peaks was measured by the ratio R of the heights of the two peaks where the heights are measured from the minimum separating the two bands. λ_{max} is the wavelength where the derivative spectrum crosses the zero axis and thereby corresponds to the wavelength of maximum extinction in the normal spectrum. This parameter is probably most closely related to the average degree of accessibility of the chromophores to solvent.

Typical derivative spectra for cardiac and skeletal Tn-C are presented in Figure 26, obtained in the absence of Ca^{2+} at pH 7.42.

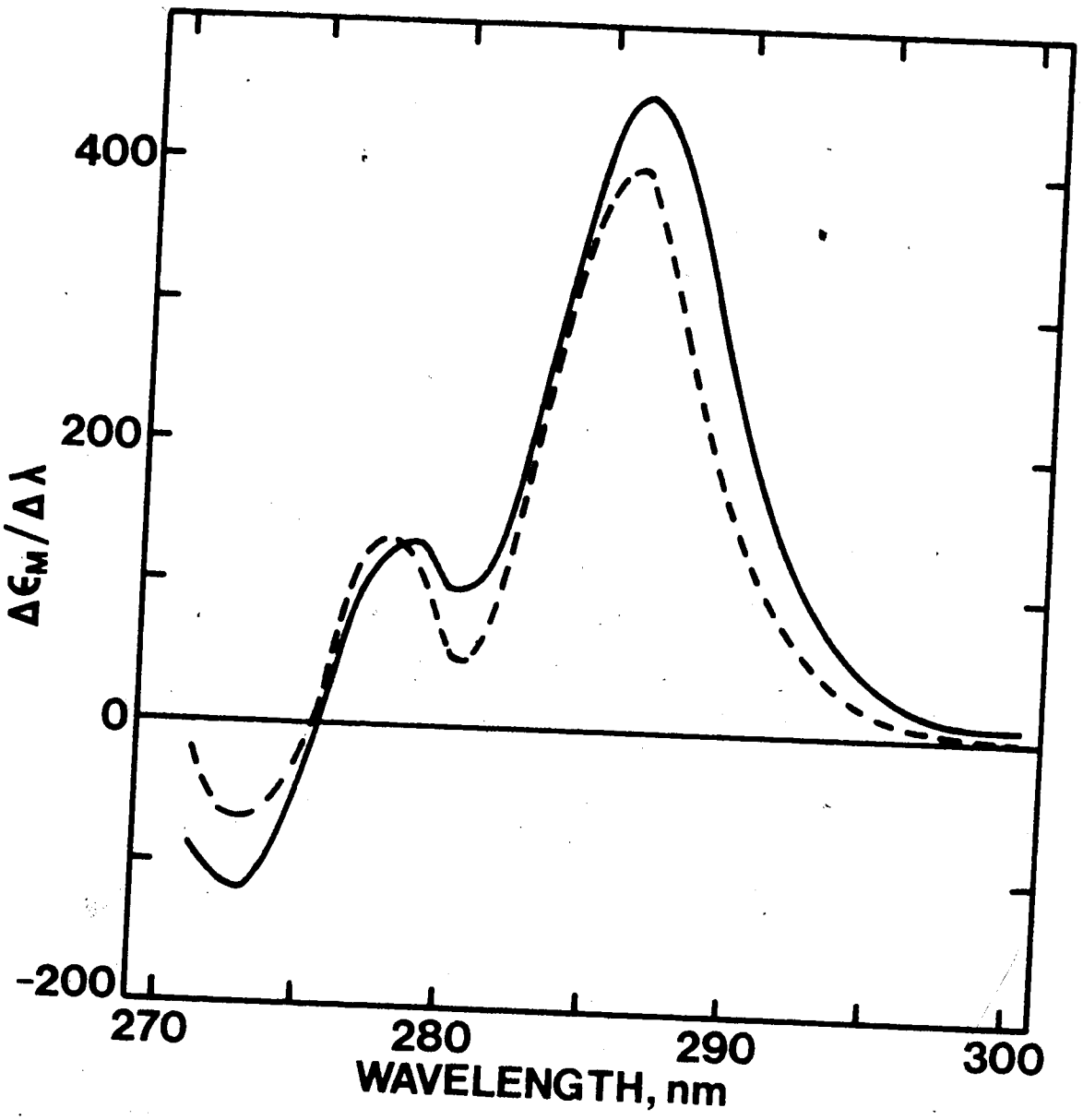


Figure 26. Derivative spectra calculated at 1 nm intervals from the absorption spectra of cTn-C (———) and sTn-C (- - -). The solvent system in each case was 0.15 M KCl, 2 mM EGTA and 10 mM cacodylate at pH 7.42.

When these have been normalized to the number of tyrosyl chromophores contributing to the spectrum (N) it is apparent that the maximum value of the derivative function ($\frac{1}{N}(\Delta\varepsilon/\Delta\lambda)_{max}$) is different for each protein. The larger value of 200 for the skeletal protein versus 150 for cTn-C suggests a greater degree of solvent exposure in the former. Comparison to data for model compounds in different media (Brandts and Kaplan, 1973) indicates the tyrosine residues in sTn-C are spectrally very homogeneous. The opposite is true for cTn-C; the tyrosine residues are very heterogeneous. Presumeably this is a reflection of environmental differences.

The λ_{max} values, 275.8 nm for cTn-C and 275.6 nm for sTn-C, indicate the tyrosine residues in each protein are fairly exposed, as compared to N-acetyl-L-tyrosine ethyl ester in solvents of differing aqueous character. Comparison of the R values for these proteins, 9.9 for cTn-C versus 4.2 for sTn-C, again indicates a greater degree of spectral heterogeneity for the tyrosyl residues in cTn-C.

Derivative spectra for these proteins were determined as a function of pH in the absence of Ca^{2+}. No trends with pH were detected and the values of the various derivative parameters are presented in Table 8.

The product of the degree of peak resolution parameter (R) and the spectral width parameter ($\Delta\lambda_{1/2}$) has been used in protein comparison studies as a monitor of spectral heterogeneity (Brandts and Kaplan, 1973). As noted in Table 8, this value for sTn-C is very similar to that of N-acetyl-tyrosine ethyl ester, suggesting complete equivalence of each tyrosine residue in this protein. The high degree of tyrosyl heterogeneity in cTn-C is evident by this technique.

The derivative spectra calculated in the presence of Ca^{2+} for each protein show little change in R or $\Delta\lambda_{1/2}$ parameters relative to

TABLE VIII

Average Spectroscopic Parameters Obtained from Derivative Spectra for Tn-C (pH Values Range from 7.4 to 5.2)

	$R=h_I/h_{II}$	$\Delta\lambda_{1/2}$	$R\cdot\Delta\lambda_{1/2}$	$\lambda_{max}(-Ca^{2+})$	$\lambda_{max}(+Ca^{2+})$
cTn-C	10.1	7.1	71.7	275.8	276.6
sTn-C	4.7	6.2	29.1	275.6	275.8
N-acetyl-L-tyrosine ethyl ester*					
H_2O	4.5	6.2	27.9		274.6
1-butanol	5.0	5.6	28.0		278.0

Solvent system was 0.15 M KCl, 10 mM cacodylate over the pH range. 2 mM EGTA was included in the minus Ca^{2+} buffers, 1 mM $CaCl_2$ for plus Ca^{2+}.

* Brandts and Kaplan, 1973.

the minus Ca^{2+} case. However, there is a red shift in the value of λ_{max} of each protein (Table 8). The red shifts indicate that in each case Ca^{2+}-binding causes the tyrosyl environment to become more non-polar. This effect is more pronounced in cTn-C, in agreement with the larger tyrosine difference spectrum produced by Ca^{2+}, relative to the skeletal case.

D. SPECTROPHOTOMETRIC TITRATIONS

Spectrophotometric titration studies to determine the pKa's of the tyrosine residues in sTn-C and cTn-C were performed. Figure 27 reveals the titration curves for cTn-C in the presence and absence of Ca^{2+}. These results indicate that in apo-cTn-C the three tyrosine residues possess similar pKa's of about 10.7. However, in the Ca^{2+}-saturated protein one or two tyrosine residues have an elevated pKa of 11.6. This is consistent with NMR evidence presented later which shows that in Ca^{2+}-saturated cTn-C a single tyrosine residue possesses an elevated pKa. The other tyrosine residues are unaffected by calcium-binding. A pKa of 10.6 is determined for them.

These results can be contrasted with those of sTn-C, where similar pKa's of 10.8 $\pm$ [illegible] are found for each tyrosine residue in the apo- and Ca^{2+}-saturated protein. This result confirms NMR data (Seamon et al., 1977) where a similar pKa was determined.

The nitrated proteins were also titrated. This modification lowers the phenolate pKa to near neutrality (the pKa of nitrotyrosine is 6.8). In apo-cTn-C, pKa's of 7.60 $\pm$ .05 were determined for the 3 nitrotyrosyl residues. Similarly, values of 7.40 were found for the residues in the apo-sTnC.

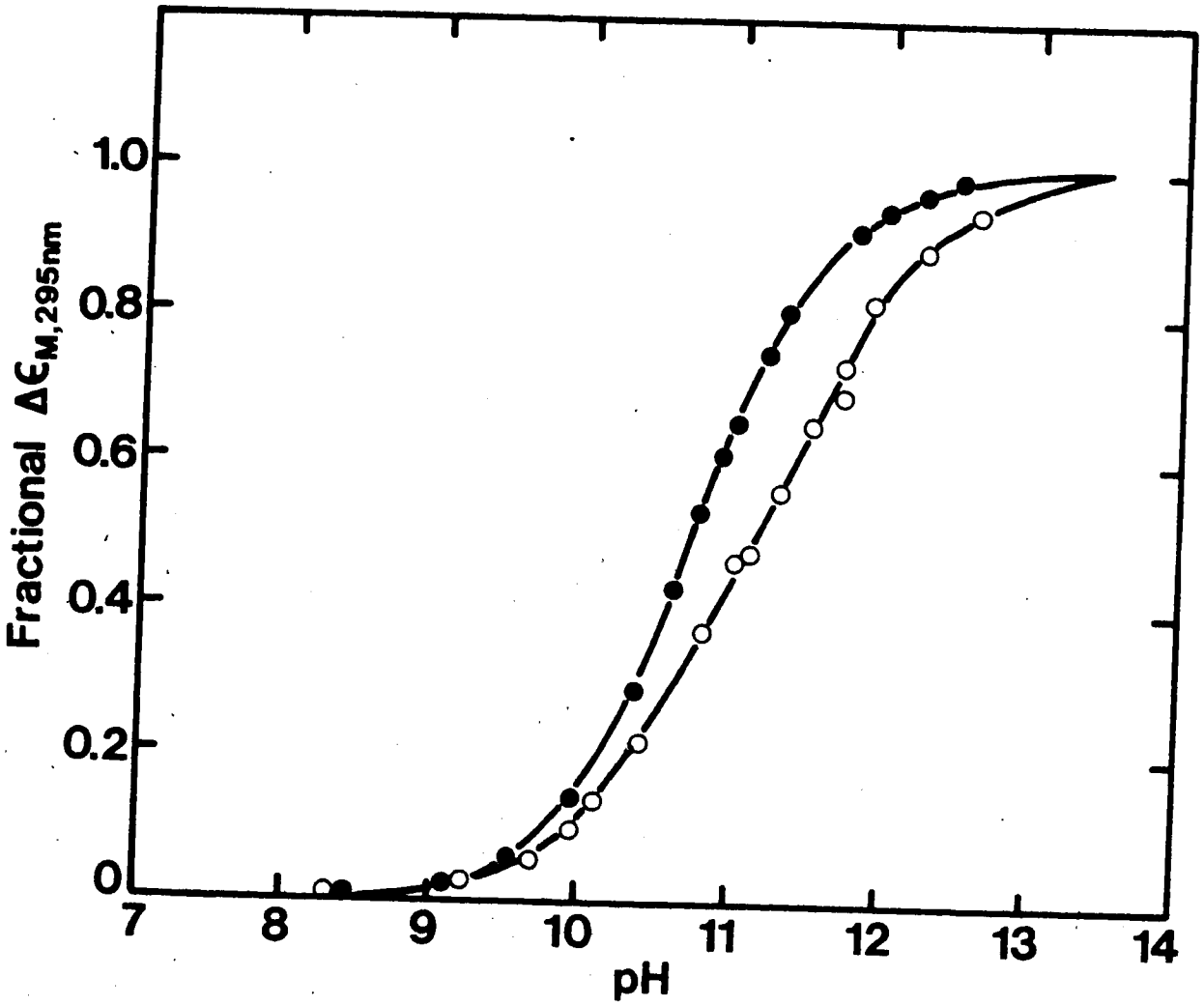


Figure 27. Spectrophotometric titration of cTn-C in the presence (○) and absence (●) of Ca^{2+}.

E. SUMMARY

Our results demonstrate that CD is a good technique for monitoring the large increase in secondary structure which is induced in cTn-C and sTn-C by Ca^{2+}-binding. The pH dependence of the CD parameters indicates that protons can compete with Ca^{2+} for binding to some carboxyl groups important in this process. It is interesting that other workers have not demonstrated this pH-dependent affinity of the high affinity sites. It has been reported that the calcium affinity of the high affinity sites of rabbit sTn-C is pH-dependent between pH 7.4 and 6.0, while the affinity of the lower affinity sites decreases as the pH is lowered within this range (Robertson, et al., 1978a). These workers came to similar conclusions regarding cTn-C (Robertson et al., 1978b). In each case tyrosine fluorescence was monitored for high affinity binding and a fluorescent sulfhydryl probe was used for the low affinity site(s) (dansylaziridine for sTn-C and IAANS for cTn-C).

It is possible that the discrepancy between our results and those of Robertson and coworkers arises from the monitoring of different conformational parameters to measure Ca^{2+} affinity. It has been demonstrated in sTn-C that the pH dependence of the proton-induced CD change is different from that of the tyrosine fluorescence enhancement (Leavis and Lehrer, 1978). These phenomena yield pKa's of 6.4 and 6.0, respectively, suggesting that they represent the neutralization of different ionizing groups. The fluorescence enhancement observed when cTn-C (Leavis and Kraft, 1978) and sTn-C (Lehrer and Leavis, 1974) bind Ca^{2+} is believed to represent the direct loss of quenching by nearby carboxylates to tyrosine 109 when the quenching groups are neutralized by H^+, Ca^{2+} or Mg^{2+}. Since the pH-induced fluorescence enhancement does not

occur in parallel with the increase in ellipticity at 222 nm (Leavis and Lehrer, 1978), the carboxyl groups whose neutralization by protons elicits the large conformational change seen by CD must be different from those whose neutralization 'dequenches' the intrinsic tyrosine fluorescence of of these proteins. This can account for a different pH dependence reported by the two methods. The competition between Ca^{2+} and H^+ for coordination to these carboxyl groups can be described with the relationship (Dixon and Webb, 1958):

$$K'_{ca} = K_{ca}/(1 + [H^+]/K_{a1} + [H^+]^2/K_{a1}K_{a2}) \qquad [11]$$

where K'_{ca} and K_{ca} are the affinities for Ca^{2+} in the presence and absence of competition respectively; and K_{ai} is the apparent proton affinity of the ionizing group with i = 1 or 2 for 1 or 2 ionizing residues involved in this equilibrium.

Data for the slight pH dependence of the high affinity sites in sTn-C has been presented (Robertson et al., 1978a). This is reproduced in Table 9, as well as the calculated affinity in the presence of competition between Ca^{2+} and H^+, described by Equation 11, for one and two ionizing groups. It can be readily seen that the experimental affinities, determined by monitoring the tyrosine fluorescence enhancement occurring with Ca^{2+}-binding, are reproduced by assuming one responsible carboxyl group possess a pKa of 5.95, as was determined for the apo-protein. Since the pH induced CD increase is described by a pKa of 6.4, a larger decrease in Ca^{2+} affinity apparently occurs over the same pH range, when compared to the data determined by tyrosine fluorescence. Our results indicate a pKa of 6.6 actually describes these groups. There is a large drop in Ca^{2+} affinity between pH 7 and 6 for cTn-C and sTn-C (Figure 19) which can be reproduced precisely with the assumption that 2 carboxyl

TABLE IX

The Influence of Proton Competition Upon Apparent Ca^{2+}-Affinity; Theoretical Calculations Based Upon Tyrosine Fluorescence Titration Data (Robertson et al., 1978a)

pH	pK'_{ca} observed (Robertson et al. 1978a)	pK'_{ca} calculated		pK'_{ca} calculated	
		1 COOH group	2 COOH groups	1 COOH group	2 COOH groups
7.5	7.16 ± .03 (pK_{ca})	-	-	-	-
6.5	7.04 ± .06	7.05	7.03	6.91	6.78
6.0	6.97 ± .09	6.88	6.73	6.61	6.17
5.5	6.62 ± .09	6.58	6.09	6.21	5.30
		pK_a = 5.95		pK_a = 6.4	

The influence of proton competition upon apparent Ca^{2+}-affinity was calculated with the expression:

$$K'_{ca} = K_{ca}/(1 + [H^+]/K_H)$$

for one carboxyl group involved in this effect, and

$$K'_{ca} = K_{ca}/(1 + [H^+]/K_H + [H]^2/K_H^2)$$

for two carboxyl groups.

TABLE X

The Influence of Proton Competition Upon Apparent Ca^{2+}-Affinity.

Theoretical Calculation for cTn-C and sTn-C,

Based Upon CD Titration Data

	BcTn-C			RsTn-C		
pH	pK'_{ca} obs.	pK'_{ca} calc.		pK'_{ca} obs.	pK'_{ca} calc.	
		1 group	2 groups		1 group	2 groups
7.106	6.97 ± .05	6.92	6.89	-	-	-
6.901	6.90 ± .04	6.87	6.80	-	-	-
6.720	6.69 ± .04	6.80	6.68	6.84 ± .05	6.93	6.81
6.388	6.47 ± .07	6.62	6.32	6.62 ± .09	6.76	6.45
6.006	5.71 ± .13	6.35	5.73	5.90 ± .08	6.48	5.87

pK_{ca} = 7.04 pK_{ca} = 7.18

The effect of proton competition upon apparent Ca^{2+}-affinity of sTn-C and cTn-C, calculated as in Table 9 for pK_a = 6.6.

groups possessing pKa's of 6.6 are responsible for this effect. These calculations are presented in Table 10. It is not clear why there is a difference between this pKa and the value determined by proton titration of the apo-proteins (6.3). Perhaps other acid groups with pKa's less than 6.6 obscure this titration, yielding a composite pKa of 6.3.

This analysis indicates that although both the CD and fluorescence parameters are responsive to Ca^{2+}-binding, their different pH dependencies reveals they do not occur via the same mechanism. Thus this evidence suggests a single carboxyl group (pKa ≈ 6.0) which is neutralized by Ca^{2+}-binding quenches the intrinsic fluorescence of tyrosine 109 in apo-sTn-C. There are also two carboxyl groups (pKa's ≈ 6.6) in both cTn-C and sTn-C whose neutralization evokes a large degree of the conformational change (CD) which occurs during Ca^{2+}-binding. This large increase in secondary structure does not however affect the quenching of tyrosine 109. It is possible some increase in secondary structure accompanies the protonation of the carboxyl group which affects the tyrosine fluorescence.

The elevated pKa of these two carboxyl groups and the observation that their neutralization mimics the CD change observed when Ca^{2+} is bound suggests their identity as the acid pairs in one or more of the high affinity binding sites. In each of the high affinity binding sites of cTn-C and sTn-C, two acid pairs serve as coordinating residues for the bound metal ion. The $\pm$ X and $\pm$ Z dentates are aspartate or glutamate residues. The Y residue is asparagine and -Y is contributed by the peptide carbonyl of tyrosine (site III) or arginine (site IV). Thus it seems likely that the neutralization of two acid pairs in one site by their each sharing one proton could elicit a large degree of the secon-

dary structural changes which normally occur upon Ca^{2+}-binding. The sharing of one proton between two acid groups would account for the elevated pKa's observed for these residues. This would not necessarily affect tyrosine 109, particularly if the effect was at the level of site IV.

A large Ca^{2+}-induced tyrosine difference spectrum is obtained for cTn-C at pH 7 which is reduced in magnitude by decreasing the pH. The sTn-C tyrosine difference spectrum is smaller and pH insensitive. The tyrosine difference spectrum in sTn-C has been attributed primarily to vicinal charge effects, (Nagy and Gergely, 1979). That is, the neutralization of neighboring carboxyl groups makes the tyrosine environment more hydrophobic. A similar phenomenon is predicted for tyrosine 111 in cTn-C as NMR evidence presented later suggests that the site III tyrosine residues of sTn-C, calmodulin and cTn-C occupy very similar environments and respond similarly when Ca^{2+} is bound. Therefore the pH sensitive alteration in tyrosyl environment observed for cTn-C can be attributed to tyrosine 150 and/or 5. This effect seems to reflect the protonation of those carboxyl groups which have such a dramatic influence upon the Ca^{2+}-affinity of the high affinity sites. However it is apparent that only cTn-C possess a tyrosyl residue (150 and/of 5) in position which is able to monitor this transition. The protonation of these groups evidently limits the ability of subsequent Ca^{2+}-binding to alter the tyrosyl environment. Thus a smaller difference spectrum is detected at the lower pH's. This effect was investigated further by ^{1}H NMR (to be discussed later).

Derivative spectroscopy indicates that both sTn-C tyrosine residues are equivalent and are fairly exposed in the apo- and Ca^{2+}-saturated

species. This interpretation is consistent with ^{1}H NMR results with this protein (Seamon et al., 1977). The chemical shifts of the ortho protons of each tyrosine are almost identical and very similar to the random coil values. These resonances are unaltered by Ca^{2+}-binding, indicating the Ca^{2+}-induced UV difference spectra represent only subtle alterations in tyrosine environment which are not detectable by NMR techniques.

On the other hand, for cTn-C the derivative spectroscopic results suggest a high degree of tyrosyl heterogeneity exists in both apo- and plus Ca^{2+}-forms. The binding of Ca^{2+} decreases the average tyrosine exposure to a greater extent than in sTn-C. This correlates with ^{1}H NMR results for this protein (to be presented later), which indicate that all tyrosine residues in apo-cTn-C have very different environments. Two tyrosine residues are very sensitive to Ca^{2+}-binding, one of which becomes extremely buried in the protein interior.

The spectrophotometric titration data supports the conclusions regarding differences between the tyrosyl environment in apo- and Ca^{2+}-saturated cTn-C. The burying of a tyrosine residue, elevating its pKa, is consistent with the large red shift in the Ca^{2+}-induced tyrosine difference spectrum. It is instructive to compare the cTn-C tyrosyl pKa's with those determined for calmodulin (Klee, 1977). In this protein pKa's of 10.1 and 12, or 10.4 and 11.9 (in the presence or absence of Ca^{2+}, respectively) were determined for tyrosine residues 99 and 138. Because of the sequence homology between cTn-C and calmodulin it is tempting to attribute the elevated pKa in Ca^{2+}-saturated cTn-C to tyrosine 150, which occupies a position homologous to tyrosine 138 in calmodulin. Indeed, the NMR evidence presented later supports this assignment. However, it is evident that although the tyrosyl environments of

site IV are similar in the Ca^{2+}-saturated proteins, as evidenced by similarly buried residues, this homology does not exist between the apoproteins.

CHAPTER VI

SPECTROSCOPIC STUDIES OF NITRATED cTn-C, sTn-C AND CALMODULIN

Tetranitromethane is a useful and, under carefully controlled conditions, specific reagent for the chemical modification of tyrosine residues in proteins (Sokolovsky et al., 1966) The nitrotyrosyl chromophore possesses optical transitions in the visible region of the spectrum and thus serves as an easily accessible and assignable probe of its micro environment. CD spectroscopy is particularly sensitive to conformational or environmental asymmetry of the nitrotyrosyl chromophore. Because of the appropriate arrangement of the tyrosine residues within cTn-C, sTn-C and calmodulin, large spectral perturbations are associated with Ca^{2+}-binding. In this section, the nitrotyrosyl chromophores in these proteins were utilized to study and compare Ca^{2+}-binding by CD and UV difference techniques (McCubbin et al., 1978).

The tyrosyl residues of cTn-C were nitrated with TNM under a variety of conditions, both in the presence and absence of Ca^{2+}. Although the initial reaction rate, as monitored by nitroformate release at 350 nm was about double for the protein in EDTA, complete nitration of the three tyrosine residues was achieved in each case. This could be easily determined by amino acid analysis as nitrotyrosine is stable to acid hydrolysis (Sokolovsky et al., 1967). In a study of this nature it is important to verify that chemical modification has not caused irreversible loss of protin structure. CD measurements determined a $[\theta]_{222}$ value for the apo-protein of -8900° at pH 7.0 in 50 mM KCl, 100 mM MOPS, 1 mM EGTA. This increased to -12,800° with Ca^{2+} saturation. These values compare well with those of -9700° and -14,450° for the native protein

under the same conditions. This suggests that nitration of cTn-C does not affect the protein folding to any great degree, in agreement with results for sTn-C (McCubbin and Kay, 1975) and calmodulin (Richman and Klee, 1979).

A. CIRCULAR DICHROISM

Nitrated cTn-C, sTn-C and calmodulin were titrated with $CaCl_2$, monitoring both $[\theta]_{222\ nm}$ and $[\theta]_{275\ nm}$. The results of a typical titration for nitrated cTn-C are displayed in Figure 28. It seems clear that the nitrotyrosyl chromophore monitors only high affinity binding. Here the solid lines depict the theoretical binding curves and are superimposed upon the experimental data. The parameters of the fit for the titrations of these proteins, n_i and K_i are summarized in Table 11. Note that the n values for the nitrated proteins are not significantly different from those of the native proteins. As well, the K values for the modified proteins, although showing some differences with the native samples (i.e. reductions of about half an order of magnitude in K_i for sTn-C and calmodulin) are within the range observed for the unmodified proteins. The similarity in the values of n and K for the derivatized and native proteins indicates that the structures of the derivatives are similar to those of the native proteins, in terms of both average secondary structure of their polypeptide chains and in their response to Ca^{2+} ions.

The utility of the nitrotyrosyl chromophore as a probe in looking at different areas of these molecules as they bind Ca^{2+} is well demonstrated by these results. In cTn-C and sTn-C it is apparent that by monitoring the Ca^{2+} titration at 275 nm, a point of maximal CD change

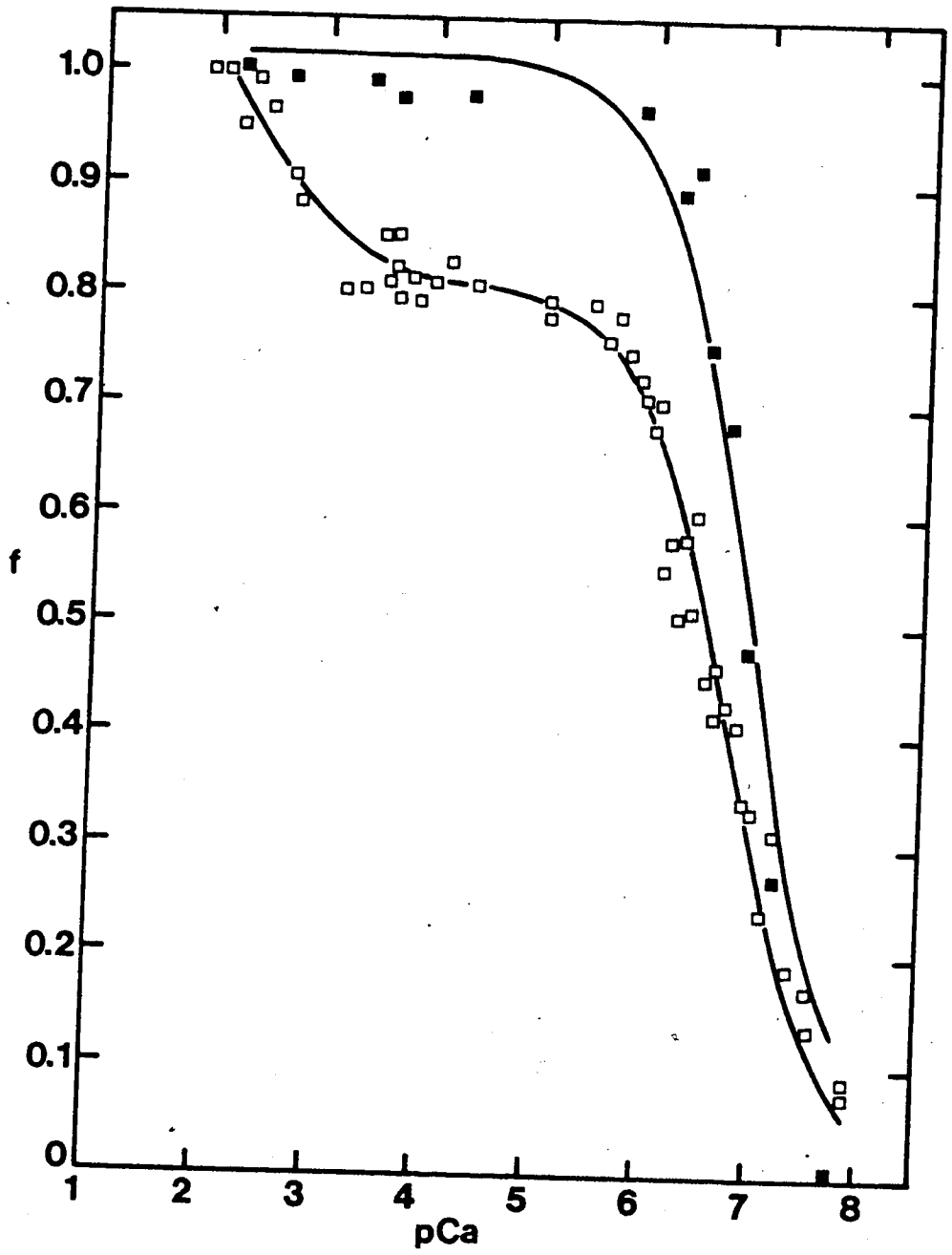


Figure 28. Ca^{2+} titration of nitrotyrosyl cTn-C at 222 nm (□) and 275 (■). The solvent system is 100 mM MOPS, 50 mM KCl and 1 mM EGTA, pH 6.87. The solid lines were derived by computer fitting to a one-term equation for the 275 nm data and a two-term equation for the 222 nm data to yield the parameters presented in Table XI.

TABLE XI

Ca^{2+} Binding Parameters for Native Proteins and Nitrated Derivatives

Protein	pH	λ*	n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})
Skeletal TN-C	7.11	222	0.634±0.016	1.73±0.18 x 10^7	0.378±0.026	3.96±0.94 x 10^3
Cardiac TN-C	6.90	222	0.763±0.015	7.86±0.69 x 10^6	0.209±0.028	1.84±0.93 x 10^3
Calmodulin	7.28	222	0.224±0.022	1.32±0.40 x 10^7	0.764±0.025	1.10±0.13 x 10^4
Nitrated skeletal TN-C	7.04	222	0.639±0.045	4.58±0.95 x 10^6	0.346±0.048	2.71±1.71 x 10^4
Nitrated skeletal TN-C	7.04	275	0.881±0.003	7.41±0.85 x 10^6	_†	_†
Nitrated cardiac TN-C	6.87	222	0.807±0.012	5.81±0.40 x 10^6	0.280±0.085	2.61±2.02 x 10^2
Nitrated cardiac TN-C	6.87	275	1.075±0.027	8.78±1.32 x 10^6	_†	_†
Nitrated calmodulin	7.04	222	0.359±0.026	5.96±1.40 x 10^6	0.610±0.032	8.54±1.68 x 10^3
Nitrated calmodulin	7.04	275	0.705±0.026	9.50±1.30 x 10^5	0.264±0.041	7.50±3.69 x 10^3

* This is wavelength at which the CD signal was monitored.

† No transition detected.

for the nitrotyrosyl chromophore, one can detect only Ca^{2+}-binding to the high affinity sites. This is a reflection of the known location of the tyrosine residues in these proteins. In sTn-C, the two tyrosyl residues are found in position 10 and 109, of which only tyrosine 109 is in a Ca^{2+}-binding region. The high affinity Ca^{2+} induced transition in $[\theta]_{275\ nm}$ is consistent with the assignment of this tyrosyl residue to the high affinity binding site III.

CTn-C has one tyrosine residue in each high affinity binding site in addition to tyrosine 5, which is not located in a Ca^{2+}-binding region. This indicates that changes in $[\theta]_{275\ nm}$ will reflect the environmental alterations of the nitrotyrosyl chromophores in these binding sites, consistent with the apparent affinity constant determined by monitoring $[\theta]_{275\ nm}$ ($8.78 \times 10^6\ M^{-1}$ at pH 6.87).

An analogous situation occurs with calmodulin where the two tyrosine residues are located at positions in the primary sequence which are equivalent to sites III and IV. The CD titration apparently confirms this homology to cTn-C but also picks up a contribution from a class of low affinity site(s). This may represent the non-specific Ca^{2+}-binding detected when monitoring $[\theta]_{222\ nm}$ in each of these proteins. The difference observed here in nitrotyrosyl sensitivity between cTn-C and calmodulin may reflect the different sensitivities of tyrosines 150 and 138 in these proteins detected by ^1H NMR. In cTn-C, the spectral changes induced in the tyrosine 150 resonances (and those of tyrosine 5) are complete when 2 moles of Ca^{2+} are bound (evidence presented later). However, tyrosine 138 in calmodulin is sensitive to the binding of all 4 moles of Ca^{2+} (Seamon, 1980). Thus in calmodulin this residue appears to be sensitive to more distant conformationsl changes,

as well as those associated solely with binding to the high affinity sites, in distinction to the case with tyrosine 150 in cTn-C.

B. UV DIFFERENCE SPECTROSCOPY

Just as Ca^{2+}-binding affects the optical activity of the nitrotyrosyl chromophore in these homologous Ca^{2+}-binding proteins, spectral changes are observed in their UV absorption spectra when Ca^{2+} is bound. These perturbations are conveniently detected by difference spectroscopy, demonstrated in Figure 29, which presents typical Ca^{2+}-induced absorption difference spectra for nitrated cTn-C, sTn-C and calmodulin at pH values close to neutrality.

The UV spectrum of nitrotyrosine contains absorption bands near 280 nm as well as bands at 355 nm and 422 nm (DiBello and Griffin, 1975). The two longer wavelength transitions reflect the ionization state of the residue. Absorption at 425 nm is due to the ionized nitrophenolate anion, while the neutral protonated chromophore absorbs at 360 nm. In the nitrated proteins near pH 7, both species exist and Figure 29 demonstrates Ca^{2+}-induced perturbation of each absorption band. These are similar for cTn-C and sTn-C; a weakly positive band with an absorption maximum near 360 nm (protonated species) and a negative band at about 440 nm which represents the ionized residue. These spectra resemble those generated upon ligand binding to staphylococcus nuclease and were interpreted in terms of a blue shift in the long wavelength absorption peak of nitrotyrosine (DiBello and Griffin, 1975). Such a blue shift is usually characteristic of an increase in the polarity of the chromophore's microenvironment (Donovan,1969).

The positive peak at 385 nm in the difference spectrum generated

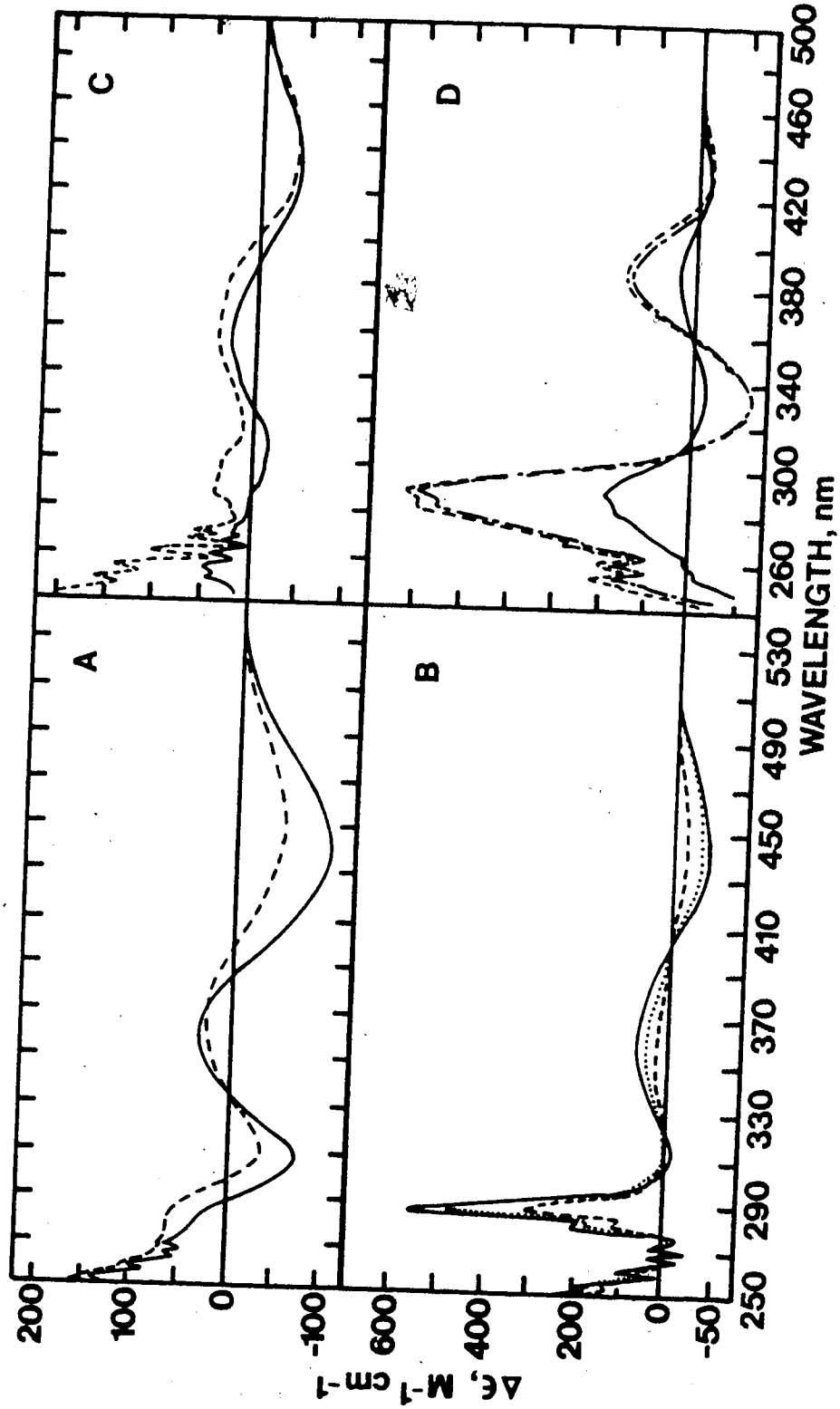


Figure 29. Absorption difference spectra of nitrated proteins. Panel A, 2.8 nitrotyrosine/cTn-C; panel B, 0.4 nitrotyrosine/cTn-C; panel C, fully nitrated sTn-C; panel D, fully nitrated calmodulin. The proteins were dissolved in 100 mM MOPS, 50 mM KCl, 1 mM EGTA at pH values close to neutrality.
Panel A: pH of solution is 6.85. (———) pCa^{2+} 4.69, (- - -) pCa^{2+} 6.66.
Panel B: pH of solution is 7.10. (———) pCa^{2+} 7.00, (·····) pCa^{2+} 6.16, and (———) pCa^{2+} 3.61.
Panel C: pH of solution is 7.04. (———) pCa^{2+} 6.30, (- - -) pCa^{2+} 3.51
Panel D: pH of solution is 7.04. (———) pCa^{2+} 6.20, (- - -) pCa^{2+} 3.38, and (-·-·-) pCa^{2+} 3.82.

by Ca^{2+} in the nitrotyrosyl chromophore of calmodulin indicates a large red shift has occurred in the absorption peak of the neutral chromophore (Figure 28, D). This result resembles that previously demonstrated for dinitrated calmodulin (Richman and Klee, 1979). The use of calmodulin selectively nitrated at tyrosine 99 or 138 allowed these authors to establish that the Ca^{2+}-induced perturbation occurred almost entirely at the level of tyrosine 138 where a red shift of 9 nm was observed. This residue possesses a pka of 8.6 and 8.1, in the presence and absence of Ca^{2+}, respectively (Richman, 1978). At pH 7.0 where our difference spectra were measured, this nitrotyrosyl residue would be almost completely protonated. Thus we detect Ca^{2+}-perturbation primarily at this absorption band. In apo-cTn-C and apo-sTn-C, the pka's of the nitrotyrosyl residues are 7.6 and 7.4 respectively. Therefore at neutral pH a significant fraction of these residues would be ionized. It is their response to Ca^{2+} which is presumably demonstrated in the large difference peak at 440 nm observed in cTn-C and sTn-C, compared to calmodulin. Since it is not possible to selectively modify the tyrosyl residues in cardiac or skeletal Tn-C, the relative contributions of the chromophores in these spectral perturbations cannot be easily assessed.

The spectral changes induced in the nitrotyrosyl chromophores of these proteins by Ca^{2+}-binding are apparently opposite in nature to those occurring in the tyrosyl chromophores of the native proteins. Ca^{2+}-binding to cTn-C and sTn-C induces a red shift in the tyrosine absorption band, while a small blue shift is seen upon Ca^{2+}-binding to calmodulin (Richman and Klee, 1978). This is to be compared with the blue and red shifts detected in the nitrotyrosyl residues of these proteins, respectively. The direction of the spectral shifts of tyrosyl

and nitrotyrosyl residues in these proteins would not necessarily be in the same direction, since the transitions involved are probably different (DiBello and Griffin, 1975). In this connection it has been reported that the long wavelength transition of 3-nitrotyrosine exhibits a blue shift in 80% methanol compared to water, in contrast to the red shift shown by tyrosine under the same conditions (Richman and Klee, 1979).

In the fully nitrated proteins a difference peak near 290 nm is also detected which represents the nitrotyrosyl chromophore. Although this peak is small in the case of cTn-C and sTn-C compared to that obtained for calmodulin, it represents the transition in which a substantial increase in optical activity is monitored as Ca^{2+} is bound. This absorption difference peak is much larger for calmodulin ($\Delta\varepsilon_{290}$ = 600 M^{-1} cm^{-1}) than in the case of either cTn-C or sTn-C. Again, this effect can be contrasted with the smaller blue shifted tyrosine difference peak ($\Delta\varepsilon_{287}$ = -170 M^{-1} cm^{-1}) observed for native calmodulin. This large alteration in nitrotyrosyl environment upon Ca^{2+}-binding is probably related to the increase in apparent pka of nitrotyrosine residue 138, from 8.1 to 8.6 noted when Ca^{2+} is bound (Richman, 1978). In native calmodulin, this residue appears to be extremely buried, possessing a pka of 11.9 in the presence and absence of Ca^{2+}.

It is only in the presence of Ca^{2+} that tyrosine 138 can be nitrated with TNM. In apo-calmodulin, this residue is resistant to this modification. No such effect is seen with cTn-C, where tyrosine 150 occupies a position in the primary sequence which is homologous to that of tyrosine 138 in calmodulin. This difference can probably be correlated with the absence of a tyrosine residue with an elevated pka in

apo-cTn-C. Thus the environment of tyrosine 138 in apo-calmodulin is such that it is resistant to nitration and possesses an elevated pka. Tyrosine 150 in apo-cTn-C does not posses such a buried environment and is modified with equal ease to the other residues. This difference between the apo-proteins surely represents part of the biological differences between these molecules.

CHAPTER VII

^{1}H NMR STUDIES ON cTn-C

In recent years, ^{1}H NMR techniques have been applied to the acidic calcium-binding proteins with great success. In part this is due to their small molecular weights and high solubilities which make them particularly suitable for NMR studies. The conformational changes which they undergo upon binding divalent cations can be monitored in great detail and with high resolution using these techniques.

Because of their smaller size and known X-ray structure, parvalbumins have been the focus of most investigations. Details of the ^{1}H NMR spectra have been studied (Birdsall et al., 1979; Cavé et al., 1979; Cavé et al., 1976), as well as the specific interaction with NMR detectable isotopes such as ^{43}Ca (Parello et al., 1978), ^{113}Cd (Drakenberg et al., 1978) and ^{23}Na (Grandjean et al., 1977). However the most promising technique for extracting structural information involves the use of paramagnetic lanthanide probes (ie. Yb^{3+}; Gd^{3+}) which bind tightly to the Ca^{2+}-binding sites and induce spectral shifts or linebroadening in the resonances of adjacent nuclei. In a recent study utilizing the shift reagent Yb^{3+} it was possible to derive a protein structure very similar to the X-ray one but with some modifications more appropriate for the solution structure (Lee and Sykes, 1980a, 1980b). sTn-C (Seamon et al., 1977; Levine et al., 1977) and calmodulin (Seamon, 1979; Seamon, 1980) have been extensively studied by ^{1}H NMR techniques. These studies of the solution conformations of each protein and their response to metal binding have underlined the obvious homology between them but also point to specific differences which must reflect their different physiological

roles.

The present work represents the first reported NMR investigation of cTn-C, in which the tyrosyl resonances were assigned to their sequence positions and the spectral alterations which occur during the Ca^{2+}-binding process were characterized. This study has provided a basis for a detailed comparison between the solution conformation of cTn-C and sTn-C and calmodulin (Hincke et al., 1981a).

A. Apo-cTn-C

The aromatic region of the ^1H NMR spectrum of apo-cTn-C is presented in Figure 30. This spectrum represents the ring protons from 9 phenylalanines and 3 tyrosines. The protein contains no histidine or tryptophan. There are a number of upfield shifted resonances, suggesting the presence of a large amount of teriary structure in the apo-protein.

The singlet resonance at 6.86 ppm represents the ortho and meta protons of one tyrosine (tyrosine A). This assignment, which accounts for the sharp, singlet nature of this peak (Snyder et al., 1975), is based on a number of observations. Slight increases in temperature (up to 43° C) cause this singlet to break up into two coupled doublets which shift to higher field (3,5 ortho protons) and lower field (2,6 meta protons) with increasing temperature. The assignment to ortho and meta protons was based upon the relative magnitudes of the Nuclear Overhauser effect (NOE) when each was decoupled. Irradiation at the meta resonance produces a larger NOE at the ortho resonance than the reverse experiment (Birnbaum and Sykes, 1978). This singlet is not coupled to any other peak in the aromatic region of the spectrum, as revealed by difference spectroscopy.

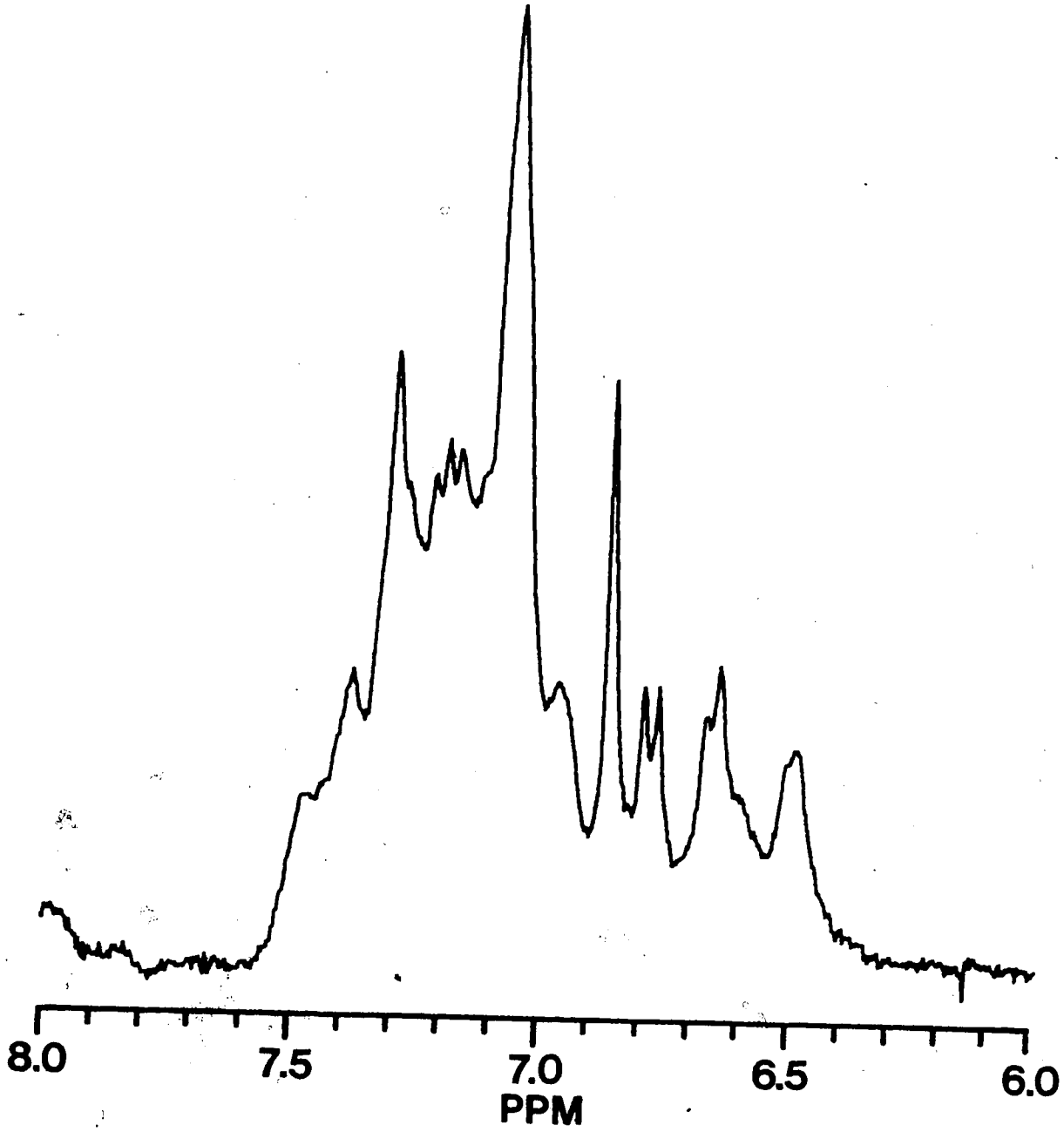


Figure 30. Aromatic region of the 270 MHz ^{1}H NMR spectrum of apo-cTn-C. 0.15 M KCl, 25 mM MOPS, 1 mM EDTA, pH 6.8.

The doublet resonance at 6.77 ppm was also assigned to a tyrosine residue (tyrosine B), based upon its decoupling pattern (see Figure 31). It is coupled to a doublet peak at 7.06 ppm, revealed by difference spectroscopy. The resonance at 7.06 ppm appears negative in the difference spectrum, which represents the difference obtained by subtracting the spectrum with the irradiation of resonance from that with the irradiation at 6.77 ppm, because of the negative NOE effect concomitant with decoupling. This can be compared to the idealized spectrum in Figure 6. Decoupling experiments which yielded a pattern of two coupled doublet resonances were unequivocally assigned to tyrosine resonances. The relative magnitudes of the NOE in these decoupling experiments suggested that the 6.78 ppm resonance represents the ortho protons, while the more downfield doublet represents the meta protons.

Increasing the temperature does not perturb the chemical shift of the tyrosine B resonances, contrary to the results with tyrosine A. This suggests that the environment of tyrosine A is within the protein and sensitive to thermally induced conformational changes. These experiments were performed on the apo-cTn-C which is known to be less stable to thermal unfolding than the Ca^{2+}-form (McCubbin et al., 1980). Tyrosine B, on the other hand, must have an environment characterized by a high degree of exposure to solvent in the apo-protein. Its chemical shifts are most similar to that of tyrosine in a random coil peptide and are unaffected by small alterations in the protein structure when the temperature is increased.

The resonance at 6.64 ppm is believed to contain the ortho protons of tyrosine C. Laser photo CIDNP experiments on apo-cTn-C at pH 6.0 (Figure 32) indicate the presence of tyrosine ortho protons at this

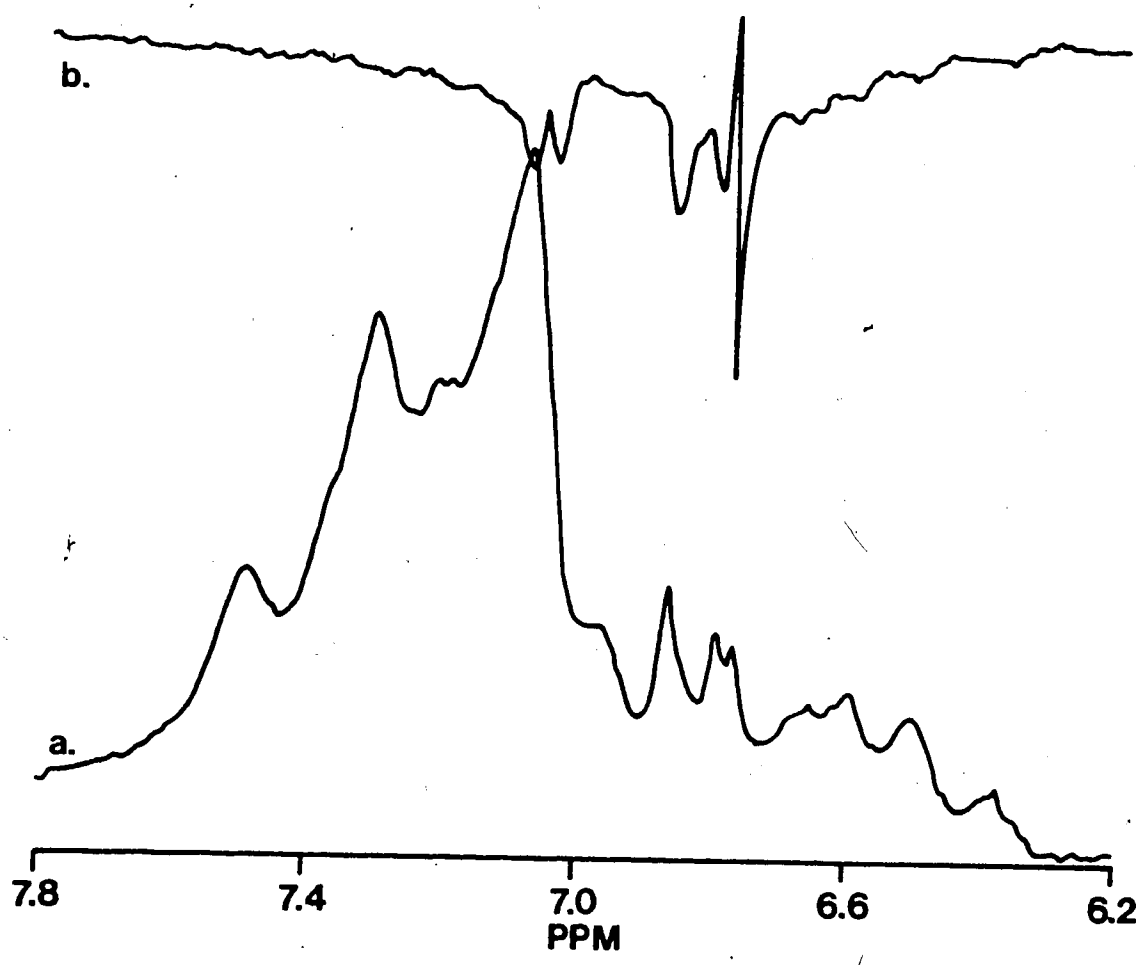


Figure 31.

A. Aromatic region of the 270 MHz ^{1}H NMR spectrum of cTn-C in 0.15 M KCl, 20 mM MOPS, pH 6.8 ∿0.6 Ca/Tn-C.

B. Difference NMR spectrum with decoupling applied at 6.77 ppm to locate the meta protons of tyrosine B. The control spectrum was obtained with the decoupler pulse applied at 5.92 ppm.

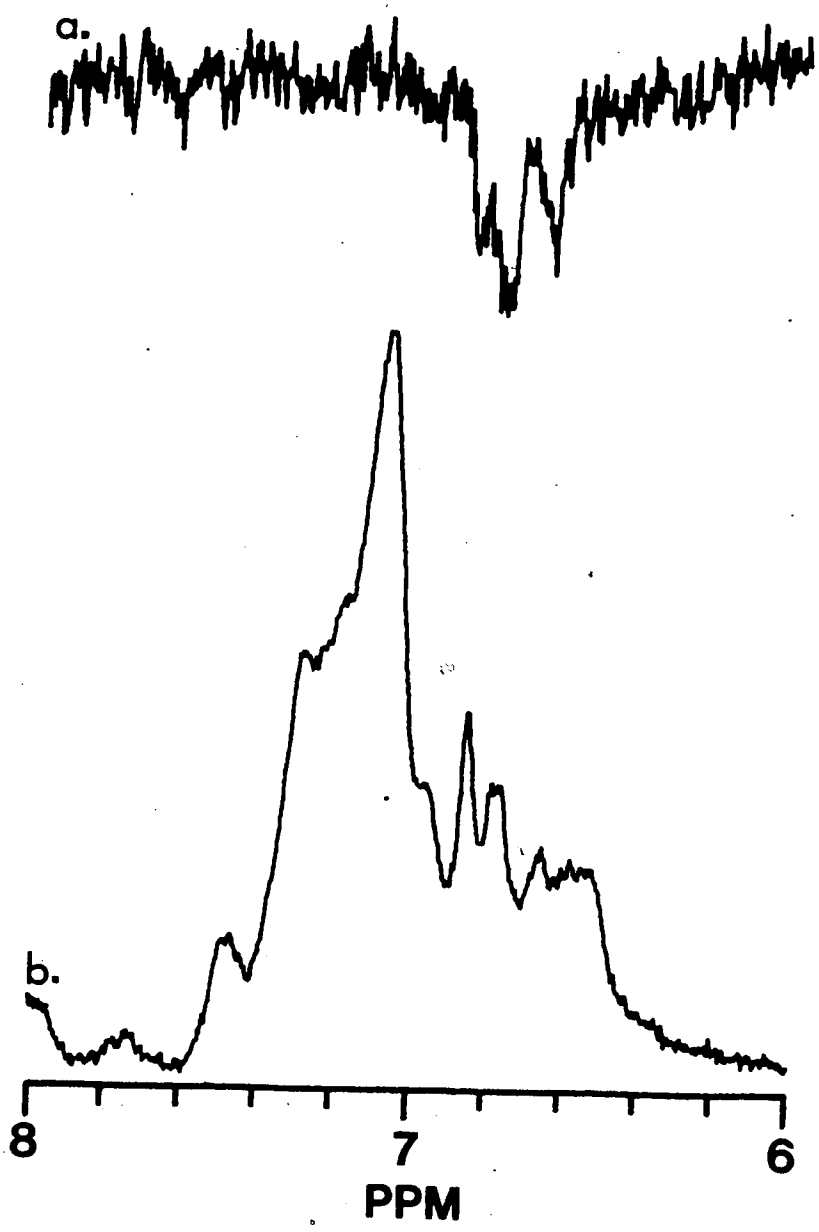


Figure 32.

A. Laser photo CIDNP difference spectrum of apo-cTn-C (1.1 mM). The difference spectrum represents the subtraction of the dark spectrum from the light spectrum (12 scans each).

B. The dark spectrum. The solvent is 0.15 M KCl, 25 mM MOPS, pH 6.04, 0.2 mM FMN.

position and also confirm the assignment of the ortho protons of tyrosine residues A and B. The CIDNP experiment demonstrates that although tyrosine B is most exposed, tyrosine A and C are also exposed to a certain extent. It should be noted, however, that comparison with results obtained with the free tryosine amino acid indicates that these tyrosines are all relatively unexposed.

During the course of pH titration of apo-cTn-C the tyrosine-C resonance at 6.64 ppm titrates upfield with the ortho resonances of the other tyrosines to a final position typical of the phenolate species in the denatured protein (see below). Decoupling experiments with irradiation at 6.64 ppm do not reveal any corresponding meta protons, even in the difference spectrum. This may indicate that they overlap the ortho protons somewhat, although not to the same extent as tyrosine A, where a sharp singlet is observed.

The aliphatic region of the apo-protein spectrum is presented in Figure 33. There are a number of upfield shifted peaks, between -0.05 and 0.3 ppm. These represent methyl groups whose environment is influenced by aromatic residues and indicate there is a well defined tertiary structure present in the apo-protein.

1. pH Titration of Apo-cTn-C

Increasing the pH of apo-cTn-C does not affect the spectrum over the pH range 7.5 to 9.5 (Figure 34). Between pH 10 and 10.2, large alterations in the phenylalanine resonances were observed as the protein began to unfold. The pKa's of the tyrosine residues, determined by the upfield titration of the ortho protons, were all about 10.5. These pKa's are similar to the value of 10.8 obtained from the spectrophotometric titration of apo-cTn-C. This would be a better indication of protein

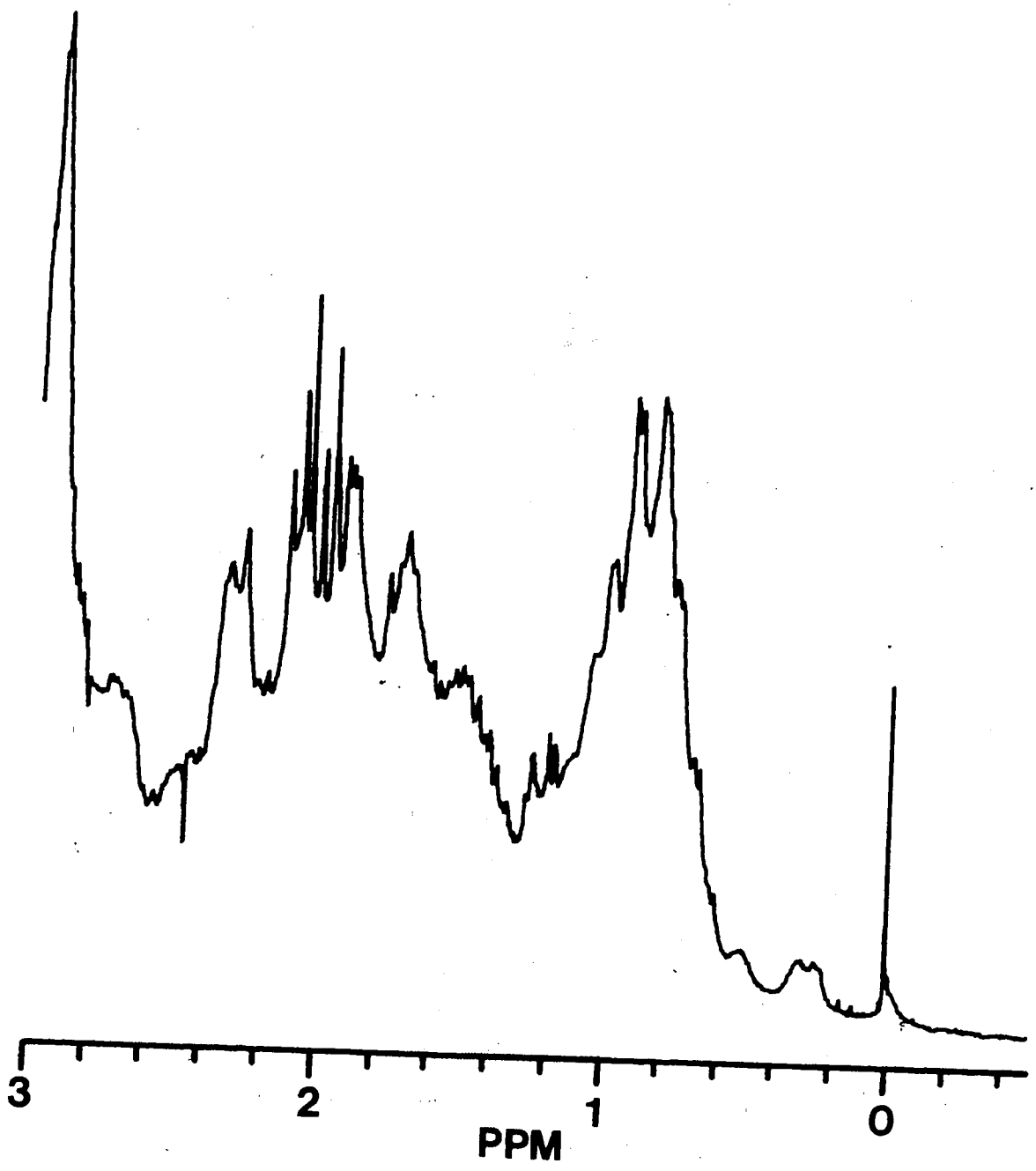


Figure 33. Aliphatic region of the 270 MHz ^{1}H NMR spectrum of apo-cTn-C. Solvent is 0.15 M KCl, 25 mM MOPS, 1 mM EDTA, pH 6.8.

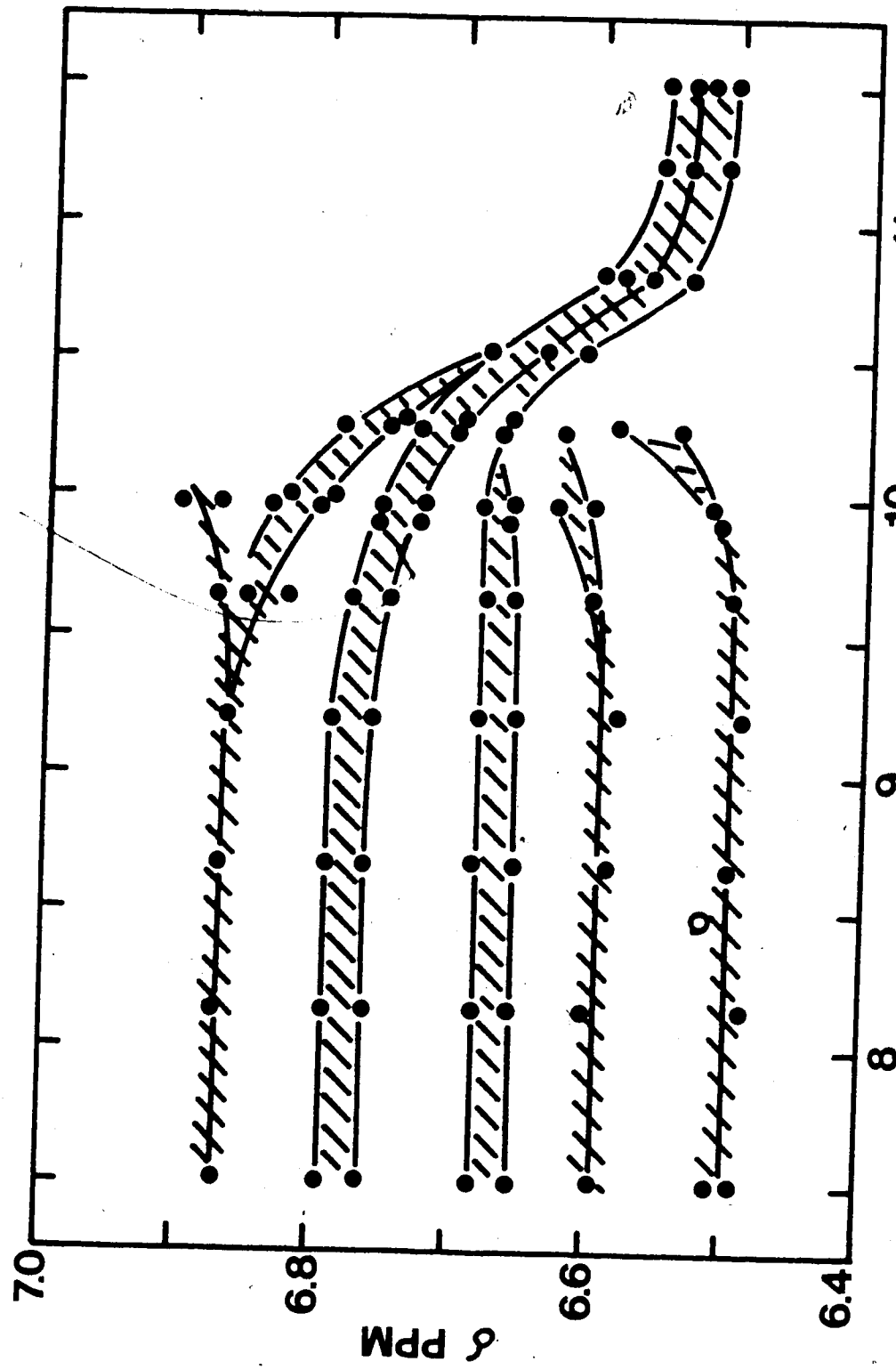


Figure 34. pH titration of apo-cTn-C. The chemical shifts of some resonances in the aromatic region of the 270 MHz ^1H NMR spectrum are plotted as a function of pH. The solvent was 0.15 M KCl, 25 mM MOPS, 1 mM EDTA. The crosshatching gives an indication of peak width.

unfolding over this pH range than tyrosine pKa. It did not appear that tyrosine B had a lower pKa, typical of an exposed tyrosine residue (10.1).

During the titration, the ortho/meta resonances of tyrosine A at 6.87 ppm split first into an apparent triplet and then two doublets as the meta resonance titrated downfield and the ortho upfield. The tyrosine B resonance at 6.78 ppm titrated upfield as a typical ortho resonance, as did the less well resolved ortho protons of tyrosine C at 6.67 ppm. The upfield peak at 6.5 ppm and the shoulder at 6.6 ppm also began to move as the protein was denatured, shifting downfield to chemical shifts typical of random coil phenylalanine residues.

The aromatic spectrum of apo-cTn-C was also investigated between pH 5.4 and 6.7, where the pH effects observed on the tyrosyl difference spectra and ellipticity of the apo-protein would be anticipated to be evident. This is shown in Figure 35. Alterations in the spectra can be seen over this pH range. The most dramatic effect is the decreasing peak height of phenylalanine resonances at 7.34 ppm as the pH is lowered. This corresponds to a decrease in solvent exposure for phenylalanine rings (Seamon, 1981) and is related to the increased α-helix detected by CD measurements. The singlet resonance of tyrosine A shifts upfield slightly (~.02 ppm) as the pH is lowered; a smaller but similar effect is seen at the tyrosine B ortho resonance. There may be an effect at the tyrosine C meta resonance (presumed to be upfield of the ortho resonance) or the alterations near 6.5 ppm may simply reflect a perturbation of the upfield shifted phenylalanine resonances. These small tyrosine effects as the pH is lowered apparently reflect the large UV difference spectra which are induced over the same pH range.

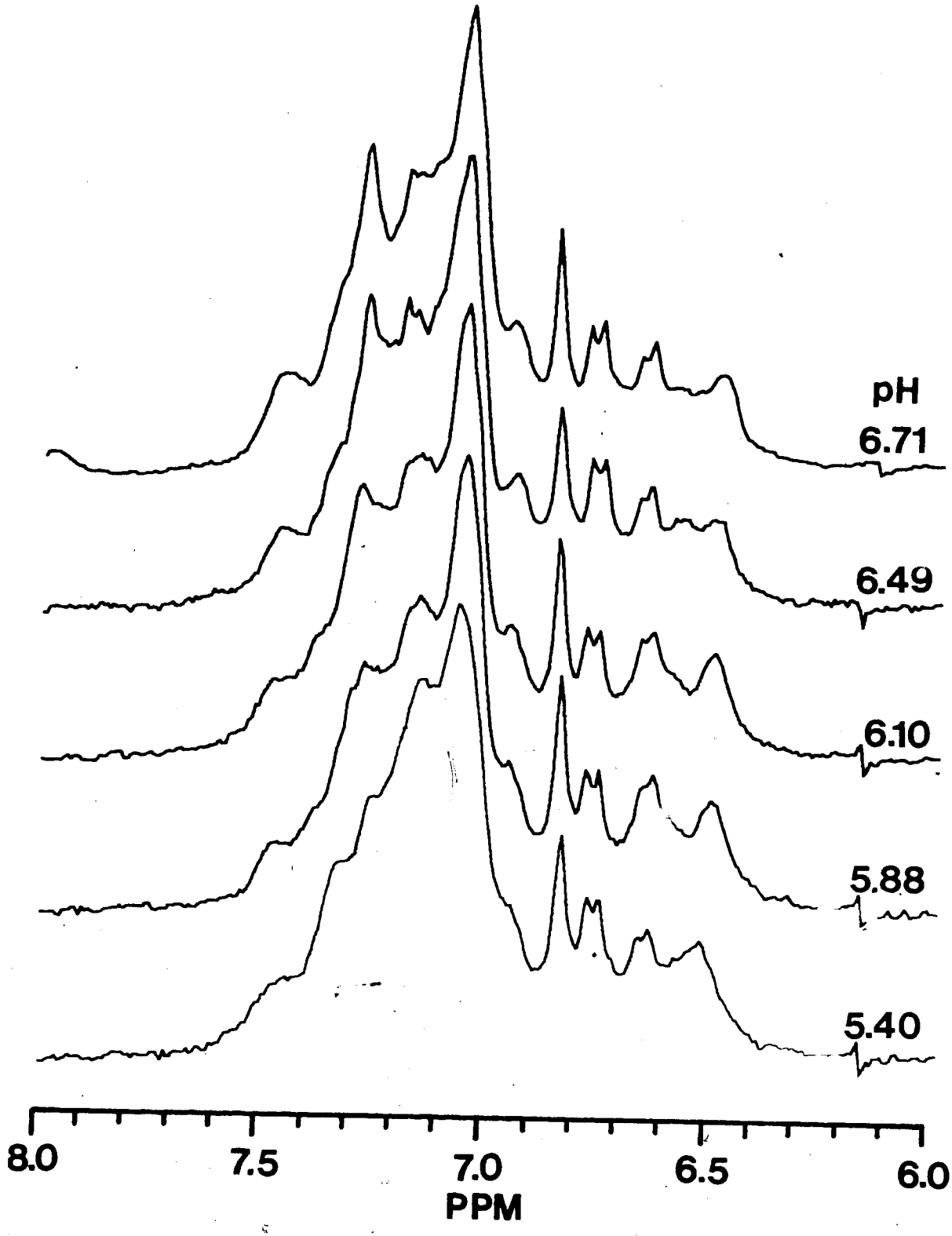


Figure 35. Aromatic region of the 270 MHz ^{1}H NMR spectrum of apo-cTn-C as a function of pH between pH 5.4 and 6.7. The solvent was 0.15 M KCl, 25 mM MOPS, 1 mM EDTA. Each spectrum is 256 scans.

B. Ca^{2+} SATURATED cTn-C

The aromatic spectrum of the calcium saturated protein is shown in Figure 36. Dramatic alterations have occurred in the phenylalanine envelope between 6.9 and 7.4 ppm with Ca^{2+}-binding. In addition, a number of overlapping doublets are observed around 6.8 ppm and there are two well-defined upfield resonances at 6.60 and 6.39 ppm. Decoupling experiments (without the NOE) demonstrated that the central doublet at 6.79 is coupled to a doublet at 7.06 ppm. For assignment purposes this was called tyrosine D. A connectivity was also revealed between the doublet at 6.72 ppm and that at 6.83 ppm (tyrosine E). Two other connected doublets were found under the peaks at 6.39 and 6.60 ppm (tyrosine F).

These decoupling experiments were carried out at temperatures ranging from 27° C to 80° C (Figure 38). At higher temperatures, the phenylalanine peaks obscuring the tyrosine doublets at 6.60 and 6.39 ppm begin to shift to lower field as the structural elements making up their environment are lost. At 65° C, each of these peaks has split into two doublets of which the upper field ones represent the coupled tyrosine doublets. At 80° C only a tyrosine doublet remains at 6.60 ppm, while a phenylalanine doublet at 6.43 ppm has clearly shifted downfield away from the tyrosine doublet at 6.39 ppm. It is obvious that a high degree of structure still remains at 80° C. As well, the chemical shifts of the two coupled tyrosine doublets at 6.60 ppm and 6.39 ppm are invariant throughout this temperature range.

Only small changes are seen at the other tyrosine resonances. Decoupling experiments at the elevated temperatures reveal that the meta resonance of tyrosine D remains at 7.06 ppm at least to 65° C. At 80° C

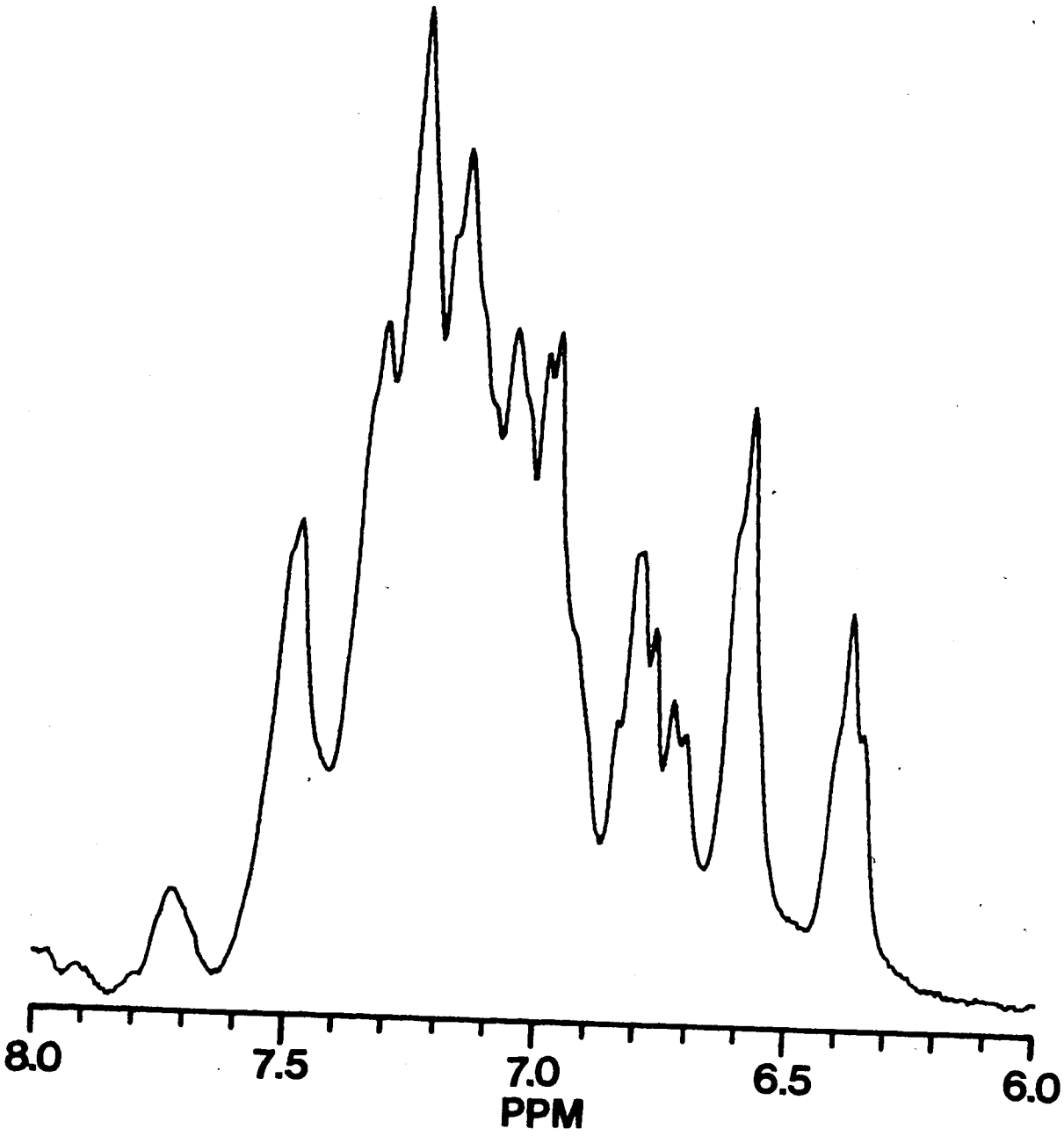


Figure 36. Aromatic region of the 270 MHz ^{1}H NMR spectrum of Ca^{2+}-saturated cTn-C in 0.15 M KCl, 25 mM MOPS, pH 6.8, 1 mM $CaCl_2$.

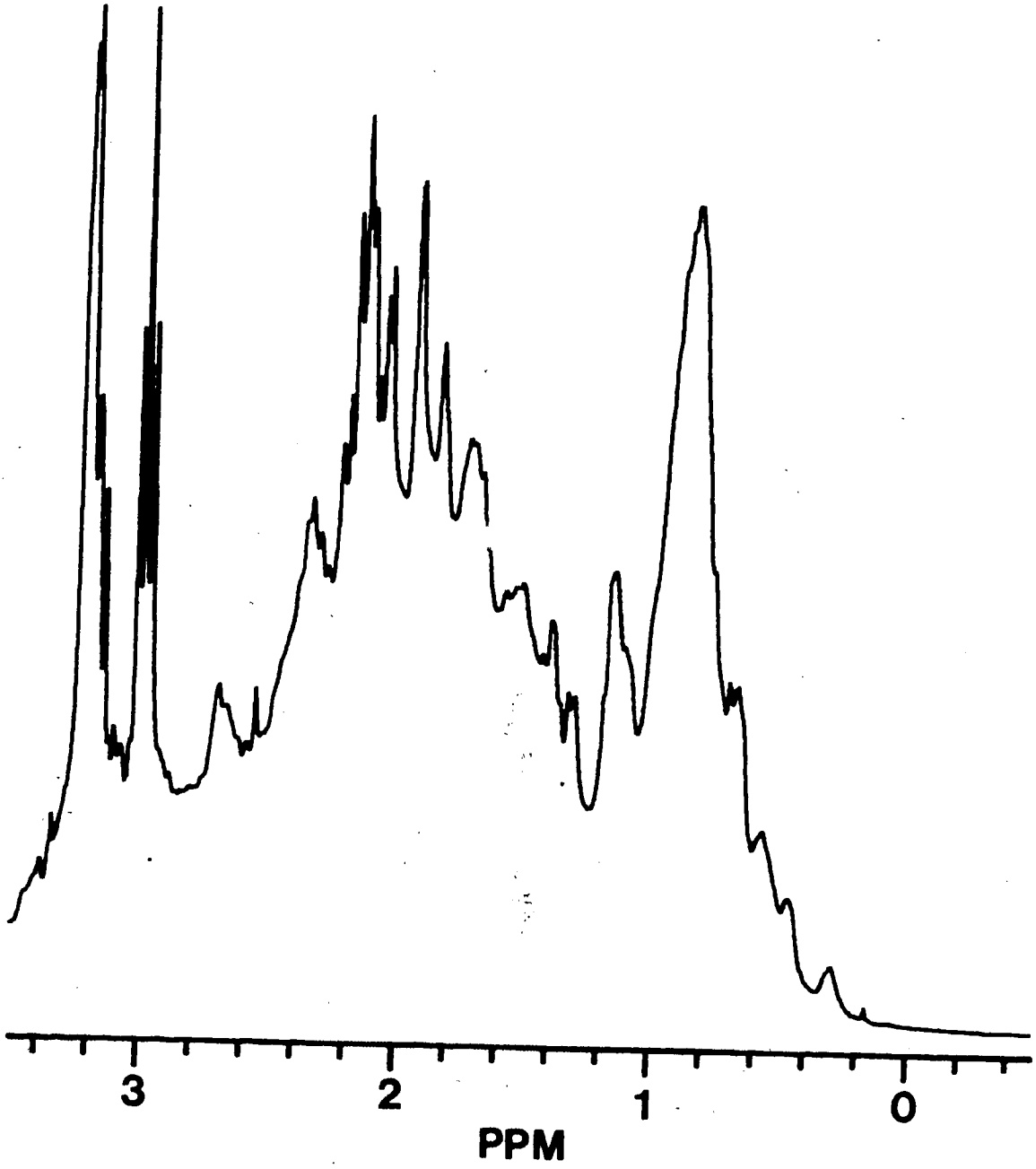


Figure 37. Aliphatic region of the 270 MHz ^1H NMR spectrum of Ca^{2+}-saturated cTn-C in 0.15 M KCl, 25 mM MOPS, pH 6.8, 1 mM $CaCl_2$ (no DSS).

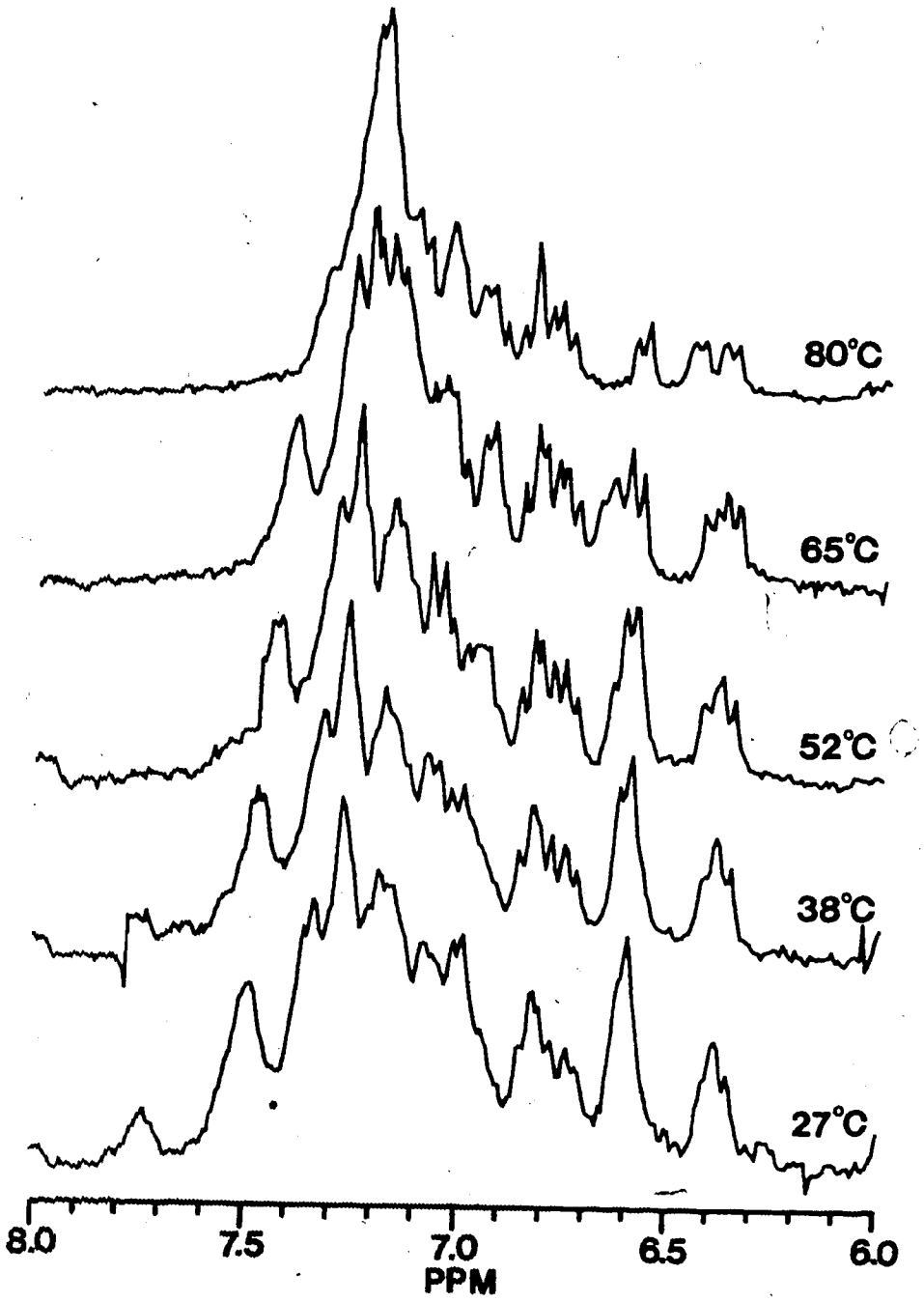


Figure 38. Thermal melting experiment of calcium-saturated cTn-C. cTn-C (1.1 mM) was dialyzed versus 0.15 M KCl, 25 mM MOPS, pH 6.78, 1 mM $CaCl_2$ prior to lyophilization. The sample was then dissolved in D_2O to the original volume. Each spectrum represents 256 scans.

it has shifted downfield to 7.10 ppm, perhaps beginning to assume a random coil chemical shift (meta 7.15, ortho 6.86; Bundi and Wuthrich, 1979).

The aliphatic spectrum of the Ca^{2+}-saturated protein is shown in Figure 37. Many changes have occurred with calcium-binding. In particular, some upfield methyl resonances around 0 ppm in the apo-protein have shifted downfield.

1. pH Titration of Ca^{2+}-Saturated cTn-C.

The pH titration of the calcium-saturated protein is presented in Figure 39. The chemical shifts of some of the peaks in the aromatic spectrum are plotted as a function of pH in Figure 40.

Increasing the pH to 10.02 causes the overlapping tyrosine doublets at 6.8 ppm to change, forming a large peak at 6.75 ppm. This represents the ortho protons of tyrosine D and E and the meta resonance of tyrosine E. The singlet nature of this peak at pH 10.7 indicates that the ortho and meta resonances of tyrosine E are overlapping and suggests that they were originally reversed. The ortho resonance is at 6.83 ppm and titrates further upfield with increasing pH than the meta resonance at 6.72 ppm. The doublet nature of this resonance at higher pH's indicates that the ortho and meta resonances of tyrosine E are no longer overlapping. Since the meta resonance does not appear as the ortho resonance titrates upfield, it might be broadened under these conditions. At 45° C the meta peak does appear as a shoulder on the downfield side of the other proton resonance during the singlet to doublet transition. The ortho proton peaks titrate upfield with increasing pH, yielding an apparent pKa of 10.6 - 10.7 for each tyrosine, a value not very different from the pKa of all three tyrosines in the apo-protein.

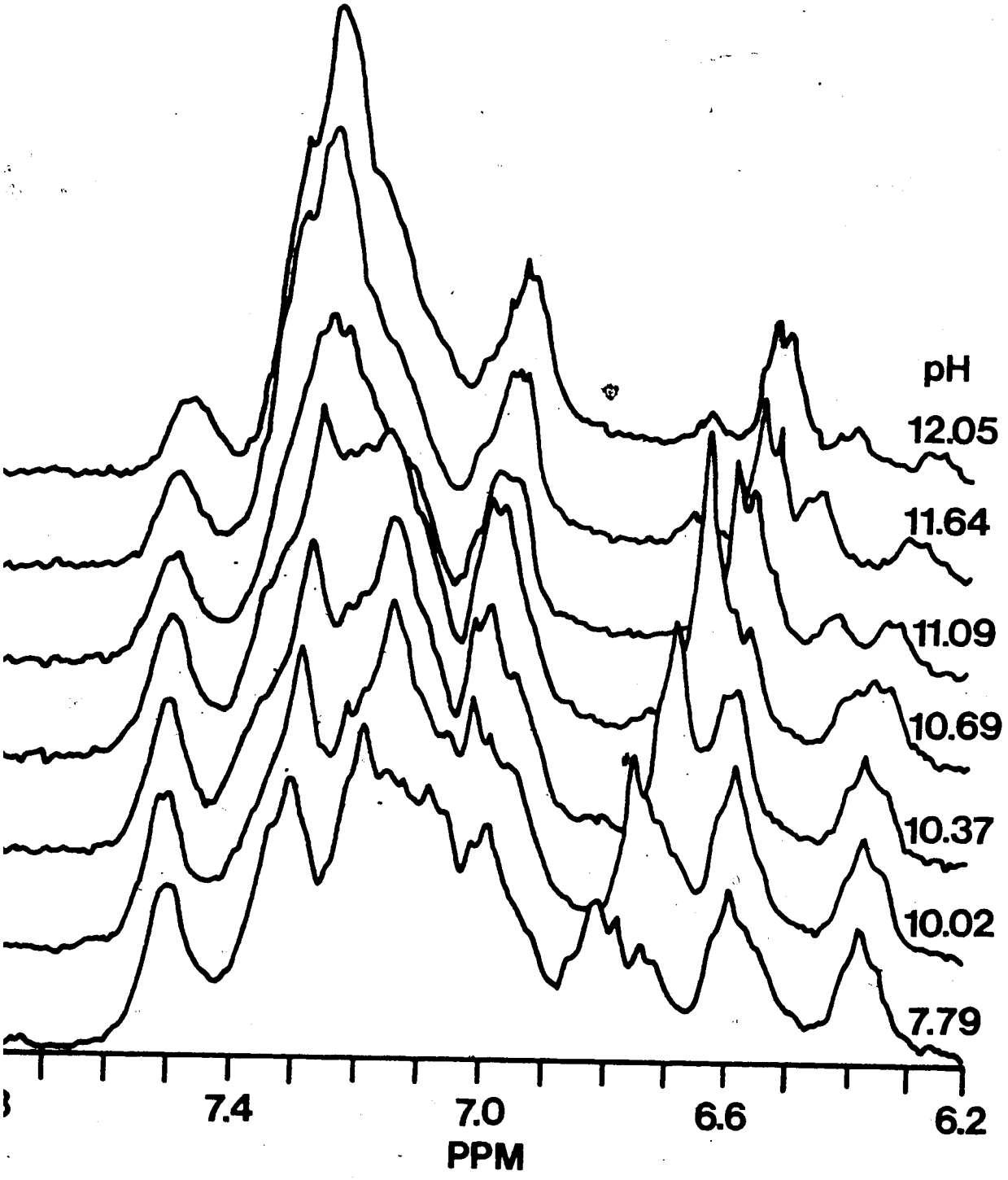


Figure 39. pH titration of calcium-saturated cTn-C. cTn-C (1.1 mM) in 0.15 M KCl, 25 mM MOPS, 2 mM $CaCl_2$. The spectra are presented at increasing pH.

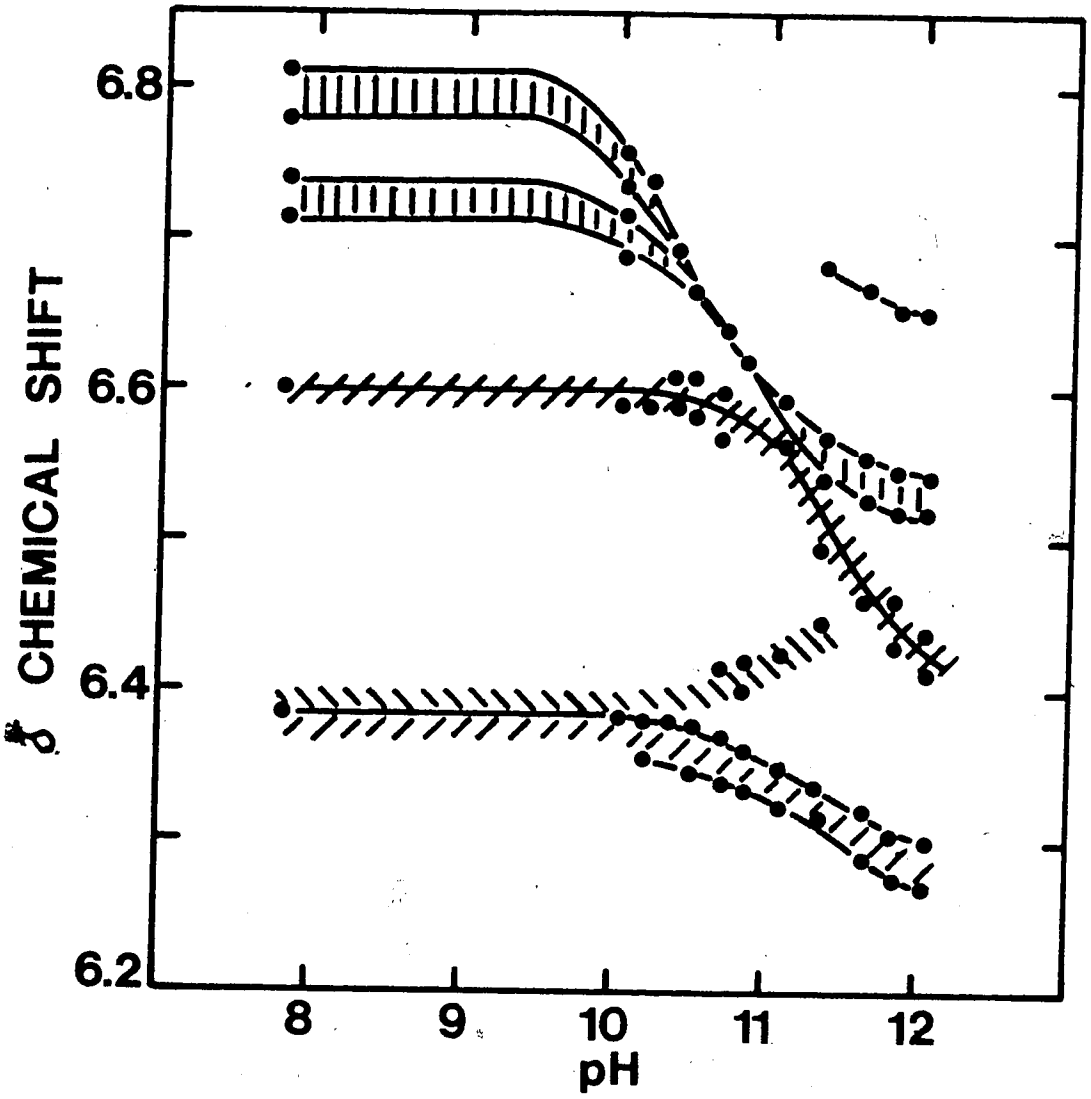


Figure 40. pH titration of calcium-saturated cTn-C. The chemical shifts of some aromatic peaks are plotted as a function of pH. The cross-hatching gives an indication of the peak width, i.e., each point represents a peak in a doublet.

It appears that these two tyrosine residues are not particularly buried during the Ca^{2+}-binding process, or that the structural elements surrounding them are not particularly stabilized by bound calcium.

However, the resonances at 6.39 and 6.60 ppm, containing the ortho and meta protons of tyrosine F, are largely unaffected by increasing pH until about pH 10.7. At this point, the tyrosine doublets begin to titrate upfield while the overlapping phenylalanine doublets shift downfield towards chemical shifts more typical for phenylalanine ring protons (7.0 - 7.4 ppm). This must be a consequence of denaturation and loss of the specific environments of the phenylalanine residues which have produced these many upfield shifted resonances. However, it is noteworthy that the environment of tyrosine F must be exceedingly stable under these alkaline conditions (also seen at high temperatures) since both ortho and meta resonances titrate upfield and remain distinct from the random coil ortho and meta phenolate resonances observed for tyrosines D and E. A pKa of 11.4 - 11.5 can be derived from this titration curve. The magnitude of the pH induced shift for these resonances can be used to assign the ortho and meta resonances, since the meta resonance will undergo a much smaller upfield shift than the ortho resonance, when the tyrosine is ionized. On this basis, the doublet at 6.39 ppm is assigned to the meta resonance and that at 6.60 to the ortho resonance.

The decreasing intensity of the tyrosine F ortho and meta doublets observed in the last stages of the titration suggests that the native structural element containing this tyrosine is in slow exchange with a denatured form at these elevated pH's. The small doublet observed at 6.65 ppm in the pH 12.04 spectrum titrates in parallel with the meta doublet of tyrosine F, suggesting that there is also a slow exchange be-

tween the native structure and some intermediate form (i.e., $N \rightleftharpoons N'$).

C. ASSIGNMENT OF TYROSINE RESONANCES

There are two aspects to assigning the tyrosine resonances elucidated in the previous section. First, the resonances in the apo-protein must be aligned with those in the calcium-bound form, and secondly, these must be assigned to the specific residues within the protein sequence. In this task, some use will be made of the large degree of sequence homology which exists between cardiac Tn-C and two other proteins - rabbit skeletal Tn-C and calmodulin.

Calmodulin possesses two tyrosines which are found within calcium-binding sites III and IV, the high affinity Ca/Mg sites. Two of the tyrosines of cardiac Tn-C are homologous to those of calmodulin; the third is found near the amino terminus of the protein at residue 5. In skeletal Tn-C, one of the tyrosine residues is at the homologous position in site III and the other is found at amino acid position 10.

Skeletal Tn-C (Seamon et al., 1977) and calmodulin (Seamon, 1980) have been extensively studied by NMR techniques. Table 12 summarizes the tyrosine assignments for these proteins and also our proposed assignments for cardiac Tn-C.

We have assigned tyrosine B in the apo protein to tyrosine D in the calcium-bound form because of the virtual equivalence of their chemical shifts. During the calcium titration (Figure 42) of cTn-C, it may be seen that the ortho resonance only changes slightly as calcium is bound. This in turn allows us to assign this residue to tyrosine 111 in site III, one of the high affinity Ca/Mg binding sites. Examination of Table I demonstrates that the homologous tyrosines in site III of

TABLE XII

Chemical Shifts of Tyrosine Resonances in Homologous Calcium-Binding Proteins

	apo			plus Ca^{2+}		
	ortho 3,5	meta 2,6	pKa	ortho 3,5	meta 2,6	pKa
Calmodulin[a,f]						
site III (99)	6.82	7.31	10.4	6.76	7.29	10.1
site IV (138)	6.70	6.72	11.9	6.55	6.36	12.0
sTn-C[b]						
N-terminal (10)	6.82	7.05[c]	10.4	6.85	7.12	NR[e]
site III (109)	6.84	7.12	10.4	6.85	7.27	NR
CB-9[d]						
site III (109)	6.83	7.06	10.6	6.64	6.51	NR
cTn-C						
N-terminal (5)	6.64	? (C)[g]	10.5	6.83	6.72(E)[g]	10.8
site III (111)	6.77	7.06(B)	10.5	6.79	7.06(D)	10.8
site IV (150)		6.86(O/M)(A)	10.5	6.60	6.39(F)	11.4

[a] Seamon, 1980.

[b] Seamon et al., 1977; Levine et al., 1977.

[c] The assignment of these coupled resonances to tyrosine 10 or 109 has not been made.

[d] Birnbaum and Sykes, 1978.

[e] NR, not reported.

[f] Calmodulin pKa's are from Klee, 1977.

[g] Tyrosine labelling used in text.

calmodulin and sTn-C possess chemical shifts resembling free tyrosine in solution and also that these chemical shifts are relatively insensitive to calcium-binding (Seamon, 1979; Seamon, 1980). These properties are shared by tyrosine (B, D). The invariance of its chemical shift with calcium binding and insensitivity to temperature increases in the apo- and calcium-bound proteins suggest that it is a fairly exposed residue. This is also implied by its pKa value which does not change markedly when calcium is bound.

It is of interest to note that the tyrosine residue in CB-9, a 52 residue CNBr fragment of sTn-C containing only site III, possesses chemical shifts in the absence of calcium which are virtually identical to those of our assigned site III tyrosine in cTn-C. Apo-CB-9 has a very extended structure (Birnbaum and Sykes, 1978), lacking tertiary features. Its tyrosine residue is probably very exposed, suggesting, in turn, that tyrosine 111 in apo-cTn-C is similarly exposed (pKa's are similar as well). However, calcium-saturated CB-9 must have a different structure from that which it would assume when it is part of the much larger sTn-C molecule. The ortho and meta resonances of tyrosine 109 in CB-9 are reversed and have very different chemical shifts compared to calcium-saturated sTn-C. The analogies can therefore only be drawn between apo-CB-9 and apo-cTn-C.

This is true as well for a 33 residue peptide analog of site III of sTn-C recently synthesized (J. Gariepy, R.E. Reid, R.S. Hodges and B.D. Sykes, privileged communication). This peptide has been synthesized with an amide C-terminal, acetylated N-terminal and cysteine 98 has been replaced by alanine. Otherwise it is identical to residues 90 - 123 of sTn-C. NMR studies have shown that the single tyrosine in the apo-peptide possesses ortho and meta chemical shifts of 6.81 and 7.07 ppm,

respectively. These values are very similar to those of apo CB-9, and probably represent a very exposed tyrosine. The lack of phenylalanine fine structure indicates a very open structure as well.

Tyrosine F in calcium-saturated cTn-C has been assigned to residue 150 in site IV, the other high affinity Ca/Mg binding site, because of the similarity between its ortho and meta chemical shifts and those of tyrosine 138 in calcium-saturated calmodulin. sTn-C has no such analogous tyrosine for comparison. This assignment is strengthened because of the reversed assignment of the ortho and meta resonances which both cTn-C and calmodulin demonstrate (the ortho peak is downfield of the meta peak). The assignment is also supported by the elevated pKa which tyrosine F possesses in calcium-saturated cTn-C. This can be correlated with the elevated pKa of tyrosine 138 in the apo- and calcium-saturated forms of calmodulin. This assignment leads, by elimination, to the conclusion that tyrosine E corresponds to tyrosine residue 5 in calcium-saturated cTn-C.

Since none of the cTn-C tyrosine residues has an elevated pKa in the apo-protein one can conclude that the homology between site IV in cTn-C and calmodulin is not absolute. There must be considerable differences in the disposition of the tyrosine residue in site IV of apo-cTn-C and apo-calmodulin. This makes the assignment of tyrosine 150 in apo-cTn-C more difficult. Because of the dissimilarities in tyrosyl pKa's in apo-cTn-C and apo-calmodulin, one would not expect the chemical shifts of tyrosine 138 of calmodulin to be comparable to those of tyrosines A or C in cTn-C for an assignment by homology. Calcium titration experiments (see next section, Figure 42) reveal the increase in intensity at 6.60 and 6.39 ppm corresponding to calcium-binding at high affinity

site IV. This can be correlated with a decrease of intensity at 6.86 ppm (tyrosine A) and also at 6.64 ppm (tyrosine C). We tentatively favor the assignment of tyrosine A to tyrosine 150, based upon an observation made when decoupling experiments were performed at the ortho and meta resonances of tyrosine 111 (tyrosine B) in apo-cTn-C. When the decoupling is applied at these resonances, a large NOE can be seen at the tyrosine A resonance. This phenomenon can be seen quite clearly in Figure 31, and is particularly obvious when the decoupling is applied at the meta position, suggesting that tyrosine 111 and tyrosine A are close in space (i.e., occupy sites III and IV).

This argument then leads, by the process of elimination, to the assignment of the resonances of tyrosine C in the apo-protein to the N-terminal tyrosine residue at position 5. It is apparent that there is a large difference between the chemical shifts of the N-terminal tyrosine residue of sTn-C and cTn-C. Those of sTn-C are representative of an exposed tyrosine (compare to CB-9) while the tyrosine near the N-terminal of cTn-C is obviously in a specia more buried, environment which is sensitive to calcium binding. Perhaps the deletion of the first calcium-binding site in the cardiac protein allows the N-terminal region to assume a different conformation than that of sTn-C, possibly one more buried in the protein interior.

D. Gd^{3+} BROADENING EXPERIMENTS

In order to test these tyrosyl assignments, the paramagnetic relaxation reagent Gd^{3+} was utilized. Because of their similar ionic radii to calcium, but greater charge, lanthanides have been used as selective probes for calcium-binding sites (Lee and Sykes, 1980a,b; Leavis et al.,

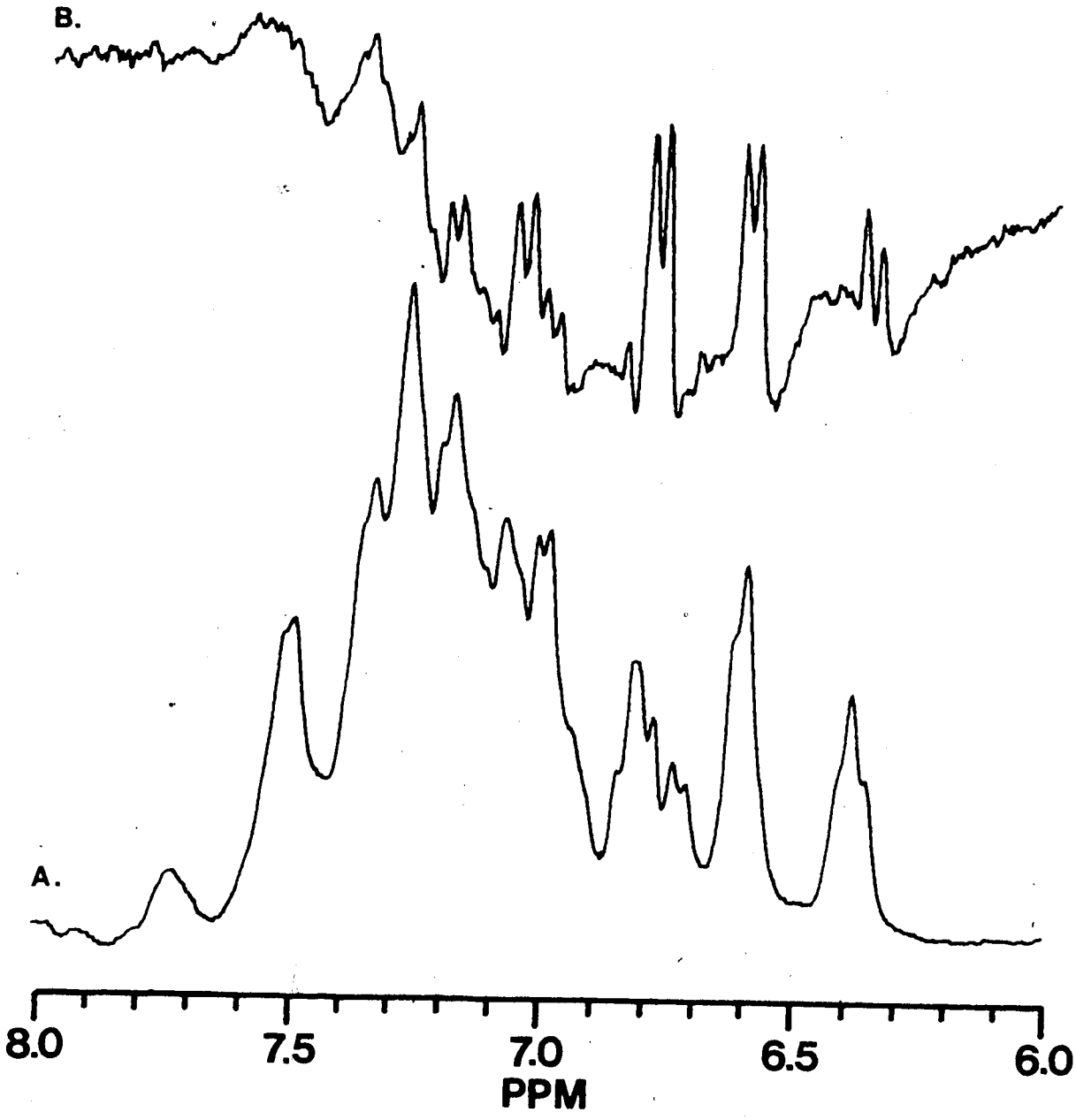


Figure 41. Gadolinium broadening experiment with calcium-saturated cTn-C. cTn-C (1.46 mM) in 0.15 M KCl, 25 mM MOPS, pH 6.8, 1 mM $CaCl_2$. Four blocks of 256 scans were summed for each spectrum. This difference spectrum represents 0.13 Gd^{3+}/cTn-C.
A. La^{3+} spectrum.
B. Difference spectrum.

1980). Gd^{3+} binding causes large perturbations in the relaxation rates of nearby protons. This effect has an r^{-6} dependence, inducing broadening of the resonances of adjacent residues, and is conveniently detected by subtracting the broadened spectrum from one to which Gadolinium has not been added. The selectively broadened resonances are then observed as sharp lines in the difference spectrum. This effect is depicted in Figure 6a.

When small aliquots of Gd^{3+} were added to calcium-saturated cTn-C, the resonances ascribed to tyrosyl residues 150 and 111 are selectively broadened. In the difference spectrum they appear as sharp doublets (Figure 41). A recent fluorescence study of terbium binding to skeletal Tn-C concluded that the lanthanides (Ho^{3+}, Tb^{3+} and Gd^{3+}) were specific probes for the high affinity Ca/Mg sites (Leavis et al., 1980). This has been verified for the cardiac protein as well (data not shown). Therefore we consider the specific broadening of these resonances as good evidence that these tyrosyl residues are in close proximity to sites III and IV, corresponding to tyrosine 111 and 150.

At higher levels of lanthanide, relaxation by Gd^{3+} broadens almost every feature of the spectrum. However the resonances of tyrosine E (position 5) are still relatively unperturbed. This suggests that the N-terminal tyrosine residue is buried within the protein interior and also is not close to the Ca/Mg high affinity sites.

E. CALCIUM BINDING

The calcium-binding process in cTn-C can be divided into two stages: those which occur as the first two moles of calcium are bound, and those which are seen when the third mole is added. Figure 42 pre-

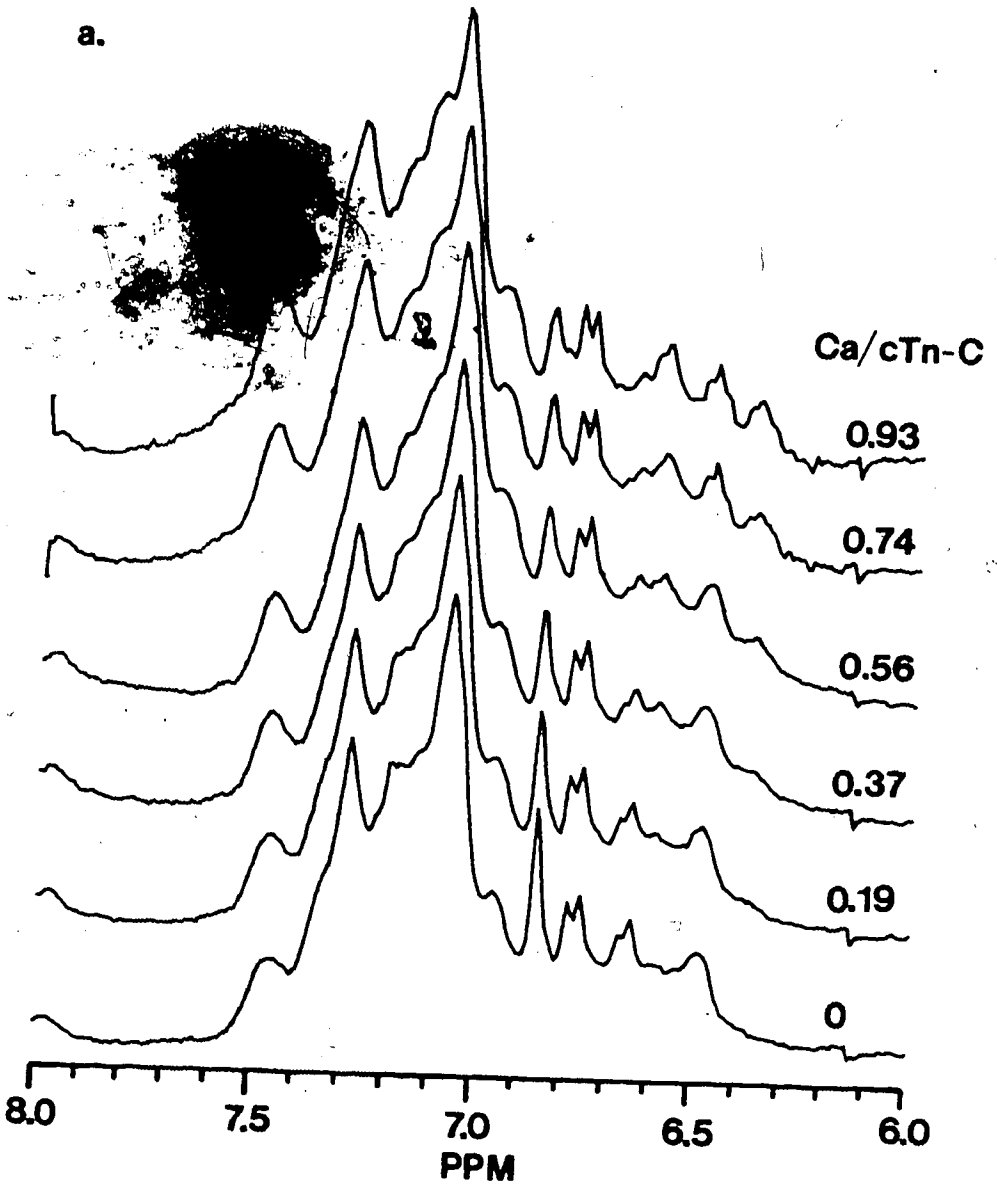


Figure 42. Calcium-binding to apo-cTn-C. cTn-C (1.07 mM) in 0.15 M KCl, 25 mM MOPS, pH 6.71 was titrated with calcium. The aromatic region of the spectrum is presented as a function of increasing calcium/ cTn-C.

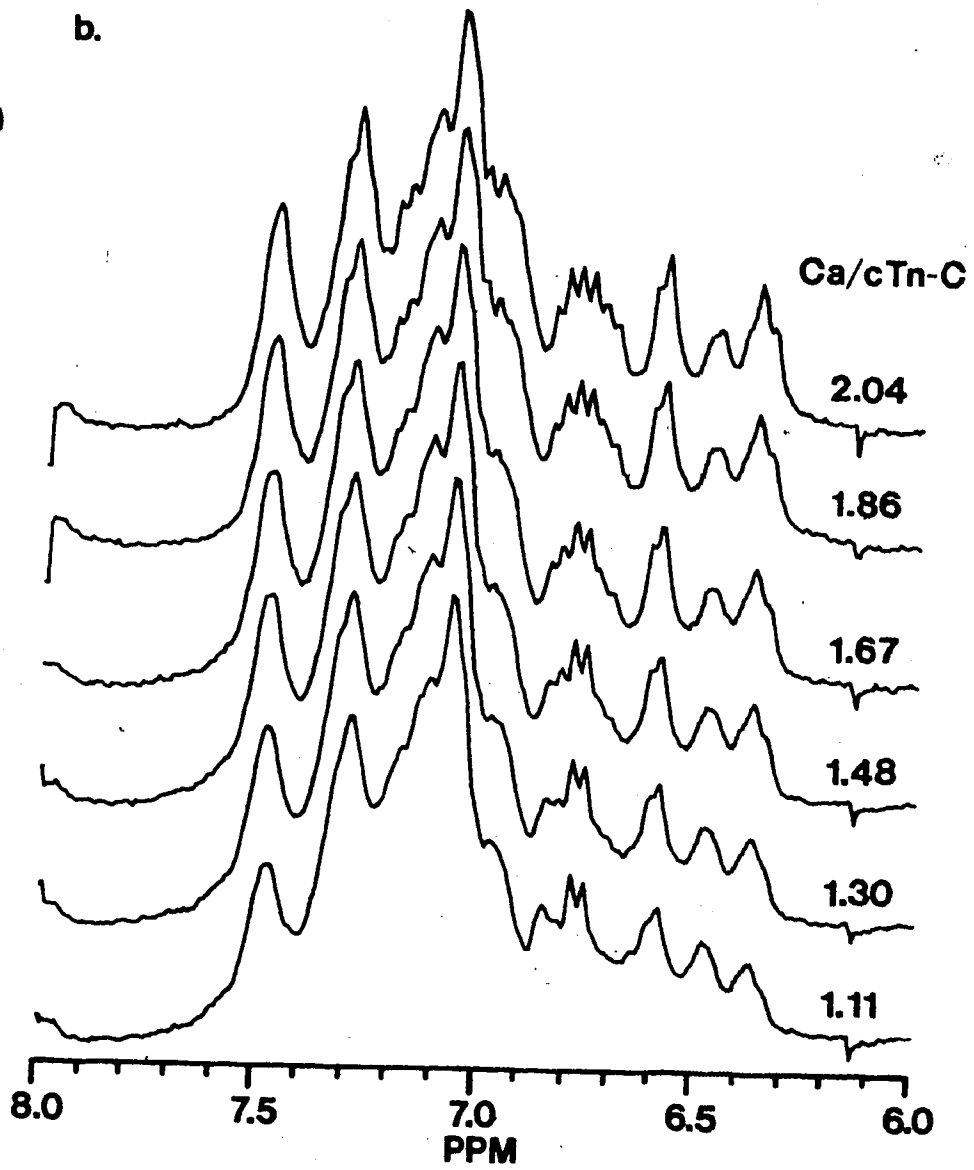


Figure 42. Calcium-binding to apo-cTn-C. cTn-C (1.07 mM) in 0.15 M KCl, 25 mM MOPS, pH 6.71 was titrated with calcium. The aromatic region of the spectrum is presented as a function of increasing calcium/cTn-C.

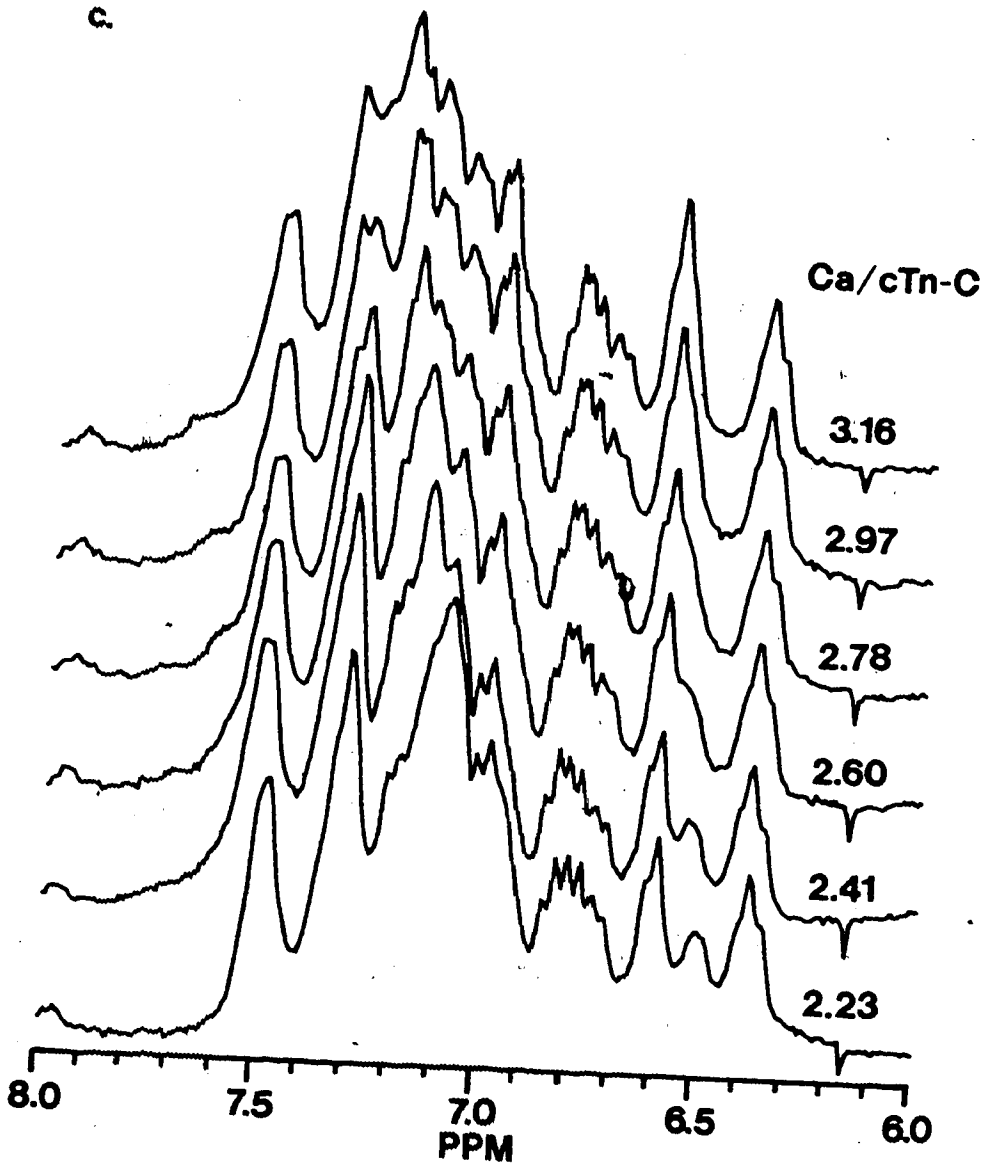


Figure 42. Calcium-binding to apo-cTn-C. cTn-C (1.07 mM) in 0.15 M KCl, 25 mM MOPS, pH 6.71 was titrated with calcium. The aromatic region of the spectrum is presented as a function of increasing calcium/ cTn-C.

sents the aromatic region of the spectrum as up to three equivalents of calcium are added to the apo-protein. In Figures 43 and 44, the peak heights and chemical shifts of some selected resonances are plotted as a function of calcium added.

A general observation is that almost all spectral changes involving tyrosine resonances are complete when two moles of calcium are bound. Because of overlapping peaks, it is only possible to completely follow the increasing peak height of the meta resonance of tyrosine 150 as calcium is added. This titration curve correlates well with the decrease in peak height of the singlet at 6.86 ppm which represents the ortho and meta resonances of tyrosine 150 in the apo-protein. The increase in intensity of the 6.60 ppm peak between 2.5 and 3 Ca^{2+}/Tn-C is due to a phenylalanine doublet which titrates upfield from 2 to 3 Ca^{2+}/Tn-C to merge with the 6.60 ppm peak.

The behavior of these tyrosine resonances as calcium is bound to the high affinity sites, where the resonances representing the apo-form decrease in intensity as the calcium-bound resonances increase, indicates that the equilibrium between these forms of the protein are in the slow exchange limit. That is, on the NMR time scale, the rate of inter-conversion is slow enough to allow each form to be seen as an individual species. This rate is $<< 2\pi\Delta$ where Δ is the frequency separation between the resonances representing the two forms of the protein. Therefore an upper limit for the exchange rate can be calculated from these data (see Table 13).

During addition of a third mole of calcium to the sample, large alterations are seen in the major phenylalanine region of the spectrum (7.0 - 7.4 ppm). As this represents the signals from 9 phenylalanine

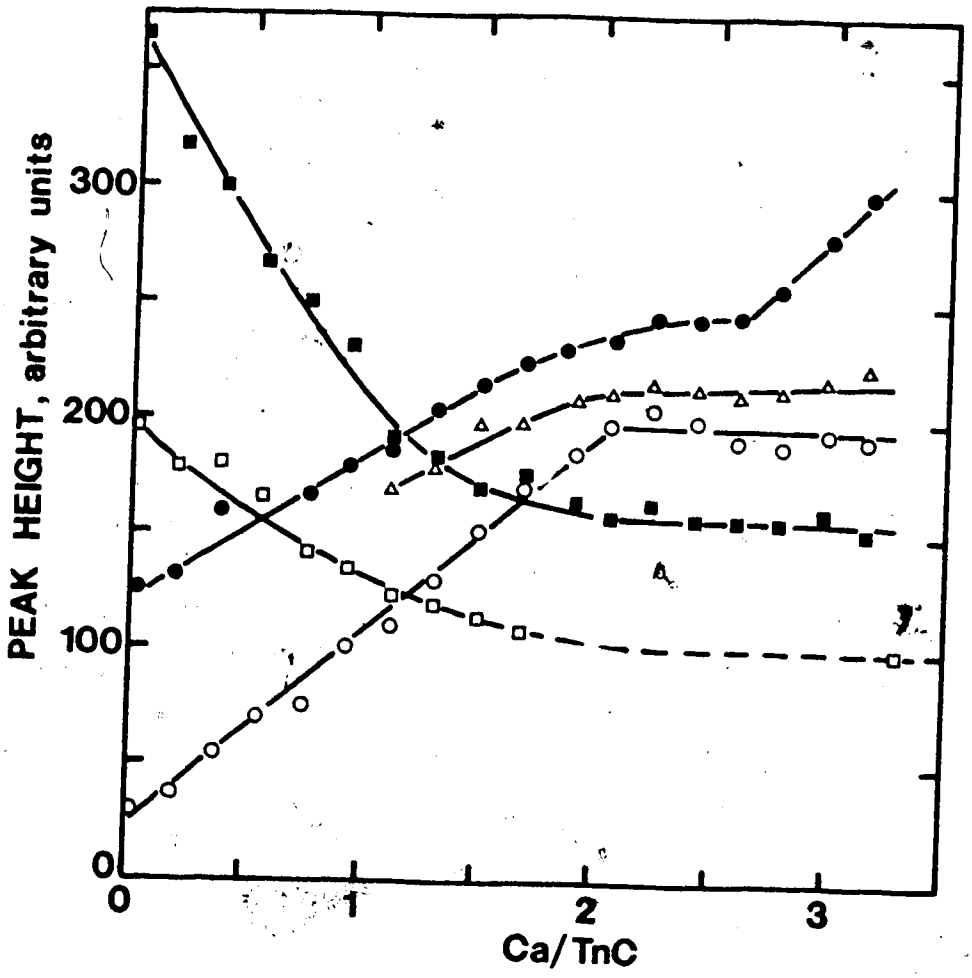


Figure 43. Plot of the peak heights of certain aromatic resonances of cTn-C as a function of Ca^{2+} added; ○, plus Ca^{2+} tyrosine 150 meta resonance; ●, plus Ca^{2+} tyrosine 150 ortho resonance; ■, apo tyrosine 150 ortho/meta resonance; □, apo tyrosine 5 ortho resonance; △, plus Ca^{2+} tyrosine 5 ortho resonance. The heights were corrected for sample dilution by using the integrated area from 6-8 ppm as a normalization constant for each spectrum.

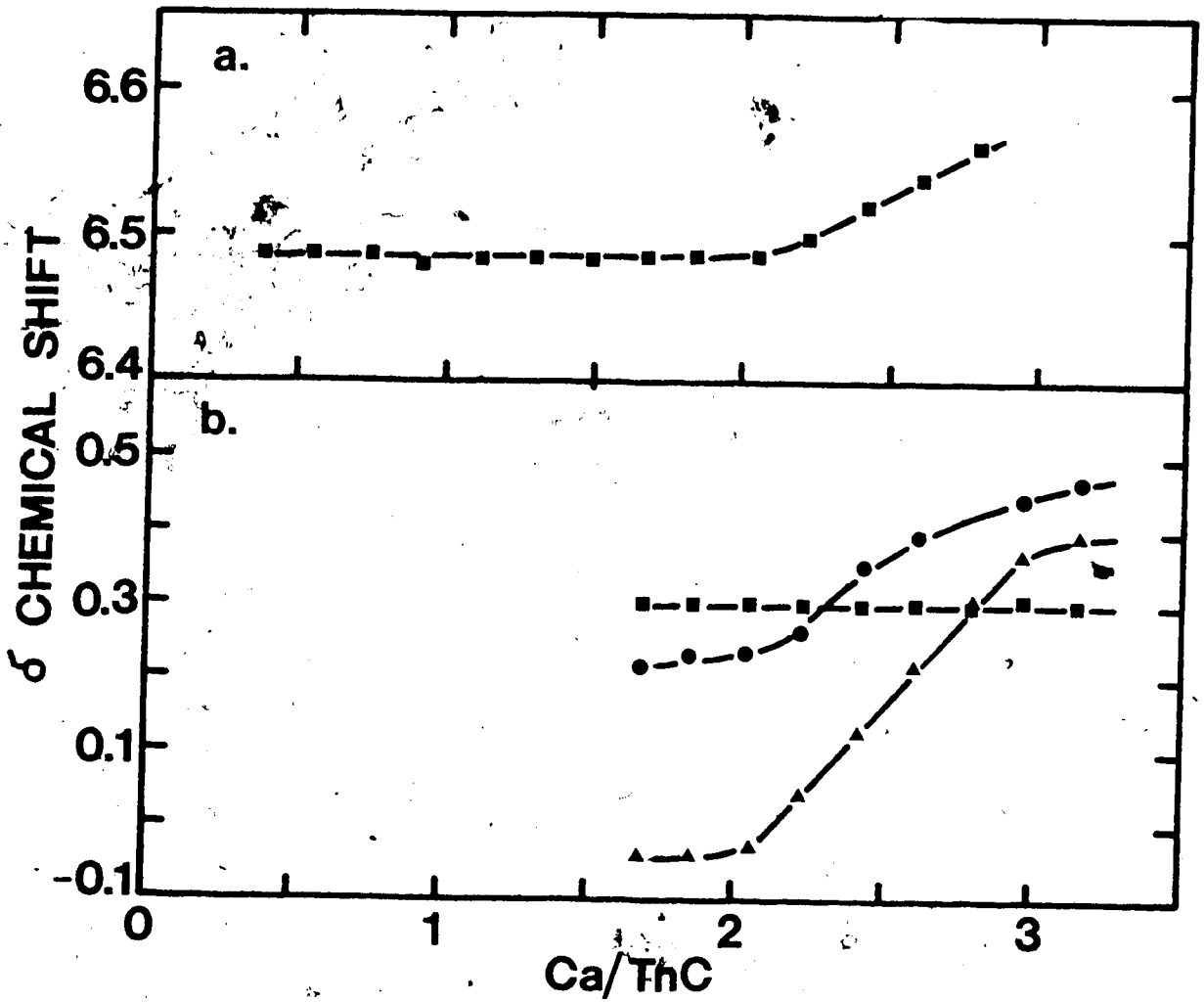


Figure 44. Several chemical shifts are plotted as a function of added calcium.
A. Phenylalanine doublet, apo δ = 6.49.
B. Upfield shifted methyl resonances.

TABLE XIII

Exchange Rates Calculated for Different Ca^{2+}-Conformations of cTn-C Based Upon the Ca^{2+}-Titration Behavior of Some Resonances

Residues	Resonance	Δ	K (S^{-1})
		Slow Exchange	
tyrosine 150	ortho protons	70 Hz	<< 440
tyrosine 150	metal protons	127 Hz	<< 798
		Fast Exchange	
phenylalanine doublet	δ = 6.49	31 Hz	>> 195
methyl resonance	δ = -0.03	116 Hz	>> 700

residues (except the upfield doublets) it signifies large alterations of protein interior accompanying the binding of a single mole of calcium to site II. A phenylalanine doublet at 6.49 ppm is observed to titrate down field as a discrete peak (Figure 42), which suggests that the rate constant for the equilibrium between the plus and minus calcium forms is in the fast exchange limit. A similar phenomenon is also observed for some upfield resonances at -0.03 and 0.22 ppm which also titrate downfield during binding of a third mole of calcium. In the fast exchange limit $K >> 2\pi\Delta$, allowing a lower limit to be calculated for the exchange rate (see Table 13).

F. SUMMARY

From the NMR data, calcium-binding to cTn-C can be differentiated into two steps. This is a consequence of the large difference in the affinity of the two sites for Ca^{2+} ($\sim 10^2$). The binding of 2 moles of calcium to the high affinity sites causes large perturbations of tyrosine residues 150 and 5. A phenylalanine doublet is shifted upfield to the same chemical shift as the meta protons of tyrosine 150 at 6.39 ppm. There is also a phenylalanine doublet at the same position as the ortho protons of tyrosine 150 at 6.60 ppm, although it is not clear whether it occurs during Ca^{2+}-binding or if the resonance is invariant with calcium-binding. Spectral changes associated with tyrosine 5, located near the N-terminus, are complete when the high affinity calcium binding sites, found in the carboxy-terminal half of the molecule, have been filled. This suggests that the N-terminal region of the molecule is folded back into the protein structure, and is therefore sensitive to calcium-induced conformational changes associated with the high affinity sites. Note

that this does not imply the N-terminus (or at least tyrosine 5) is physically close to either calcium binding site. Such a possibility is ruled out by the Gd^{3+}-broadening experiments which demonstrate that only tyrosine 111 and 150 are close to metal ions bound at sites III and IV (within about 15 Å).

It is interesting in this regard that an analysis of cTn-C sequences from different sources (rabbit and bovine) indicates that the mutation rate in the N-terminal region is not different from that for the rest of the molecule (Wilkinson, 1980). This suggests that although site I in cardiac troponin-C's does not bind calcium, it nevertheless provides an important element of structural stability to the protein and is not subject to less stringent structural requirements than the rest of the molecule. The implication is that there may be subtle differences in the manner that cardiac and skeletal troponin-C respond to increases in cytoplasmic free calcium, related to structural differences at the N-terminus. The different environments of tyrosine 5 in cTn-C and tyrosine 10 in sTn-C would be a reflection of this suggestion.

Tyrosine 111, found in site III, a high affinity binding site, displays relatively little change in chemical shift of ortho or meta resonances upon calcium binding. This point may be taken as good evidence that the environment of this residue, which the NMR data suggests is quite exposed to solvent, does not change when calcium is bound. The peptide carbonyl group of this residue is believed to be a coordinating group (-Y coordinate) to the bound metal ion in site III, based upon the X-ray structure derived for carp-MCBP (Kretsinger and Nockolds, 1973). Thus, it is not unexpected that the phenolic ring could be directed away from the binding site toward the solvent. Similar behavior is observed for

the homologous tyrosine residue in site III of calmodulin and sTn-C, suggesting that the teriary folding around this binding site in each protein is also conserved.

However, there are subtle differences between these three proteins. The tyrosine 109 resonances in sTn-C are insensitive to calcium-binding (Seamon et al., 1977), as are those of tyrosine 111 in cTn-C. However, the tyrosine 99 ortho proton resonance in calmodulin shifts upfield and broadens, then narrows as the first two moles of calcium are added (Seamon, 1980). The environment of this tyrosine residue in calmodulin makes it a sensitive probe for the binding of the first 2 moles of calcium. It is not clear if this represents conformational transitions which are unique to calmodulin or if tyrosine 99 has a more sensitive environment than does the homologous tyrosine in cTn-C or sTn-C.

There are important differences in the positions occupied by tyrosine 150 and 111 in the calcium binding loops of sites IV and III. The peptide carbonyl of tyrosine 111 is a dentate specifying a geometry for this residue which is further immobilized by the constraints of the other liganding residues within the loop. The evidence suggests that this results in considerable solvent exposure for this phenolic side chain. An entirely different behavior is observed for the tyrosine 150 residue in cTn-C and 138 in calmodulin. These are not coordinating residues but are part of a region which has been postulated to become helical when calcium is bound at the high affinity sites (Nagy and Gergely, 1979; Reid et al., 1980; Reid and Hodges, 1980). In fact, this would result in the clustering of hydrophobic residues and the tyrosyl residue, merely from helix formation. As well, sequence homology with carp-MCBP predicts that this tyrosine residue will occupy a position in the helical region

connected to the S_4 binding loop and form part of the hydrophobic core of the protein.

The NMR data indicates that in the calcium saturated proteins tyrosines 150 and 138 possess very similar environments and are buried in the hydrophobic core of the protein. Such a suggestion is based upon similar ortho and meta chemical shifts and elevated pKa's. Seamon (1980) has predicted that tyrosine 150 in cTn-C would exhibit the same spectral behavior as tyrosine 138, reflecting the hydrophobic interactions of this residue within the protein interior. However, it is important to note that while the ortho and meta resonances of tyrosine 150 in cTn-C are sensitive solely to high affinity calcium-binding, the chemical shift of the ortho protons of tyrosine 138 undergoes an upfield shift as the first two moles of calcium are added, and also as the fourth calcium is bound. The meta resonance of tyrosine 138 is only sensitive to the first two moles of calcium. This subtle restructuring of the environment of this tyrosine in calmodulin, when the low affinity site is occupied, is not displayed by tyrosine 150 in cTn-C. This phenomenon may represent a conformational change which is unique to calmodulin.

In apo-calmodulin, tyrosine 138 possesses an elevated pKa and is resistant to chemical modification by tetranitromethane (Klee, 1977), criteria implying that it is buried and inaccessible to solvent. However, no such behavior is found for tyrosine 150 in apo-cTn-C. It can be nitrated to the same extent as the other tyrosine residues and possesses the pKa of a relatively exposed residue. Comparison of the two apo-proteins by the CIDNP technique suggests that tyrosine 138 in calmodulin is buried while tyrosine 150 in cTn-C is almost as exposed as tyrosine 111. The binding of calcium buries this residue. These differences are re-

flected in the chemical shifts of these tyrosine residues in the apo-proteins and suggest that there may be large structural differences in this region of the molecule between the two apo-proteins.

Comparison of the aromatic spectrum of apo-cTn-C with those of apo-sTn-C (Seamon et al., 1977) and apo-calmodulin (Seamon, 1980) indicates that sTn-C probably has the most open structure of the three proteins. The majority of phenylalanine resonances are undifferentiated and are found at ~7.34 ppm, a typical chemical shift for solvent exposed phenylalanine rings (Seamon, 1981). Calmodulin does not possess this feature; about two-thirds of the phenylalanine resonances are upfield shifted from this position (Seamon, 1981). This correlates with the unique and exceedingly stable environment of tyrosine 138 in the apo-protein and suggests apo-calmodulin is less unfolded than apo-sTn-C. Similar conclusions can be drawn for cTn-C. At neutral pH the aromatic phenylalanine spectrum is highly differentiated, indicating a large number of the phenylalanine residues experience non-exposed environments, which also correlates with the unique chemical shifts of tyrosine residues 150 and 5 in the apo-protein. The phenylalanine resonances do not alter dramatically as Ca^{2+} is bound until the low affinity site begins to be filled (Figure 42). However this conformational change is distinct from that induced by proton-binding (Figure 35). The alteration in phenylalanine environment appears to be completely different from that elicited by Ca^{2+} ions, corresponding neither to high or low affinity binding. The large H^{+}-induced tyrosine difference spectrum, which mimics the effect of Ca^{2+}, probably arises from a different perturbation of the tyrosine residues.

There are small alterations in the spectrum of apo-sTn-C as the pH is lowered from pH 7 to 5 (Levine et al., 1977). Small upfield shifts occur in the phenylalanine resonances at 6.42 and 6.64 ppm. No effect at the level of the tyrosine ortho resonances is mentioned, but these are not sensitive to Ca^{2+}-binding either (Seamon et al., 1977). Apparently the UV difference technique is capable of detecting very subtle alterations in tyrosyl environment, changes which are not seen in the NMR spetrum. The highly exposed nature of the tyrosine residues in sTn-C makes them very poor indicators of the conformational changes occurring in the molecule when Ca^{2+} (or protons) are bound. Entirely the opposite effect is observed for cTn-C and calmodulin.

The pH titration of apo-calmodulin (Seamon, 1981) reveals many subtle alterations in the aromatic region of the spectrum as the pH is reduced from 7.44 to 5.61. The largest effect is seen at the ortho and meta proton resonances of tyrosine 138. An upfield shift of the ortho proton resonance is similar to that seen during the first state of the Ca^{2+}-induced conformational transition, as 2 moles of Ca^{2+} are bound. However the downfield movement of the meta proton resonance in no way resembles the behavior of the resonance as Ca^{2+} is bound. It is suggested that the environment of tyrosine 138 in apo-calmodulin is determined somewhat by free carboxyl groups which are affected by proton binding. However, Ca^{2+}-binding induces a large conformational change which is not elicited by proton binding, leading to a different effect on these resonances. An effect is seen at the ortho resonance of tyrosine 99 and significant alterations in the phenylalanine resonances between 7.4 and 7.1 ppm also occur as the pH is reduced, indicating the structural changes induced by proton binding are more extensive than localized effects at

tyrosine 138.

In sTn-C the phenylalanine resonances are most sensitive to the proton-induced conformational change. This must reflect the more exposed nature of tyrosine 150, as compared to tyrosine 138 in calmodulin. Only a slight upfield shift is seen in the resonance of tyrosine 150 as the pH is decreased, in contrast to the larger effect upon tyrosine 138.

Examination of apo-sTn-C fragments by NMR (Evans et al., 1980) and comparison with the spectrum of apo-sTn-C has suggested that upfield phenylalanine doublets (δ = 6.65, 6.43) are due to ring current shifts amongst phenylalanine residues 19, 23, 26, 72 and 75. The authors claim that these resonances represent hydrophobic contacts between the regions containing phenylalanine residues 72 and 75 and the region with residues 19, 23 and 26, i.e., site I and site II. These upfield resonances are present in apo-sTn-C, a thrombin fragment (residues 1 - 20) and a tryptic fragment (residues 8 - 84). Upfield shifted methyl resonances (around 0 ppm) in the apo-sTn-C spectrum are also present in these fragments, presumably arising from the same source. In sTn-C the upfield phenylalanine resonances titrate downfield towards the main aromatic peak as the low affinity sites are filled (Seamon et al., 1977). This is correlated with the downfield movements of methyl resonances at about 0.15 and -0.15 ppm. Cardiac Tn-C and calmodulin possess the 5 phenylalanine residues (19 - 75) in homologous positions to those in sTn-C. In calmodulin a phenylalanine resonance at 6.49 ppm is sensitive to calcium-binding, titrating downfield during the addition of 2 moles of calcium to the apo-protein and also as a fourth mole of calcium is bound (Seamon, 1980). As well, upfield shifted methyl resonances shift downfield and disappear into the main methyl resonance during calcium binding. These

resonances reflect similarities in the tertiary structure of apo-sTn-C and apo-calmodulin and also in the folding which occurs when calcium is bound. The above observations can be extended to cTn-C. The phenylalanine resonance at 6.49 ppm is insensitive to high affinity calcium binding but titrates to lower field as the low affinity site is filled, which can also be correlated with the downfield movement of some methyl resonances at 0.21 and -0.03 ppm (Figure 44).

This phenylalanine resonance at 6.49 ppm seems to represent a homologous phenylalanine residue with a similar magnetic environment in calmodulin, sTn-C and cTn-C. Fragment studies (Evans et al., 1980) localize this residue in site I or II and suggest that its unique environment arises from interactions between the aromatic residues at sites I and II. If this is so, it implies that the lack of a low affinity calcium binding site in the site I region of cTn-C does not affect some interactions in the N-terminal portion of the molecule which are also present in sTn-C and calmodulin. This result is anticipated, as cTn-C and sTn-C can substitute for each other in a biological assay system. The calcium sensitive upfield shifted methyl resonances may also be representative of these interactions.

Analysis of the data suggests that the rate of exchange between the apo-protein and the protein with two moles of calcium bound at the high affinity sites is in the slow exchange limit, while the rate of exchange of calcium at the low affinity site is in the fast exchange limit. The rates calculated in Table II suggest that the slow exchange rate is less that 440 S^{-1}, while the rate of fast exchange is greater than 700 S^{-1}. These rates represent either the actual calcium off-rate, or the rate constant for the relaxation of the protein structure when

the metal ion comes off, whichever is slower. NMR studies on sTn-C, where similar phenomena are observed, have indicated that the conformational change is the rate limiting step (Levine et al., 1977). Unfortunately, absolute values for the exchange rates cannot be determined from these data. Stopped flow fluorescence studies utilizing cTn-C labelled with the fluorescent probe IAANS have yielded similar results, in that the Ca^{2+} off-rate from the low affinity site is 230 - 350 S^{-1} and calcium release from the high affinity sites is much slower (Johnson et al., 1978). Similar experiments with sTn-C have suggested that the Ca^{2+} off-rate from the high affinity sites is too slow to allow these sites to regulate contraction (Johnson et al., 1979). The low affinity sites, however, have an exchange rate which is fast enough to regulate striated muscle contraction (350 - 750 S^{-1}). These two proteins are similar, then, in that the high affinity sites appear to play mainly a structural role while the low affinity site(s) is/are the regulatory ones. The reorganization of the protein interior, as evidenced by the dramatic phenylalanine changes, during the binding of calcium to the low affinity site in cTn-C must represent the most important structurally significant changes in this molecule.

CHAPTER VIII

LASER PHOTO CIDNP STUDIES OF cTn-C, sTn-C AND CALMODULIN

A technique has recently been introduced which allows NMR signals to be selectively detected from histidine, tyrosine and tryptophan residues which are on the protein surface. This method is based on the laser photochemically induced dynamic nuclear polarization (CIDNP) of the aromatic protons of these residues (Kaptein, 1978). A flavin dye, excited to its triplet state by brief laser irradiation, reacts reversibly with any accessible histidine, tyrosine or tryptophan residues. This leads to transient radical formation causing nuclear polarization in the side chains of these residues. The resulting spectral changes, positive or negative enhancements of aromatic resonances, can most easily be detected by subtracting a normal spectrum ('dark') from one obtained immediately after illumination with the laser ('light'). This technique has been used to probe the surface exposure of these aromatic residues in a number of different protein systems, including dihydrofolate reductase (Feeney et al., 1980), bovine pancreatic phospholipase (Egmond et al., 1980) and bovine α-lactalbumin (Berliner and Kaptein, 1980).

The Tn-C's and calmodulin are interesting candidates for photo-CIDNP experiments because of the appropriate location of the residues which are sensitive to this technique. A tyrosine residue is found in site III in each protein while only cTn-C and calmodulin possess a tyrosine residue within the domain of the other high affinity site IV. The single histidine residue of calmodulin and sTn-C is located between sites III and IV in each case.

As previously discussed, each of these proteins has been exten-

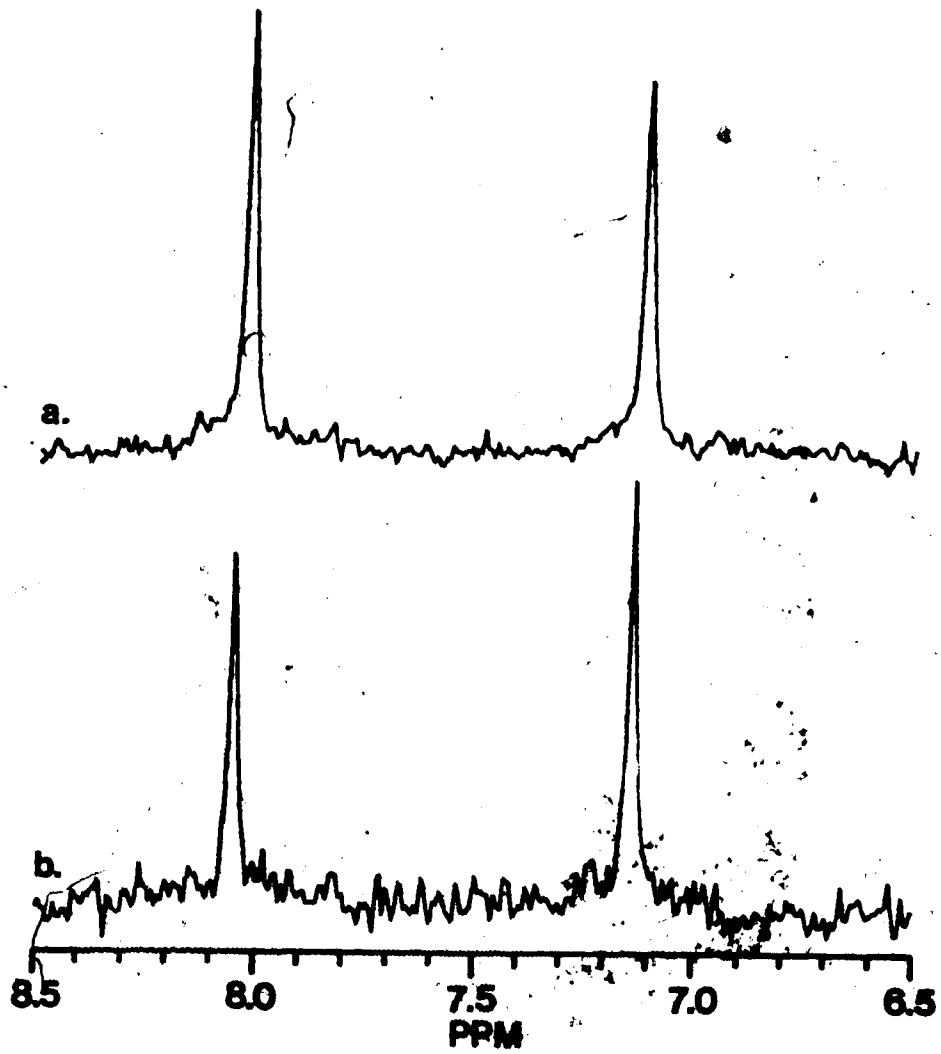


Figure 45. 1.0 mM Gly-His-Gly in 5 mM MOPS, 20 mM KCl, pH 7.00, 0.08 mM FMN.

A. CIDNP difference spectrum (light-dark) after 8 scans (1.0 s irradiation) with a 20 s delay between spectra.

B. 270 MHz spectrum, 128 scans. Here the CIDNP effect represents a 16-fold enhancement.

Figure 46. N-acetyl-L-tyrosinamide (1.1 mM) in 5 mM MOPS, 20 mM KCl, pH 7.0, 0.2 mM FMN.

A. CIDNP difference spectrum as in Figure 45, the magnitude of the enhancement is 1.7-fold for the 2,6 meta protons and 28-fold for the 3,5 ortho protons.

B. 270 MHz dark spectrum, 128 scans.

sively investigated by ^{1}H NMR techniques. Laser photo-CIDNP experiments were anticipated to provide additional information, allowing a direct comparison of tyrosyl exposure in each protein and permitting this to be monitored as Ca^{2+} was bound. The previous assignments of the tyrosyl residues in cTn-C would allow the fullest interpretation of the results (Hincke et al., 1980, 1981b).

A. NATIVE PROTEINS

The CIDNP effect in model compounds is demonstrated in Figures 45 and 46. The peptide Gly-His-Gly exhibits positive enhancement of the C-2 and C-4 histidine protons (Figure 45), while with tyrosine there is positive enhancement of the meta (2,6) protons and much larger negative enhancement of the ortho (3,5) protons (Figure 46).

The accessibility of histidine and tyrosine residues in the native proteins was first investigated. Data for sTn-C, seen in Figure 47, reveal the positive enhancement of the C-2 and C-4 histidine protons and also the negative enhancement of tyrosine ortho protons. No effect is seen for the tyrosine meta resonances; this is typically much smaller and can be cancelled out by cross relaxation. As Ca^{2+} is added to the protein the CIDNP effect diminishes, implying at least one of the tyrosines is being buried. [It was not possible to fully interpret the histidine CIDNP effect, as this proved to be much more variable, as noted below.]

Similar results are obtained with cardiac Tn-C (Figure 48), where the location of the three sets of ortho protons in the apo-spectrum is indicated very convincingly. The tyrosine residues to which these have been assigned are indicated (Hincke et al., 1981a). Calcium-binding

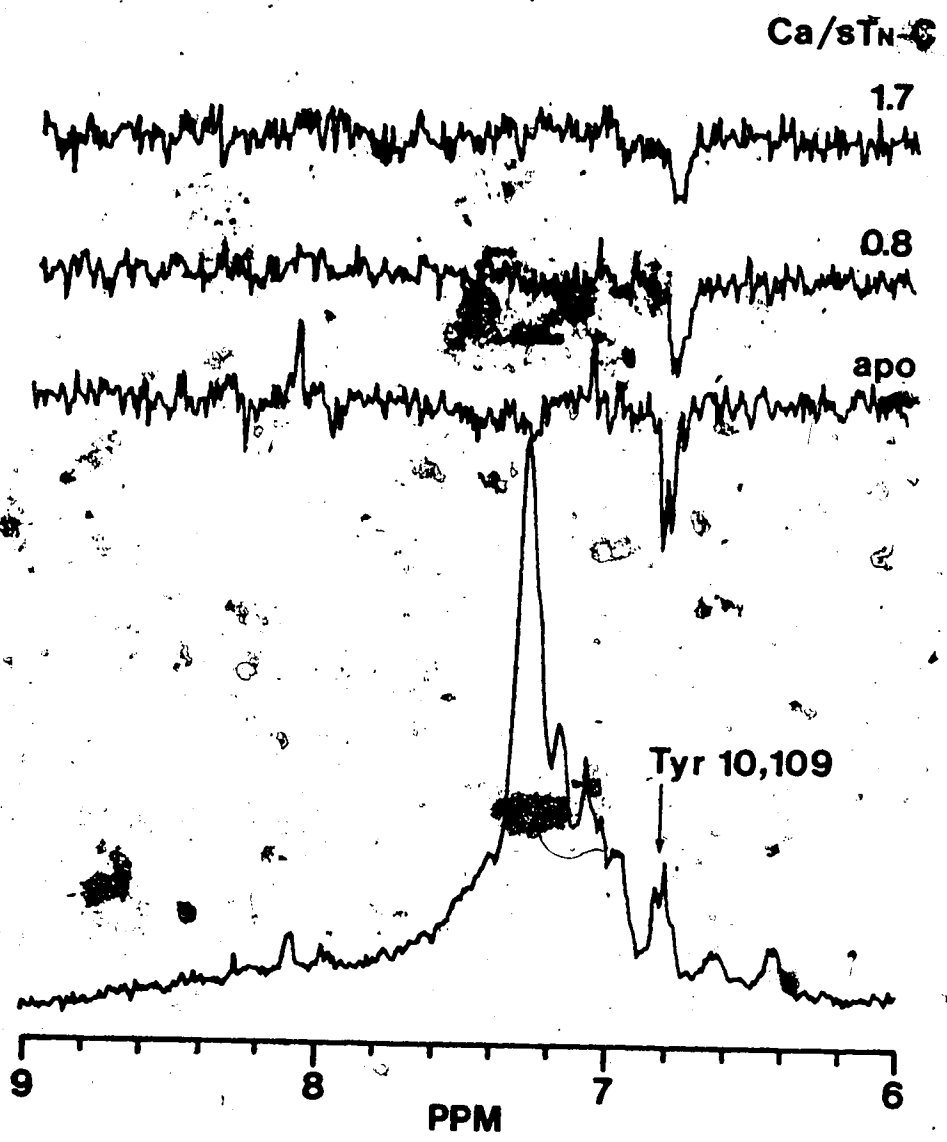


Figure 47. CIDNP difference spectra of native sTn-C (0.4 mM) in 0.1 M KCl, 20 mM MOPS, pH 7.4. Each spectrum represents 8 light and dark scans (as in Figure 45). The dark spectrum of apo-sTn-C is presented. 5 λ FMN (50 mM) was added before each spectrum. The ortho resonances of tyrosine residues 10 and 109 are indicated.

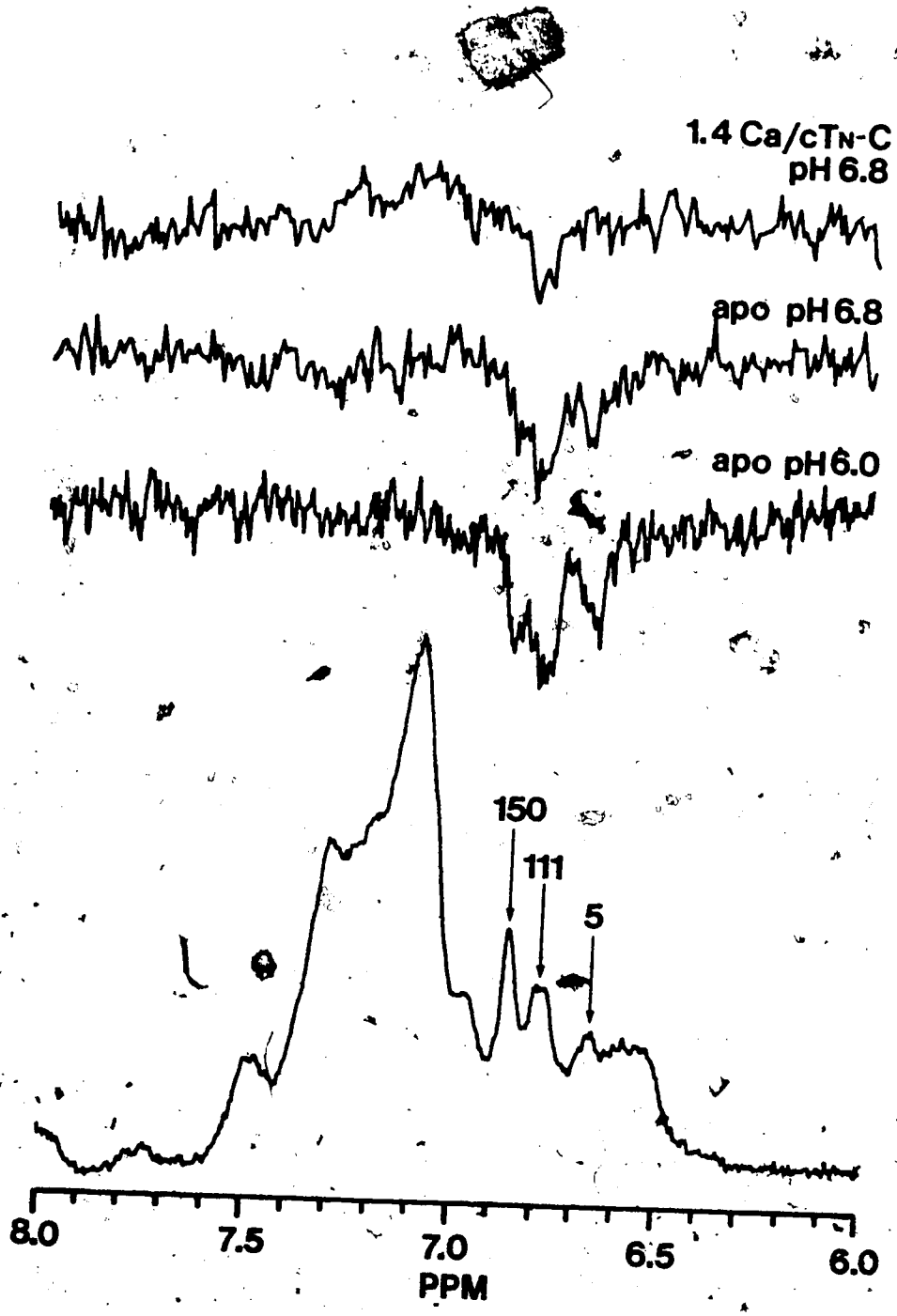


Figure 48. CIDNP difference spectra of native cTn-C (1.1 mM) in 20 mM MOPS, 0.1 M KCl. Each difference spectrum represents 12 light and dark scans (as in Figure 45). The dark spectrum of apo-cTn-C, pH 6.0 is presented as a reference spectrum with assigned tyrosine ortho resonances indicated. 5 λ FMN (50 mM) were added before each spectrum.

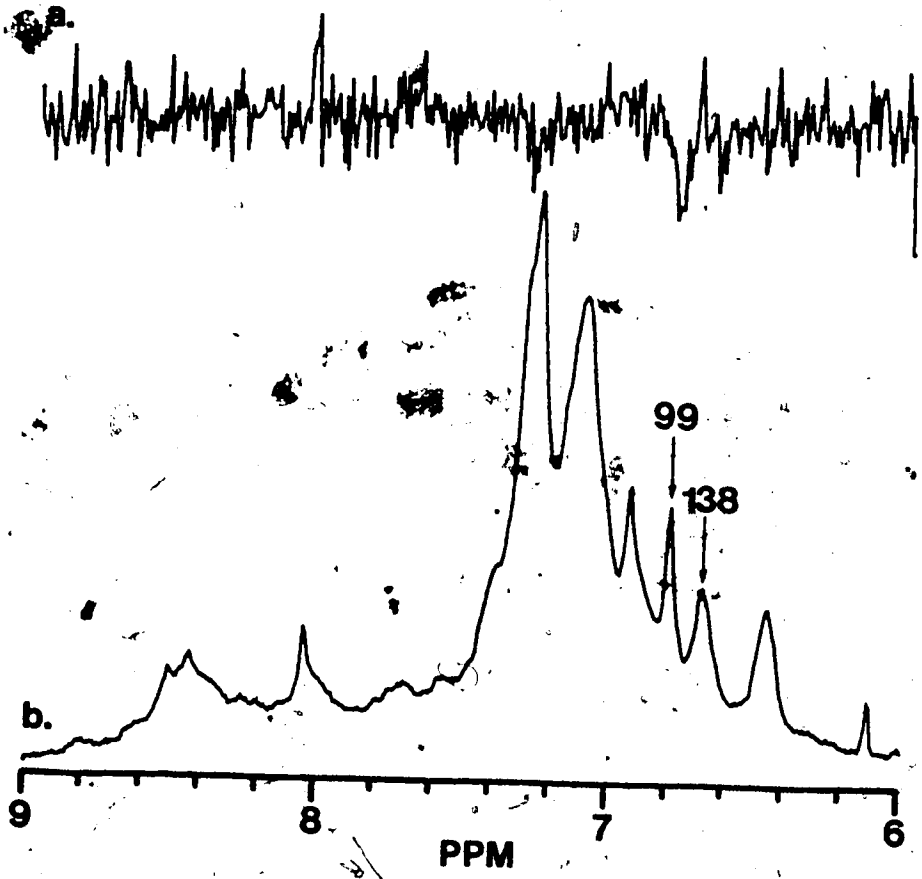


Figure 49. Apo-calmodulin (1.3 mM) in 25 mM MOPS, 0.15 M KCl, pH 7.0, 0.18 mM FMN.
A. CIDNP difference spectrum (4 scans).
B. 270 MHz dark spectrum, 256 scans. The ortho resonances of tyrosine residues 99 and 138 are indicated.

alters the chemical shifts of residues 5 and 150; the CIDNP data suggests these tyrosine residues are not accessible in their Ca^{2+}-bound conformation. Tyrosine 111 also becomes less exposed as Ca^{2+} is bound. cTn-C does not possess any histidine residues.

Apo-calmodulin displays a different behavior (Figure 49). Only slight enhancement is observed at the C-2 histidine resonance; there is no effect at the C-4 histidine. Only a small effect occurs for the ortho protons of tyrosine 99. Apparently in the apo-protein this residue is much less exposed than are the homologous tyrosine residues in cTn-C or sTn-C. Tyrosine 138 is not accessible to the dye under these conditions. This agrees with the buried nature of this residue deduced by other techniques. For example, it possesses a pK_a of 12.0 and 11.9 in the absence and presence of Ca^{2+} respectively (Klee, 1977).

B. UREA UNFOLDED PROTEINS

In concentrated urea solutions a high degree of tyrosyl exposure is observed for each of these three proteins. In Figure 50 the tyrosyl CIDNP effect obtained with cTn-C in 4.2 M urea is demonstrated. Notice that the magnitude of this phenomenon is calcium-dependent, decreasing as the protein binds Ca^{2+}. The protein does indeed bind Ca^{2+} under these conditions, as evidenced by the appearance of aromatic resonances which represent features of the Ca^{2+}-bound spectrum. This effect may be seen in Figure 51 and also occurs in 6 M urea. These results suggest a native plus Ca^{2+} species is being reformed and indicates the tremendous stabilization of the protein structure by Ca^{2+} ions. Similar phenomena are observed with sTn-C and calmodulin. In each case, in 6 M urea solutions, Ca^{2+}-binding decreases the accessibility of the tyrosine residues. With

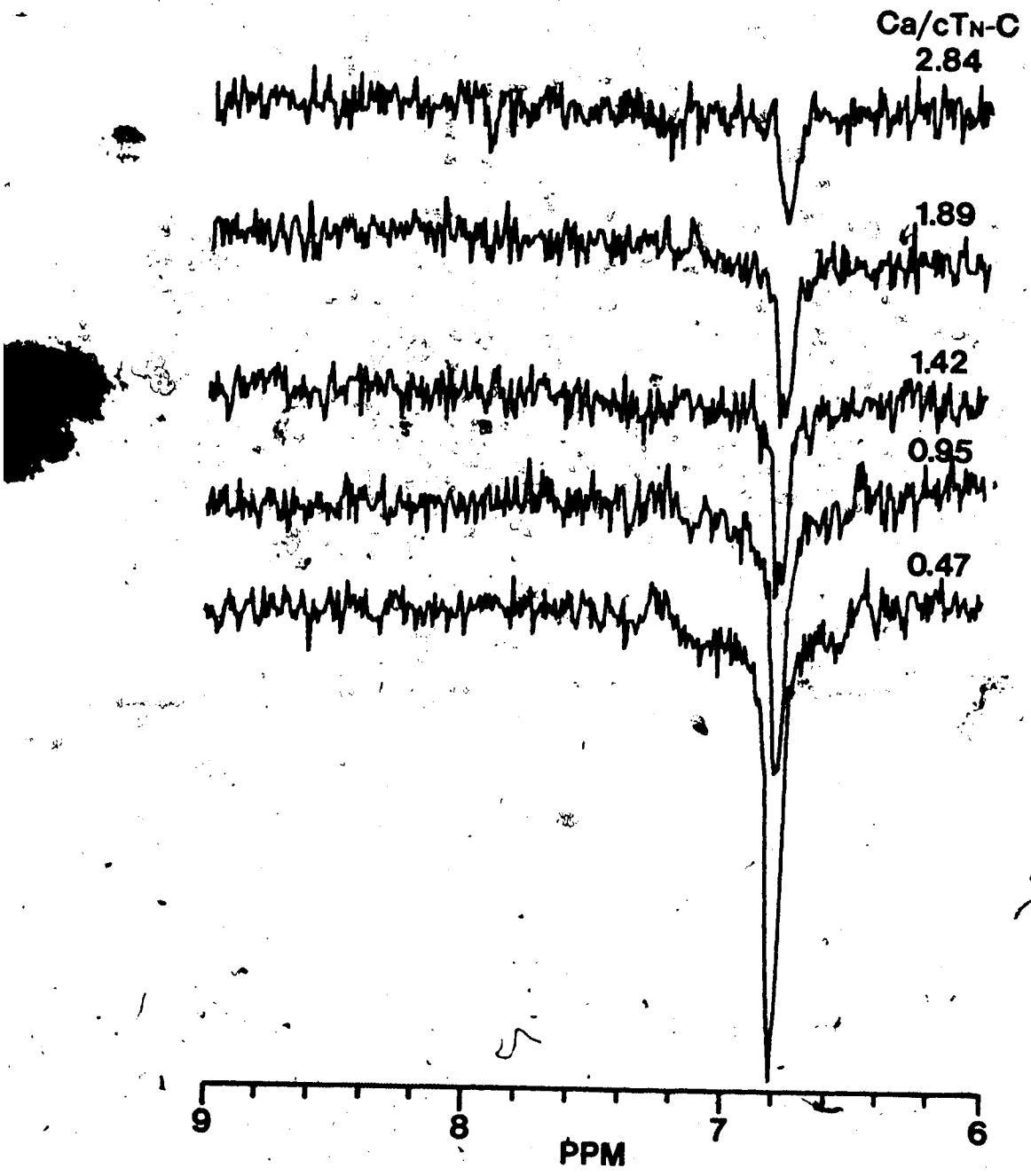


Figure 50. CIDNP difference spectra of cTn-C in 4.2 M urea as a function of added Ca^{2+}. Apo-protein was dissolved in 25 mM HEPES, pH 7.0, 0.15 M KCl. 5 λ FMN (50 mM) was added before each spectrum (8 light, dark scans) was collected.

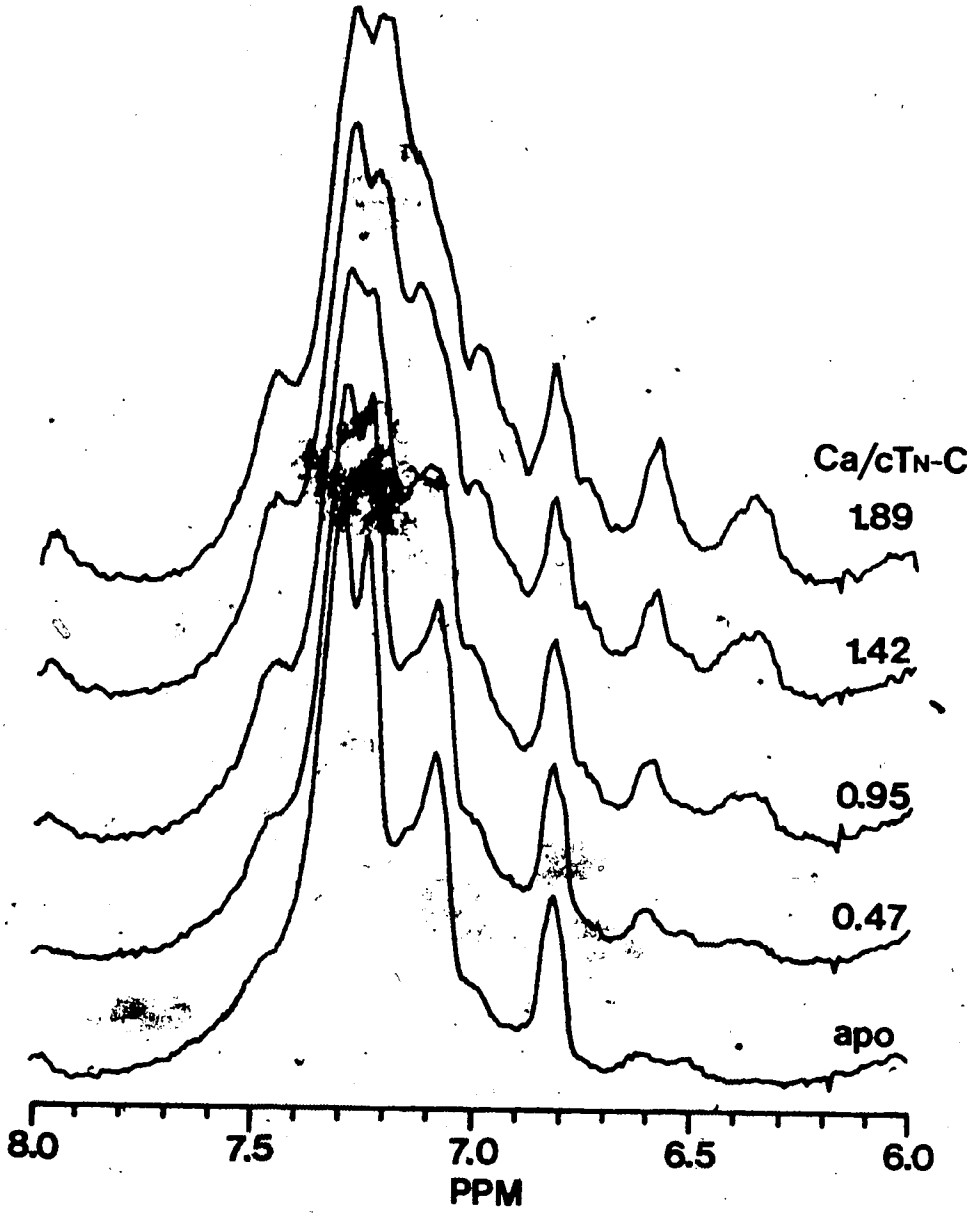


Figure 51. Aromatic region of the 270 MHz dark spectrum of cTn-C in 4.2 M urea as a function of added Ca^{2+}. Apo-protein was dissolved in 25 mM PIPES, pH 7.0, 0.15 M KCl. Each spectrum was collected after the 8 light and dark scans of the CIDNP spectrum (Figure 5) and represents 256 scans.

cTn-C it is evident that the Ca^{2+}-bound residues do not contribute to the CIDNP effect. That is, the chemical shifts of the residues in the plus Ca^{2+} protein are different from those of the same residues in the apo-unfolded structure; no CIDNP signal is observed from these 'plus Ca^{2+}' resonances. It is difficult to understand the small C-2 histidine CIDNP effect observed for calmodulin unfolded in 6 M urea (about the same magnitude as for the native protein), which disappears as Ca^{2+} is bound. No histidine CIDNP signal was observed from sTn-C in 6 M urea. The CIDNP effect at the C-2 and C-4 protons could be readily demonstrated for 1 mM Gly-His-Gly in 6 M urea.

In a study of this nature it is important to ensure that the decrease in intensity of the tyrosyl CIDNP effect is not due to destruction of the exposed tyrosyl residues or of the dye. The most convincing experiments would be with fresh protein samples for each point in a Ca^{2+} titration. This would be extremely demanding in terms of protein. We have attempted to 'reuse' the samples in that each point in a titration represents the cumulative addition of FMN as Ca^{2+} is sequentially added to the same sample. At the conclusion of an experiment a sample has been irradiated many times. It is important that control experiments allow the effects of Ca^{2+}-binding to be discerned from those of tyrosine or dye destruction, which would also decrease the CIDNP intensity due to progressive loss of chromophore. Control experiments with N-acetyl-L-tyrosinamide in 6 M urea are shown in Figure 52. It is apparent that when the experiment is conducted in this manner, some process reduces the magnitude of the CIDNP effect with each subsequent series of irradiations. The presence of urea has little effect on these controls (not shown) and there is also no effect from adding Ca^{2+} with each FMN addition. Histi-

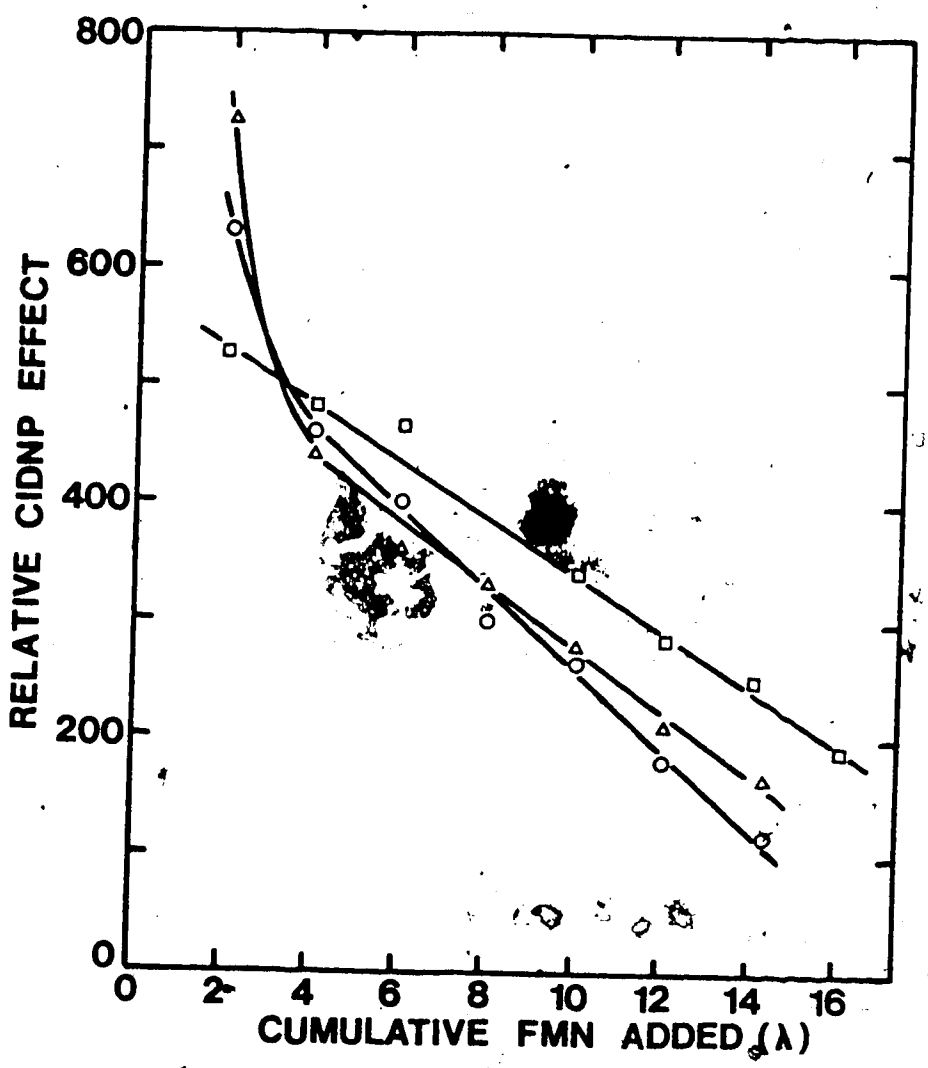


Figure 52. Magnitude of the CIDNP effect (arbitrary units) of ortho tyrosine protons as a function of irradiation and FMN added. N-aceyl-L-tyrosinamide (1.1 mM) in 6 M urea, 5 mM MOPS, 20 mM KCl, pH 7.0 (1.20 ml).

A. ○, 8 scans (1.0 s x 3.5 watts, delay = 20 s).

B. △, as in A. but 2 λ Ca^{2+} (0.131 M) added with each FMN addition.

C. □, as in B. but only 2 scans for each point (normalized by multiplying by 4).

dine controls (Gly-His-Gly) yielded similar results under these conditions (not shown). Under gentler irradiation conditions (2 irradiations per point) less tyrosine destruction is noted, as a larger CIDNP effect can be obtained at the higher cumulative amounts of FMN (Figure 52, curve c). The destructive process was less dramatic for protein samples; perhaps some protein functional groups scavenge or quench the free radical which are responsible for the diminished effect.

Figure 53 demonstrates the Ca^{2+} titration of calmodulin in 6 M urea. In Figure 53b data from 2 different titration curves have been plotted together. Although one set of data represents many more irradiations and much more FMN added than the other because more points were taken, the titration curves are virtually superimposable. As an additional feature, the control data for N-acetyl-L-tyrosinamide in 6.0 M urea, obtained under similar conditions (2 scans per point, see Figure 52), has been normalized and plotted with the calmodulin data. These results suggest Ca^{2+}-dependent burying of tyrosine residues is occurring in the protein, supported by the decreasing intensity of the resonance representing the unfolded tyrosine residues as Ca^{2+} is added (Figure 53a). Other spectral features appear at the same time which represent the plus Ca^{2+} protein. The increasing peak height of a resonance at 6.31 ppm is also plotted in Figure 53a.

Controls were also performed with protein samples, in which only FMN but no conformation-altering Ca^{2+} ions were added. These substantiated our conclusions that Ca^{2+} is primarily responsible for the decreasing tyrosine CIDNP effect. A control experiment in which a similar sample of cTn-C was titrated with FMN (4 scans per point) is plotted with the Ca^{2+}-titration data in Figure 54b. It is apparent that there is a

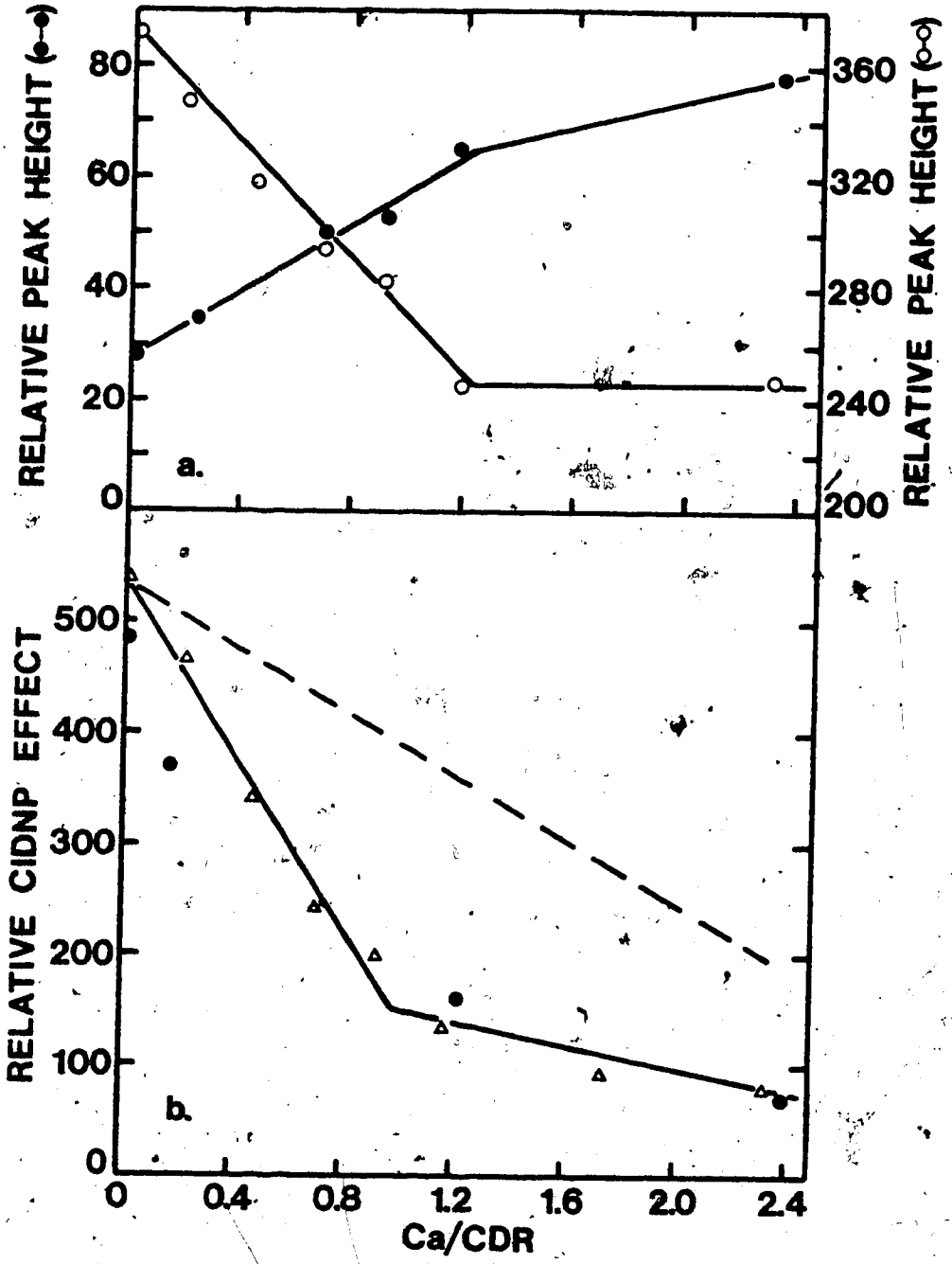


Figure 53. Calmodulin (0.94 mM) in 6.0 M urea, 25 mM MOPS, pH 7.0, 0.15 M KCl.

A. Plot of the peak height (arbitrary units) of some aromatic resonances in the 270 MHz dark spectrum (256 scans) as a function of added Ca^{2+}. ●, 6.31 ppm; ○, 6.82 ppm.

B. Tyrosyl CIDNP effect (arbitrary units) as a function of added Ca^{2+}. 2 λ FMN (50 mM) were added for each point which is 2 scans (1.0 s x 3.5 watts). △,● represent different titration curves which have not been normalized. (- - -) is curve C from Figure 52.

Ca^{2+}-dependent burying of the tyrosyl residues which is complete when about 1 mole of Ca^{2+} has been added. The addition of solid urea to 8 M unfolds the protein somewhat, increasing the CIDNP signal, even when the protein is saturated with Ca^{2+}. The protein spectrum at this point suggests that there are still features representing the 'native' plus Ca^{2+} protein. This would explain why 8 M urea does not return the CIDNP signal to control levels, and attests to the large degree of stabilization afforded by Ca^{2+}-binding. In Figure 54a the appearance of native 'plus Ca^{2+}' resonances in the 6 M urea spectrum of cTn-C as Ca^{2+} is added is presented. While some of the decreasing peak height at 6.84 ppm is probably due to tyrosyl destruction, as noted in the control experiment (Figure 54b), a large degree of it must correspond to the increasing peak intensity at 6.60 and 6.76 ppm, which have been previously assigned to the ortho protons of tyrosine 150 and 111 in the native plus Ca^{2+} spectrum. The resonances at 6.35 and 6.41 ppm represent the meta protons of tyrosine 150 and a phenylalanine doublet, respectively (Hincke et al., 1981a). The appearance of this phenylalanine resonance is associated with Ca^{2+}-binding to the high affinity sites, as are the tyrosine 150 ortho and meta resonances at their 'plus Ca^{2+}' chemical shift positions. In the native protein, Ca^{2+} titration of these resonances indicates they are responsive solely to Ca^{2+}-binding to the two high affinity sites (Hincke et al., 1981a). The titration curves in 6 M urea indicate there is only one high affinity site. Since Ca^{2+}-binding to this site restores tyrosine 150 and tyrosine 111 to their native conformations, it is not clear if the metal ion is binding to site III or IV.

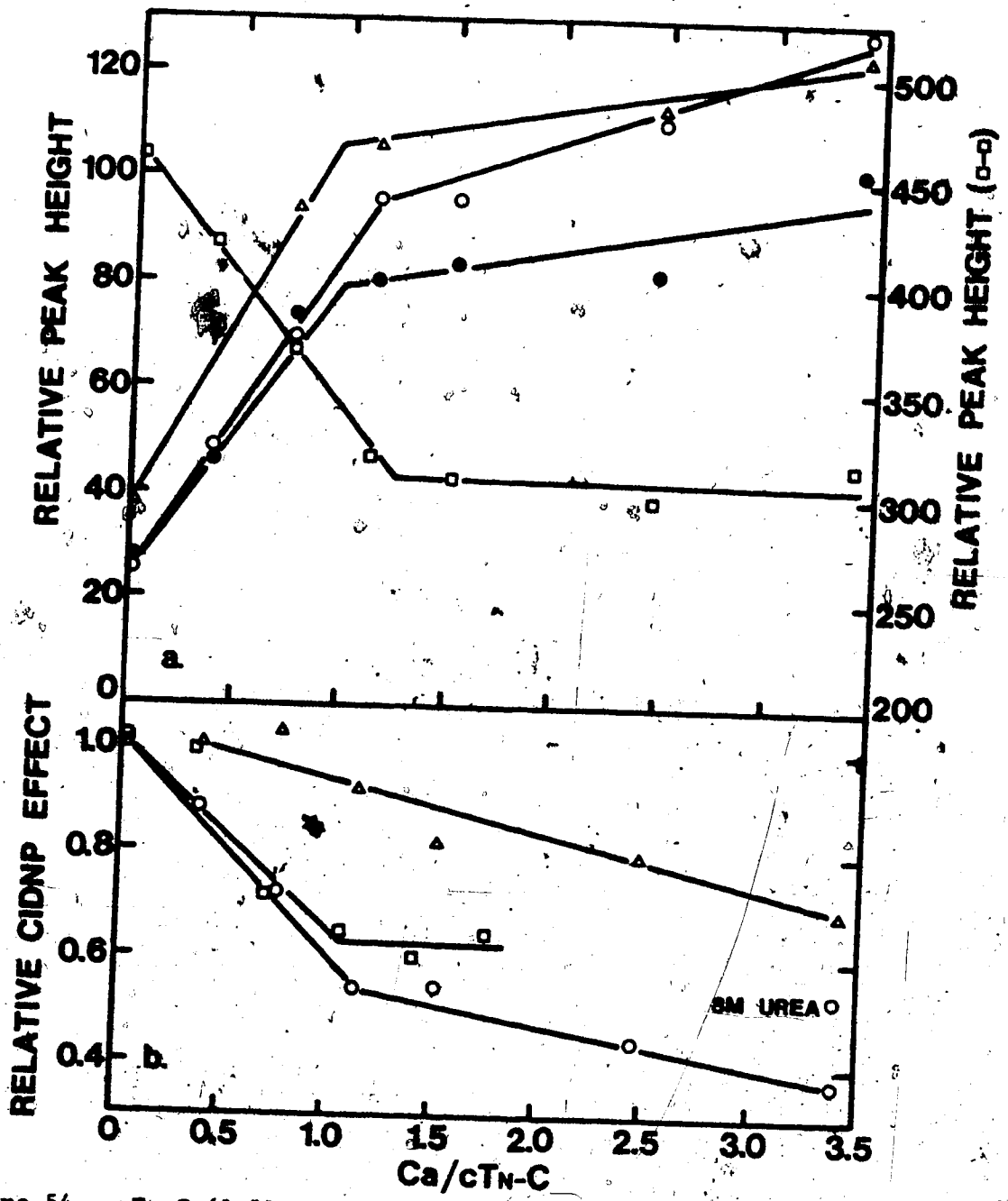


Figure 54. cTn-C (0.53 mM) in 6.0 M urea, 25 mM MOPS, 0.15 M KCl, pH 7.0.

A. Plot of the peak height of some aromatic resonances as a function of added Ca^{2+} in the 270 MHz dark spectrum (256 scans). □, 6.84 ppm; ●, 6.35 ppm; ○, 6.60 ppm; △, 6.76 ppm.

B. Tyrosyl CIDNP effect (arbitrary units) as a function of added Ca^{2+}. 2 λ FMN (50 mM) were added for each point (4 scans). △, cTn-C control, no Ca^{2+} added for each point. ○, cTn-C titration, Ca^{2+} added for each point. □, sTn-C (0.62 mM) in 6.0 M urea, titrated as for cTn-C with Ca^{2+}, except that each point is 8 scans.

The tyrosine CIDNP results for sTn-C appear to be similar to those of cTn-C. That is, Ca^{2+}-binding to the protein in 6 M urea buries the tyrosine residues (Figure 54b). The effect levels off at about 1 Ca^{2+}-bound per molecule. In native sTn-C, the tyrosine resonances are not markers for Ca^{2+}-binding. That is, tyrosine residues 10 and 109 exhibit very similar ortho proton chemical shifts which are not altered by Ca^{2+}-binding (Seamon et al., 1977). These chemical shifts are almost identical to those of the tyrosyl residues in urea-unfolded sTn-C (6.82 ppm) and it is not possible to make inferences about the return of 'native' plus Ca^{2+} tyrosine conformations in these experiments, as was done for cTn-C.

The results for calmodulin are interesting as they indicate that although tyrosine residue(s) are buried as one mole of Ca^{2+} is bound, the 'plus' Ca^{2+} resonances of tyrosine 138 are not evident in the aromatic dark spectrum (Seamon, 1980). The resonance at 6.31 ppm which appears in the spectrum as Ca^{2+} is bound may represent a phenylalanine residue or a tyrosine residue, either of which is occupying a slightly altered environment in the plus Ca^{2+} protein in 6 M urea. This is somewhat surprising in view of the extensive amino acid homology between Tn-C, and calmodulin (Vanaman et al., 1980) and also the similar tyrosyl environments of the Ca^{2+}-bound proteins deduced by NMR studies (Hincke et al., 1981a). A recent report suggests that the high affinity Ca^{2+} binding sites in calmodulin are sites I and II, in contrast to the case with Tn-C (Kilhoffer et al., 1980). If correct, this might account for the differences observed here in the response of the tyrosine residue in site IV of calmodulin and cTn-C to Ca^{2+}-binding in 6 M urea.

C. SUMMARY

The laser photo CIDNP experiments with cTn-C, sTn and calmodulin presented in this section have demonstrated that the tyrosine residues in the native apo-proteins are not completely homologous in terms of solvent exposure. Tyrosine 138 in calmodulin appears to be inaccessible to the FMN probe, in agreement with other techniques which have demonstrated the buried nature of this residue (Klee, 1977) and tyrosine 99 shows a smaller enhancement than tyrosine 111 in cTn-C, for example. Ca^{2+}-binding by cTn-C selectively buries residues 5 and 150 while tyrosine 111 remains exposed to the solvent. In sTn-C, the resonances of the two tyrosine residues overlap and therefore cannot be distinguished.

Even in cardiac and skeletal Tn-C, where some exposure of the tyrosyl residues is indicated in the apo-protein, comparison with model compounds such as N-acetyl-L-tyrosinamide (Figure 46) suggests a much lower degree of accessibility in the former. Possibly the degree of enhancement can be correlated with the slightly elevated pKa's that the tyrosine residues possess in each protein. In apo-cTn-C, each of the three tyrosines has a pKa of about 10.8, as do those in apo-sTn-C (McCubbin et al., 1979). Comparison with values for exposed tyrosine residues in protein (9.5 - 10 (Nozaki and Tanford, 1957)) indicates these residues are probably not freely exposed on the protein surface. However, the pKa of tyrosine 99 in calmodulin (10.4 and 10.1 in the absence and presence of Ca^{2+}) indicates it is an exposed residue (Klee, 1977). It is difficult to correlate this with the apparent low degree of exposure indicated by the CIDNP results.

The three apo-proteins are largely unfolded by 6 M urea but native-like features in the aromatic spectrum reappear as Ca^{2+} is bound. Titration of these resonances, representing the plus Ca^{2+} protein, indicates that in 6 M urea only one high affinity site remains in each protein. Ca^{2+}-binding to the single high affinity site in cTn-C induces a native protein structure at the level of tyrosine residues 111 and 150. Whether binding to site III or site IV is responsible for the phenomenon, this observation emphasizes the importance of long-range interactions in Ca^{2+}-binding to the individual sites. These have been postulated to play a key role in the mechanism of Ca^{2+}-binding to the homologous (to MCBP) proteins (Reid and Hodges, 1980). This evidence also suggests that the structural changes observed upon Ca^{2+}-binding to urea-unfolded cTn-C also occur when the native protein binds Ca^{2+}, and by implication, when sTn-C binds Ca^{2+}.

Other spectroscopic studies on Ca^{2+}-binding to sTn-C in 6 M urea have also demonstrated that only one of the high affinity sites binds Ca^{2+} under these denaturing conditions, and shown that this evokes almost all of the CD and fluorescence changes which Ca^{2+}-binding to the native molecules elicited (Leavis et al., 1980; Nagy and Gergely, 1979). It was concluded that the sole high affinity site in 6 M urea corresponded to site III, primarily because the increase in tyrosine fluorescence observed when sTn-C binds Ca^{2+} was assumed to originate from tyrosine 109, located within the Ca^{2+}-binding loop of this site. The large increase in tyrosyl fluorescence observed when the sTn-C fragment CB-9, containing only site III, binds Ca^{2+} (Nagy et al., 1978) was considered to support this assignment (Nagy and Gergely, 1979). However, it has been demon-

strated by ^{1}H NMR (Birnbaum and Sykes, 1978) that the environment of tyrosine 109 in Ca^{2+}-bound CB-9 is completely different from that in the plus Ca^{2+}-native sTn-C molecule. This can be illustrated by comparing the chemical shifts of tyrosine 109 under these different conditions (Table XIV). Examination of Table XIV reveals that although the chemical shifts of tyrosine 109 are similar in apo-CB-9 and apo-sTn-C, reflecting an exposed environment (Hincke et al., 1981a), Ca^{2+}-binding induces a large alteration in the environment of tyrosine 109 in CB-9 but very little in the case of sTn-C. This difference suggests the fluorescence changes induced by Ca^{2+} in sTn-C and CB-9 might not be attributed to the same source, although a similar fluorescence enhancement is observed in each case.

Other evidence also exists which casts doubt upon the assignment of the single high affinity site in 6 M urea to site III. X-ray structural studies of MCBP, containing two high affinity Ca^{2+} binding sites which exhibit sequence homology with sites III and IV in troponin-C's, have indicated the carbonyl group of phenylalanine residue 57 serves as a donor to Ca^{2+}-bound to the CD loop. However the sidechain of this aromatic residue is within 5 Å of the Ca^{2+} atom bound to the EF loop (Donato and Martin, 1974). The EF loop is homologous to site IV, while the CD loop has sequence homology to site III in Tn-C in which phenylalanine 57 has been replaced by tyrosine 109 in sTn-C and tyrosine 111 in cTn-C. As well, models of sTn-C based on the X-ray structure derived for Ca^{2+}-bound MCBP have indicated tyrosine 109 would be in close proximity to the metal ion bound at site IV (Kretsinger and Barry, 1975).

Spectroscopic studies have indicated there is energy transfer from the Phe 57 sidechain to Tb^{3+} bound at the Ca^{2+} binding site in the EF loop (Moews and Kretsinger, 1975). Furthermore, terbium luminescence studies with sTn-C and MCBP (Miller et al., 1975) and cTn-C (Brittain et al., 1976) demonstrate near identity in circularly polarized emmission (CPE) line shape and dissymmetry factors, indicating closely similar binding sites for Tb^{3+} in these proteins. These studies concluded that in each protein a homologous aromatic residue was involved in the energy transfer, this being the -Y residue of the CD Ca^{2+}-binding loop which was in close proximity to the metal ion bound in the EF loop.

Additional evidence to support the proposed orientation of tyrosine 111 relative to site IV stems from Gd^{3+}-binding experiments utilizing cTn-C (Hincke et al., 1981a). When Gd^{3+} was added to the Ca^{2+} saturated native protein, the resonances representing the ortho and meta protons of tyrosine residues 111 and 150 were selectively broadened, indicating their proximity to the paramagnetic ions bound at the high affinity sites. The ortho resonances of tyrosine 111 were broadened to a greater extent than the meta resonances, indicating that the ortho protons are closer to the bound metal ion than are the meta protons. In Ca^{2+}-binding to site III, tyrosines 109 and 111 in sTn-C and cTn-C are proposed to occupy the -Y ligand position, in which the metal ion is coordinated to the peptide carbonyl group. This specifies a geometry with the aromatic side chain directed away from the bound ion. It is difficult to conceive of an orientation in which the ortho protons of this residue could be closer to the metal ion than the meta protons and one cannot be demonstrated utilizing a model of sites III and IV constructed from coordinates based on the parvalbumin X-ray structure (Kret-

singer and Barry, 1975). However the predicted position of this residue in relation to the metal ion bound at site IV (within 5Å) readily accomplishes this.

Energy transfer from a tyrosine residue in sTn-C to bound Tb^{3+} has been demonstrated for the native protein and the protein unfolded in 6 M urea (Leavis et al., 1980). The stoichiometry for Tb^{3+} binding is 2 moles/Tn-C in the native protein but only one binding site remains in 6 M urea, as is observed for Ca^{2+}. Because a similar degree of Tb^{3+} fluorescence is seen in each case, these workers suggested site III and not site IV was the remaining high affinity site in 6 M urea, since in a largely unfolded protein the other binding sites were considered to be too far away from tyrosine for energy transfer to occur. However, Nagy and Gergely (1979) demonstrated that under both native and denaturing (6 M urea) conditions three α-helical segments, each containing 9 to 10 residues, are formed when Ca^{2+} is bound to sTn-C. Evidently large conformational changes occur in regions of the denatured molecule which are distant from the single Ca^{2+} binding site. It is equally likely that this extensive conformational change in urea is brought about by Ca^{2+}-binding to site IV, bringing tyrosine 109 into close proximity with a metal ion bound at site IV even when binding at site III itself was abolished.

An initial premise for this proposal is that the chemical shifts of tyrosyl residues 111 and 150 are dependent upon their environment, which is determined by metal binding to site IV in the native plus Ca^{2+} protein, where 2 moles of Ca^{2+} are bound. The results presented here indicate that the same environment is created for these residues when only one mole of Ca^{2+} is bound to the protein in 6 M urea. It seems

more likely that Ca^{2+}-binding to site IV in cTn-C is responsible for this effect. A similar situation probably occurs in sTn-C, although the resonances of tyrosine 109 are not suitable for monitoring the phenomenon.

This conclusion stresses the fundamental nature and importance of long range interactions between residues of sites III and IV in Ca^{2+}-binding to each site. The presence of these interactions is also indicated by studies with fragments of sTn-C, specifically those containing site III (CB-9), site IV (TH2) and both sites III and IV (TR2) (Leavis et al., 1978). The Ca^{2+}-affinity of TR2 was similar to that of the high affinity sites of native sTn-C, with an affinity of 5×10^7 M^{-1} for 2 moles of Ca^{2+}. However the affinities of the peptides containing the single sites were much reduced; 2.2×10^5 M^{-1} for CB-9 and 2.5×10^4 M^{-1} for TR2. Clearly, important interactions exist between these two regions of the molecule which affect Ca^{2+}-binding at each site.

The similar decrease in exposure of tyrosyl residues observed when the Tn-C's and calmodulin bind Ca^{2+} in 6 M urea has some interesting implications. This phenomenon was monitored by the decreasing tyrosyl CIDNP effect and the decrease in magnitude of the exposed tyrosyl ortho proton resonance as Ca^{2+} was added and indicated only one high affinity site was responsible for the effect in each protein. It did not seem that 'native' plus Ca^{2+} resonances were appearing in the calmodulin dark spectrum as Ca^{2+} was added, as opposed to the case with cTn-C. As mentioned earlier, this might be due to sites I and II being the high affinity sites in calmodulin (Kilhoffer et al., 1980). Since the tyrosine residue(s) are still affected by Ca^{2+}-binding in 6 M urea, a conformational change must be transmitted from site I or II to tyrosine 99 or 138, or, in 6 M urea at least, the high affinity Ca^{2+} binding site corresponds to

the same site as in cTn-C (site IV). The specific conformational features which would cause sites I and II to be the highest affinity sites in the native molecule might not be operative in the urea unfolded molecule.

TABLE XIV

Chemical Shifts of Tyrosine 109 in Apo- and Plus Ca^{2+} CB-9 and sTn-C

	apo ortho, meta	plus Ca^{2+} ortho, meta
sTn-C[a]	6.84, 7.12	6.85, 7.72
CB-9[b]	6.83, 7.06	6.64, 6.71[c]

[a] Seamon et al., 1977; Levine et al., 1977.

[b] Birnbaum and Sykes, 1978.

[c] Note the reversed nature of these resonances.

CHAPTER IX

CONCLUDING DISCUSSION

In general, the cardiac regulatory mechanism appears to be quite similar to that operating in skeletal muscle, as shown previously (Burtnick and Kay, 1976). This is evident in the common features of the Tn-I - Tn-T interaction in cardiac and skeletal tissue. However differences can also be demonstrated between the interactions of the cardiac and skeletal contractile proteins, specifically at the level of the conformational change each Tn-C component induces in the Tn complex, and the inhibitory ability of each Tn-I.

A major effort was directed toward studying the Ca^{2+} induced conformational change in cardiac and skeletal Tn-C by a variety of spectroscopic techniques. These included CD, UV difference, derivative spectroscopy, spectrophotometric titration of tyrosine residues, ^{1}H NMR and laser photo-CIDNP. The CD and UV difference techniques point to overall similarities between these proteins at the level of high affinity sites; particularly with respect to carboxyl groups possessing elevated pKa's. A somewhat artificial difference is observed in that the tyrosine residues of cTn-C are very good probes for high affinity binding, in contrast to the case for sTn-C. Of course sTn-C does not possess a residue corresponding to tyrosine 150 at site IV. Consideration of this residue indicates significant conformational differences exist between apo-cTn-C and apo-calmodulin. Presumeably sTn-C is more similar to cTn-C in this respect. ^{1}H NMR reveals tyrosine 5 in cTn-C is also a useful monitor of high affinity binding. Possibly the defunct site I in cTn-C is responsible for the sensitivity of this residue, in contrast to tyrsoine 10 in

sTn-C. Tyrosine 5 apparently experiences the protein interior and is sensitive to Ca^{2+}-binding at sites III and IV, indicating the N-terminal regions of sTn-C and cTn-C may possess different conformations, perhaps related to their physiological functions or specific interactions with Tn-I or Tn-T. Techniques such as UV difference and first derivative spectroscopy also indicate the large environmental alterations in residues 150 and 5 upon Ca^{2+}-binding. However these effects, in contrast to those detected by NMR, represent the averaged change and it is almost impossible to interpret them in terms of a specific residue.

NMR monitors the environmental alterations at specific tyrosine residues and the comprehensive effect upon phenylalanine and aliphatic resonances as Ca^{2+} or H^{+} ions are bound. The well resolved and assigned tyrosine resonances are of most value, but the other resonances, although overlapping and difficult to interpret also allow discrimination between the conformational changes produced by Ca^{2+} or H^{+}. NMR readily reveals that quite distinct protein structures are induced by these ions, although the change in an 'averaged' parameter such as CD or UV absorption is qualitatively similar for each. This difference could be inferred by the CD and UV difference methods from the different pKa's deduced for the carboxyl groups involved in each process. However the NMR results are more easily interpreted.

It is anticipated that a detailed interpretation of the spectroscopic evidence and conclusions presented here regarding sTn-C and cTn-C will soon be possible. Recently preliminary X-ray data on rat testis and bovine brain calmodulin (Cook et al., 1980; Kretsinger et al., 1980) have been presented, as well as similar results for sTn-C from the rabbit and chicken (Strasburg et al., 1980). These crystallographic stud-

ies will provide the most conclusive evidence for the structural differences and similarities between these homologous proteins.

In order to extend these X-ray studies, the most relevant investigation would be of whole troponin. In our laboratory, attempts to crystallize rabbit skeletal troponin have been unsuccessful for a number of years. This possibly results from the highly aggregated nature of troponin in concentrated solutions (Lovell and Winzor, 1977). A similar phenomenon has been noted for bovine cardiac troponin (Byers et al., 1979). Insight into the structure of the thin filament at this point has largely come from three-dimensional image reconstruction of data from low angle X-ray studies of frog muscle (Huxley, 1972) and of electron micrographs from the reconstituted thin filament (Wakabayashi et al., 1975). This information has been coupled with biochemical evidence to yield models such as that presented in Figure 4 (Potter and Gergely, 1974). However there seems to be a problem with high level resolution of the subtle details of the thin filament interactions.

One promising technique appears to be the specific cross-linking of various components of the thin filament, thereby defining their relationships within the 'regulation unit'. A recent study (Sutoh, 1980; Sutoh and Matsuzaki, 1980) has elegantly demonstrated the possibilities of this method. A heterobifunctional reagent was incorporated into Tn-C or Tn-I prior to reconstitution of the troponin or thin filament complex. Flash photolysis generated a reactive nitrene, resulting in crosslinks to adjacent components which were analysed by quantitative gel electrophoresis. In reconstituted troponin, Tn-C formed crosslinks with both Tn-I and Tn-T, while Tn-I crosslinked to Tn-C and Tn-T. These results are in agreement with known interactions between these components. It

is relevant that no effect by Ca^{2+}-binding upon the crosslinking patterns could be demonstrated. Reconstitution of the thin filament caused changes in the crosslinking pattern, and this was affected by Ca^{2+}. One unexpected observation was that crosslinking between Tn-I and actin occurred in the presence and absence of Ca^{2+}, indicating these proteins interact under each condition. This finding contradicts results obtained from cosedimentation analysis of Tn-I - Tn-C binding to F-actin in the presence and absence of Ca^{2+} (Potter and Gergely, 1974). Here the interaction could only be demonstrated in the absence of Ca^{2+}, implying the Tn-T component was necessary for maintaining the troponin complex on the thin filament in the presence of Ca^{2+}, as depicted in Figure 3.

Studies of this nature have the potential for probing details of the interactions of the thin filament components which are dependent upon the presence of all the subunits. Data gathered from investigations with the isolated subunits should not be casually extrapolated to the whole thin filament. However, we anticipate that the differences revealed between the cardiac and skeletal troponin subunits during this study will be represented in the regulation process in each muscle.

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