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Purification and Characterization of Troponin C from Pike Muscle: A Comparative Spectroscopic Study with Rabbit Skeletal Muscle Troponin C†

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ABSTRACT: The conformation of troponin C (TN-C) isolated from the white muscle of pike (*Esox lucius*), in the Ca^{2+} and metal-free states, was studied by circular dichroism, absorption difference spectroscopy, solvent perturbation difference spectroscopy, intrinsic fluorescence, thiol titration, and ^1H nuclear magnetic resonance spectroscopy. In addition, the molecular weight of the protein was determined by sedimentation equilibrium and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The composition of the protein was established by amino acid analysis. The resulting data were compared with those from the widely studied analogue isolated from rabbit skeletal muscle. The results indicate near equivalence in many of the properties of pike and rabbit TN-C, such as molecular weight, the magnitude of the calcium-induced conformational change, and urea- or thermal-induced denaturability. However, the pike protein has five additional potential carboxyl groups, and there is good evidence from NMR, solvent perturbation, and fluorescence studies for the presence of a buried tyrosine residue in the apo state.

Troponin C (TN-C),[1] the Ca^{2+}-binding subunit of the troponin complex from mammalian skeletal muscle, is an acidic protein, isoelectric point of 4.1–4.3, with a molecular weight as established from amino acid sequence studies of 17 965 (Collins et al., 1977). The molecule has no tryptophan residues but possesses a high ratio of phenylalanine to tyrosine residues, which results in a distinctive UV absorption spectrum similar to that noted for other Ca^{2+}-binding proteins such as parvalbumin and calmodulin.

An equilibrium dialysis study (Potter & Gergely, 1975) showed that TN-C has two distinct classes of Ca^{2+}-binding sites: two sites that have a high affinity for Ca^{2+} but also bind Mg^{2+} on a competitive basis (Ca^{2+}–Mg^{2+} sites, $K_{Ca^{2+}} = 2.1 \times 10^7\ M^{-1}$; $K_{Mg^{2+}} = 5 \times 10^3\ M^{-1}$) and two sites that bind Ca^{2+} specifically but with a lower affinity, $K_{Ca^{2+}} = 3.2 \times 10^5\ M^{-1}$. The four Ca^{2+}-binding sites have been numbered I–IV from the N terminus. A chemical modification study, which included the generation of peptide fragments (Sin et al., 1978), established that the two sites found closest to the N terminus are the low-affinity sites (I and II) and the high-affinity sites are located in the C-terminal half of the molecule (III and IV). This was further confirmed by Ca^{2+}-binding studies on specific fragments of the molecule (Leavis et al., 1978).

Ca^{2+} binding to TN-C has been studied by a number of spectroscopic techniques. These include an increase in the helical content of the protein, measured by CD (Murray & Kay, 1972), and changes in intrinsic tyrosine fluorescence (Kawasaki & van Eerd, 1972) and in changes in the environment of hydrophobic residues as probed by ^1H NMR (Levine et al., 1977; Seamon et al., 1977). It has generally been demonstrated that most of the structural changes take place when Ca^{2+} or Mg^{2+} bind to the high-affinity sites. Fluorescence studies employing the dansylaziridine probe at

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[1] Abbreviations: acto-HMM, a complex of fibrous actin and heavy meromyosin; ATPase, adenosinetriphosphatase; CD, circular dichroism; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; pCa^{2+}, the negative logarithm of the free calcium ion concentration; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Mops, 4-morpholinepropanesulfonic acid; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); UV, ultraviolet; TN-C, troponin C; TN-I, troponin I; r^2, the square of the radial distance; Me_2SO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight.

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methionine-25 have monitored the effects of Ca^{2+} binding to low-affinity sites I and II (Johnson et al., 1978a). A number of other workers have demonstrated additional changes in helical content, in tyrosine fluorescence, and in the environment of hydrophobic residues accompanying low-affinity Ca^{2+} binding (Seamon et al., 1977; Johnson & Potter, 1978). It has been demonstrated by stopped-flow fluorescence experiments that it is probably the low-affinity sites that regulate muscle contraction (Johnson et al., 1978b, 1979), since the time scale of Ca^{2+} exchange from these sites and the associated changes in the protein structure are fast enough to occur during one contraction cycle. Ca^{2+}-binding exchange at the high-affinity sites of TN-C, on the other hand, is too slow to regulate contraction and probably fulfills a structural role only.

Two papers have appeared in which TN-C has been isolated from reptiles and fish muscle (Demaille et al., 1974) and the Pacific dogfish (Malencik et al., 1975). These studies describe the isolation and purfication as well as a comparison of some of their chemical and biological properties with those of the rabbit skeletal protein. It was felt that it would be of interest to extend these observations by isolating and characterizing TN-C from the white muscle of the pike (*Esox lucius*). This freshwater teleostean fish was chosen as it is considered to be more primitive in the evolutionary ladder of actinopterigian stock when compared with fish species already studied.

The purified TN-C was subjected to detailed analysis using such techniques as CD, intrinsic tyrosine fluorescence, absorption difference and solvent perturbation spectroscopy, sulfhydryl titration, and ^1H NMR. Thermal denaturation of the protein was followed by fluorescence changes. Urea denaturation was monitored by CD methods. The effect of Ca^{2+} binding on these spectroscopic properties was also established. In addition, the amino acid composition was evaluated, and the molecular weight was determined by sedimentation equilibrium and $NaDodSO_4$–polyacrylamide gel electrophoresis. These observations will be the major focal points of this paper.

Materials and Methods

Specimens of pike (*E. lucius*) were caught in a nearby lake, killed by a blow to the head, and filleted. The fillets were immediately placed on ice and preparative methods started within 2 h.

Troponin was isolated from an acetone powder prepared from the residue remaining after a low ionic strength extract; 10 mM Tris-HCl, pH 7.5, was used to isolate pike parvalbumins (unpublished work from this laboratory). The powder (~140 g) originating from 1 kg of fresh fillets was extracted overnight at 4 °C with 10 volumes of a solution of 0.6 M LiCl–50 mM Tris-HCl (at pH 7.5)–0.5 mM DTT with gentle overhead stirring. The pH of the supernatant after centrifugation was slowly lowered to pH 4.6 by addition of 1 N HCl, and the precipitate of crude tropomyosin was centrifuged down. The pH of the supernatant was brought back to 7.5 by addition of a solution of 2 M Tris and an ammonium sulfate fractionation carried out. The material of interest was collected in the range of 37–55% saturation. The protein pellet was dissolved in 10 mM NH_4HCO_3–1 mM DTT, pH 8, and dialzyed overnight against 2 changes of 150 volumes of water–0.1 mM DTT. The material was then freeze-dried.

Pike troponin was resolved into its subunits by applying the freeze-dried material to a column of DEAE-Sephadex A-25 equilibrated with 6 M urea–25 mM Tris-HCl (pH 7.5 at 4 °C)–0.5 mM DTT–2 mM EDTA; the subunits were eluted with a linear NaCl gradient from 0 to 0.5 M. Urea (Fisher) was purified by stirring with a mixed-bed resin (Bio-Rad AG-50 1-X8). The pooled fractions corresponding to TN-C were dialyzed against 100 mM NaCl–25 mM Tris-HCl (at pH 7.5)–2 mM EDTA for removal of the urea and then applied to a DEAE-Sephadex A-25 column equilibrated with the same solvent. The protein was eluted with a linear NaCl gradient from 0.1 to 0.6 M, dialyzed against 5 mM NH_4HCO_3, and freeze-dried. The yield of pure pike TN-C thus obtained is ≈400 mg. Rabbit skeletal TN-C is obtained in an essentially similar manner.

Protein concentrations were routinely measured by employing extinction coefficients determined by the synthetic boundary procedure of Babul & Stellwagen (1969). For both pike TN-C and rabbit TN-C, a value of $A_{276nm}^{1mg/mL} = 0.175$ cm^{-1} has been found. All optical measurements were made on a Cary 118C recording spectrophotometer, while CD spectra were taken on a Cary 60 spectropolarimeter, with an attached 6001 CD accessory according to Oikawa et al. (1968). Fluorescence spectra were measured on a Perkin-Elmer MPF 44B recording spectrofluorometer, operating in the ratio mode and equipped with a thermostated cell assembly. Protein samples intended for spectroscopic analysis were routinely clarified by centrifugation in a Beckman Model L ultracentrifuge. Dialysates used for reference base lines were passed through Millipore filters (pore size 0.45 μm). Sedimentation equilibrium studies were performed at 20 °C on a Beckman Spinco Model E analytical ultracentrifuge equipped with Rayleigh interference optics in accordance with methodology described by Chervenka (1969).

Amino acid analyses were performed on a Durrum D-500 automated amino acid analyzer. Samples were hydrolyzed in constant-boiling 6 N HCl for 24 or 48 h. Phenol (1%) was included to minimize degradation of tyrosine residues. Polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ was performed by the methodology of Shapiro et al. (1967). Samples were dissolved in either 2% or 10% $NaDodSO_4$ containing 1 mM DTT and heated in a boiling water bath for 2–5 min. Either 10% or 15% polyacrylamide gels were used. Gels were stained with Coomassie Brilliant Blue and destained in 7% acetic acid–7.5% methanol (v/v).

For solvent perturbation and Ca^{2+}-induced absorption difference spectroscopy, the Cary 118C 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. In addition, the reference and sample cells were thermostated by circulating water from a Lauda K 2R thermoregulator. For generation of the Ca^{2+}-induced difference spectra, a base line was drawn with both sample and reference cells containing equal volumes of the same protein solution. An aliquot of concentrated (50 mM) $CaCl_2$ solution was added to the sample cell while an equivalent volume of solvent was added to the reference cell. The contents were carefully mixed and allowed to come to thermal equilibrium, and the difference spectrum was measured. This procedure was repeated to full saturation of the protein. Solvent perturbation difference spectral measurements employed split compartment tandem cells (Hellma) (0.874-cm path length). The base line for this procedure is generated with separated protein solutions in both reference and sample compartments of the spectrophotometer. The other compartments in both cases contained a solution of the perturbant at twice the desired concentration. In the actual results presented here, 40% Me_2SO was employed since the effects were maximal with this particular solvent. The contents of the sample cell were then mixed to expose the protein to 20% Me_2SO (v/v), and the perturbation difference spectrum was recorded. Finally, the contents of the reference cells were

mixed and another base line was generated. If this did not match the original one, the results were discarded and the experiment repeated.

Ca^{2+} removal of solvents was achieved by passing them slowly through a column of Chelex 100 (Bio-Rad). Apo-TN-C's were prepared by prolonged dialysis against water containing Chelex 100, followed by a final dialysis against the Ca^{2+}-free buffer of choice. All solutions were stored, handled, or transferred in plasticware that had previously been washed in 6 N HCl and then deionized water.

Titration with DTNB (Ellman, 1959) was used to quantitate the sulfhydryl content of pike TN-C and to check the reactivity of thiol groups in the absence and presence of Ca^{2+}. To ensure complete unfolding of the protein, with consequent full exposure of all -SH groups in the unknown pike TN-C, we incorporated 8 M urea into the buffer system. Reactions were carried out in 100 mM NaCl with either 25 mM Pipes at pH 6.8 or 25 mM Mops at pH 7.2.

^1H NMR spectra were recorded at 270 MHz on a Bruker HXS-270 spectrometer operating in the Fourier transform mode with quadrature detection. Typical instrument settings were a sweep width = ±2000 Hz, 8196 data points, and a 9.5-μs pulse (90°). The spectra were digitally filtered by using the Lorentzian to Gaussian conversion subroutine provided with the Nicolet 1180 computer (DM = 5). All spectra are reported relative to the methyl resonance of DSS and were taken at 300 K. ^1H NMR spectra were run in a 100 mM NaCl–10 mM Pipes buffer, pH 6.91, in D_2O. All pH measurements in D_2O were made on a Radiometer Model PHM62 pH meter equipped with an Ingold electrode (6030-40) and are reported as direct meter readings, uncorrected for the deuterium isotope effect.

Results

The elution profile of the pike troponin subunits from the DEAE-Sephadex column in 6 M urea is essentially identical with to that obtained for rabbit skeletal troponin in this laboratory (data not shown). For both the pike and rabbit systems material corresponding to the pooled TN-C fractions displayed, on occasion, a small percentage (up to 2%) of a component of apparent M_r 37 000–40 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. A second DEAE-Sephadex chromatography carried out in benign medium removed this contaminant. Pike and rabbit TN-C's prepared in this fashion have a lower optical density in the 290–310-nm region than before and display a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis, when up to 50 μg of protein was loaded on either 10% or 15% gels. Mobilities were identical and molecular weight estimates yielded a value close to 18 000.

Sedimentation equilibrium studies on pike TN-C were carried out in a solvent system consisting of 100 mM NaCl with either 25 mM Mops at pH 7.2 or 25 mM Pipes at pH 6.8. In both instances, linear plots were obtained of ln c vs. r^2 from which a weight-average molecular weight of 18 500 ± 500 was obtained, well within the range obtained for rabbit skeletal TN-C.

Amino Acid Analysis. The amino acid composition of pike TN-C is listed in Table I along with the compositions of several selected TN-C's. It is clear that the protein from pike muscle presents the same distinctive features as found in other TN-C molecules, viz., a high ratio of phenylalanine to tyrosine (9:2), a low level of proline, no histidine, and a high ratio of (aspartic plus glutamic acid) to (lysine plus arginine). With direct comparison to rabbit skeletal TN-C, the pike protein possesses five additional potential carboxyl functions and minor differences at the level of isoleucine, leucine, and alanine.

Table I: Amino Acid Compositions of Several TN-C's Isolated from Various Species

	no. of residues/molecule				
amino acids	pike[a]	rabbit[b]	dog-fish[c]	hake[d]	chicken[e]
aspartic acid	24.9	22	26.9	34.5	25
threonine	6.1	6	8.6	3.1	7
serine	7.3	7	6.7	7.9	6
glutamic acid	32.9	31	39.6	30.6	30
glycine	13.0	13	14.1	10.1	13
alanine	10.1	13	11.3	9.8	13
valine	5.5	7	7.9	6.2	6
methionine	8.8	10	9.0	7.4	11
isoleucine	7.8	10	8.9	8.4	11
leucine	14.0	9	12.7	15.0	10
tyrosine	1.9	2	1.9	1.5	0
phenylalanine	9.2	10	10.7	8.7	11
histidine		1	1.0	0.3	1
lysine	8.2	9	11.4	11.8	10
arginine	7.3	7	5.5	7.2	6
half-cystine	1.0	1	0.8	0.5	1
proline	1.0	1	1.6	1.8	1
tryptophan	0	0	1.8	0	0

[a] Average values of 24- and 48-h hydrolysis times. [b] Sequence data of Collins et al. (1977). [c] Malencik et al. (1975). [d] Damaille et al. (1974). [e] Sequence data of Wilkinson (1976).

Ca^{2+}-Induced Conformational Changes. (a) Circular Dichroism. A number of publications have arisen from this laboratory describing the utility of CD for following the vast increase in secondary structure that accompanies Ca^{2+} binding to TN-C (Murray & Kay, 1972; McCubbin & Kay, 1973). All these studies have included EGTA in the buffer system to maintain a Ca^{2+}-free state, which as necessitated the use of complex calculations for the free Ca^{2+} concentration. In this work, the apoprotein was prepared by exhaustive dialysis followed by Chelex 100 treatment. Ellipticity values obtained were comparable to those found in the presence of 2 mM EDTA, so it was concluded that Ca^{2+} removal was essentially complete: e.g., in a solvent of 100 mM NaCl–25 mM Mops at pH 7.2 $[\theta]_{221nm}$ for rabbit skeletal TN-C is −10 500 ± 300 deg cm^2 dmol^{-1}. In the presence of sufficient Ca^{2+} to saturate all four Ca^{2+}-binding sites, $[\theta]_{221nm}$ rises to −15 900 ± 300 deg cm^2 dmol^{-1}. Some representative far-UV CD data for pike TN-C is shown in Figure 1. The inset shows the Ca^{2+}-induced conformational change whereby $[\theta]_{221nm}$ rises from an initial value of −10 200 ± 300 deg cm^2 dmol^{-1} to a final value of −15 800 ± 300 deg cm^2 dmol^{-1}. The main plot displays the increase in α-helical content that accompanies Ca^{2+} binding. In agreement with data for the rabbit skeletal protein, most of the structural change is associated with the binding of the first mole of Ca^{2+}. The aromatic CD spectrum of pike TN-C (data not shown) is very similar to the published data for rabbit skeletal TN-C (Murray & Kay, 1972).

Several studies have indicated that protons are able to mimic, to a certain extent, the ability of Ca^{2+} to evoke a conformational change in skeletal TN-C and that this effect is due to an increase in the value of the initial ellipticity (Hincke et al., 1978; Leavis & Lehrer, 1978; Lehrer & Leavis, 1974). Figure 2 shows the titration of apo pike and rabbit TN-C with protons over the pH range 5–8.5. For the rabbit protein, groups with pK_a values of 6.2–6.3 are responsible for the increase in ellipticity, while for the pike protein, the pK_a values are nearer 5.9. The final ellipticity, after Ca^{2+} addition to both proteins, is essentially constant over this pH range, in agreement with earlier observations (Hincke et al., 1978).

(b) Absorption Difference Spectra. Typical Ca^{2+}-induced difference spectra for pike and rabbit TN-C are displayed in

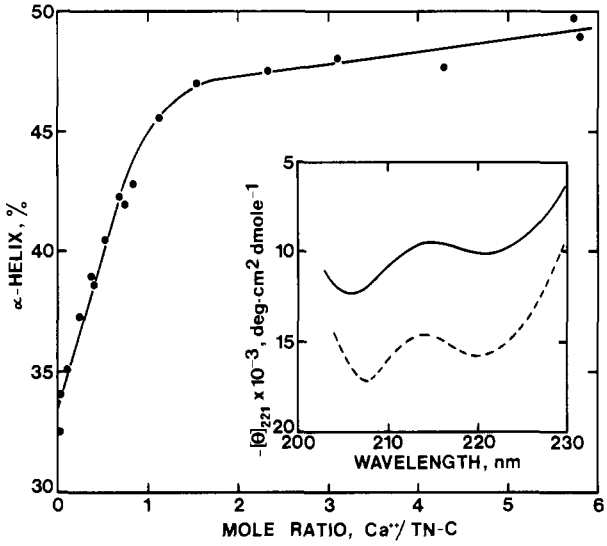

FIGURE 1: Ca^{2+} titration of pike TN-C. The solvent was 100 mM NaCl–25 mM Mops, pH 7.2. The protein concentration was 0.92 mg/mL. The main figure shows α helix [calculated from the equations of Chen et al. (1974)] plotted against moles of Ca^{2+} bound to TN-C. The inset displays portions of the far-UV CD spectra for apo-pike TN-C (—) and the Ca^{2+}-saturated form (---).

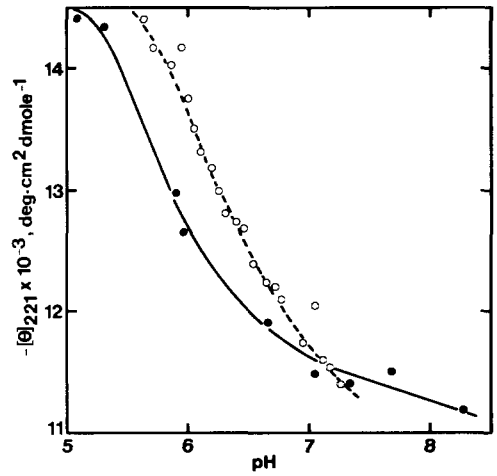

FIGURE 2: Ellipticity at 221 nm of apo pike TN-C (●) and apo rabbit TN-C (○) plotted as a function of pH. Concentration of protein in both cases was ≈1 mg/mL in 100 mM NaCl–10 mM Pipes. The path length of the cell used was 0.05 cm.

Figure 3. These spectra were obtained by addition of sufficient Ca^{2+} to the sample cell to saturate both the high- and low-affinity sites. An equal volume of solvent was added to the reference cell. With respect to the rabbit protein, the spectra were comparable to those obtained earlier (Hincke et al., 1978) with some differences in peak amplitudes. The enhanced contribution from phenylalanine residues, as well as the diminished tyrosine signal, should be noted for the pike TN-C.

The far-UV (i.e., from 250 to 210 nm) Ca^{2+} difference spectra of pike and rabbit TN-C were measured in a similar manner, except that cells of path length 0.05 cm were employed. The results are shown in Figure 4, and the positive peaks are consistent with the transfer of phenylalanine and tyrosine residues from solvent water to a hydrophobic medium. The inset shows a titration of the magnitude of the peaks at 234 and 219 nm, as a function of Ca^{2+} concentration, for pike TN-C.

(*c*) *Solvent Perturbation Difference Spectra.* The extent of exposure of the phenolic chromophore of the two tyrosine

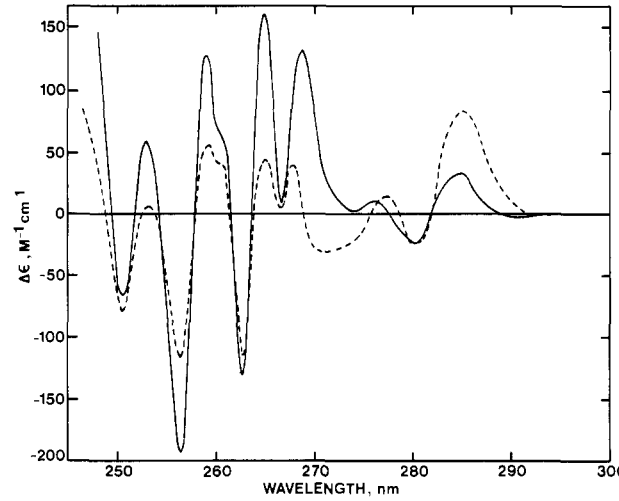

FIGURE 3: This figure shows typical Ca^{2+}-induced absorption difference spectra for pike TN-C (—) with 4.9 mol of Ca^{2+}/mol of Tn-C and rabbit skeletal TN-C (---) with 4.7 mol of Ca^{2+}/mol of Tn-C. The solvent in both cases was 100 mM NaCl–25 mM Mops at pH 7.2. The protein concentration in both cases was 2.2 mg/mL.

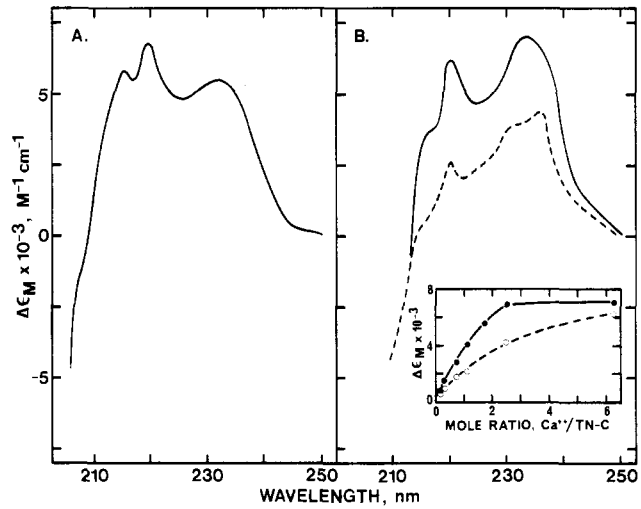

FIGURE 4: This figure shows Ca^{2+}-induced absorption difference spectra in the wavelength region 250–210 nm for rabbit skeletal TN-C (panel A) with 4.9 mol of Ca^{2+}/mol of TN-C. Panel B shows two sets of data for pike TN-C: (---) at 2 mol Ca^{2+} and (—) at 4 mol of Ca^{2+}/mol of TN-C. The inset in panel B shows titrations of the difference spectral peaks as functions of Ca^{2+} ion concentration: (●) 234-nm peak and (○) 219-nm peak. The solvent in both cases was 100 mM NaCl–25 mM Mops at pH 7.2. The protein concentration was 2.2 mg/mL. The optical path length was 0.05 cm.

residues in TN-C in the absence and presence of Ca^{2+} may be determined by solvent perturbation spectroscopy, following the procedures of Herskovits & Laskowski (1962a,b). The perturbant of choice in this study is Me_2SO, used at a final concentration of 20% (v/v). The degree of chromophore exposure is determined by comparison with data from the model compound *N*-acetyltyrosine ethyl ester, obtained under the same experimental conditions. The results for several TN-C samples, as well as the nonmuscle Ca^{2+}-binding protein calmodulin, are listed in Table II. In apo rabbit skeletal TN-C only partial exposure of the two chromophores is noted, which is somewhat reduced in the presence of Ca^{2+}. All three tyrosines are considered exposed in apo bovine cardiac TN-C, while addition of Ca^{2+} seems to bury one residue. Calmodulin shows only fractional exposure of one residue, which is slightly reduced by Ca^{2+} addition. Pike TN-C behaves rather like calmodulin in that only fractional exposure of one residue is

Table II: Degree of Exposure of the Tyrosyl Side Chains of Several Ca^{2+}-Binding Proteins As Determined by Solvent Perturbation in 20% Me_2SO

protein	no. of Tyr	minus Ca^{2+} $\Delta\epsilon_{M279}$ (nm)	minus Ca^{2+} $\Delta\epsilon_{M286}$ (nm)	minus Ca^{2+} fractional exposure	plus Ca^{2+} $\Delta\epsilon_{M279}$ (nm)	plus Ca^{2+} $\Delta\epsilon_{M286}$ (nm)	plus Ca^{2+} fractional exposure[a]
rabbit TN-C	2	215	335	1.39	149	260	1.08
bovine cardiac TN-C	3	477	769	3.20	264	487	2.03
calmodulin	2	134	209	0.87	80	147	0.61
pike TN-C	2	63	198	0.83	61	117	0.49

[a] The fractional exposure was calculated from the ratio of the change in molar extinction coefficients at 286 nm ($\Delta\epsilon_{286nm}$) noted for the various proteins and the model compound *N*-acetyltyrosine ethyl ester, which gave a value of $\Delta\epsilon_{286nm} = 240\ M^{-1}\ cm^{-1}$ when measured under identical conditions.

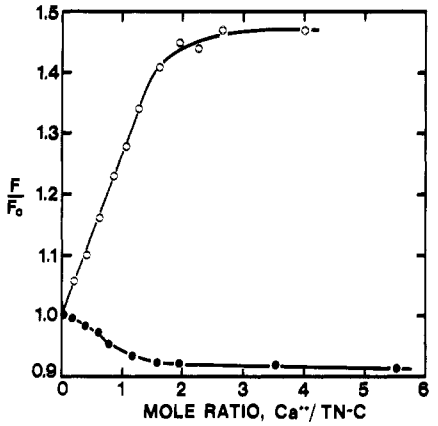

FIGURE 5: Fluorescence titrations of pike TN-C (●) and rabbit skeletal TN-C (○) with Ca^{2+}. The ratio of the fluorescence with added Ca^{2+} (F) and the fluorescence without added Ca^{2+} (F_0) is plotted against the mole ratio of Ca^{2+} to protein. Excitation was at 276 nm and the emission monitored at 304 nm. Temperature is 20 °C. The solvent is 100 mM NaCl–25 mM Mops, pH 7.2. The pike TN-C concentration was 1.22×10^{-5} M and the rabbit TN-C concentration was 2.3×10^{-5} M.

seen in the apo form, which is further reduced by Ca^{2+} addition.

(*d*) *Thiol Titration.* Rabbit skeletal TN-C (prepared by the methodology described) routinely shows 0.93–0.98 mol of thiol/mol of TN-C, when assayed by DTNB titration. The reaction is practically instantaneous in the absence of Ca^{2+}, but in the presence of this cation, the rate is slowed down somewhat. No kinetic analysis has been performed, but after 5 min of reaction about 0.85–0.90 mol of thiol/mol of TN-C can be detected. Titration of apo pike TN-C in 8 M urea–100 mM NaCl–25 mM Pipes (at pH 6.8)–2 mM EDTA shows the instantaneous exposure of one thiol group per mole of protein. The reaction rate in benign medium in the absence and presence of Ca^{2+} seemed to be comparable to that observed for the rabbit protein.

(*e*) *Fluorescence.* Since neither pike nor rabbit TN-C has any tryptophan residues, the intrinsic fluorescence of both proteins is due entirely to the tyrosine residues. The emission spectrum of apo pike TN-C in saline buffer at neutral pH is essentially the same as the spectrum of rabbit skeletal TN-C and need not be reproduced here. Upon excitation at 276 nm both proteins display an emission maximum near 304 nm. The relative quantum yield for the pike protein is somewhat lower than that for the rabbit protein: $R_{Tyr} = 0.32$ compared with $R_{Tyr} = 0.40$ at 24 °C (Lehrer & Leavis, 1974), suggesting a reduction in the relative exposure to solvent for one or both of the tyrosine residues in the fish protein.

The effect of Ca^{2+} on the tyrosine fluorescence of pike and rabbit TN-C is shown in Figure 5. The properties of the two proteins diverge dramatically when studied by this approach.

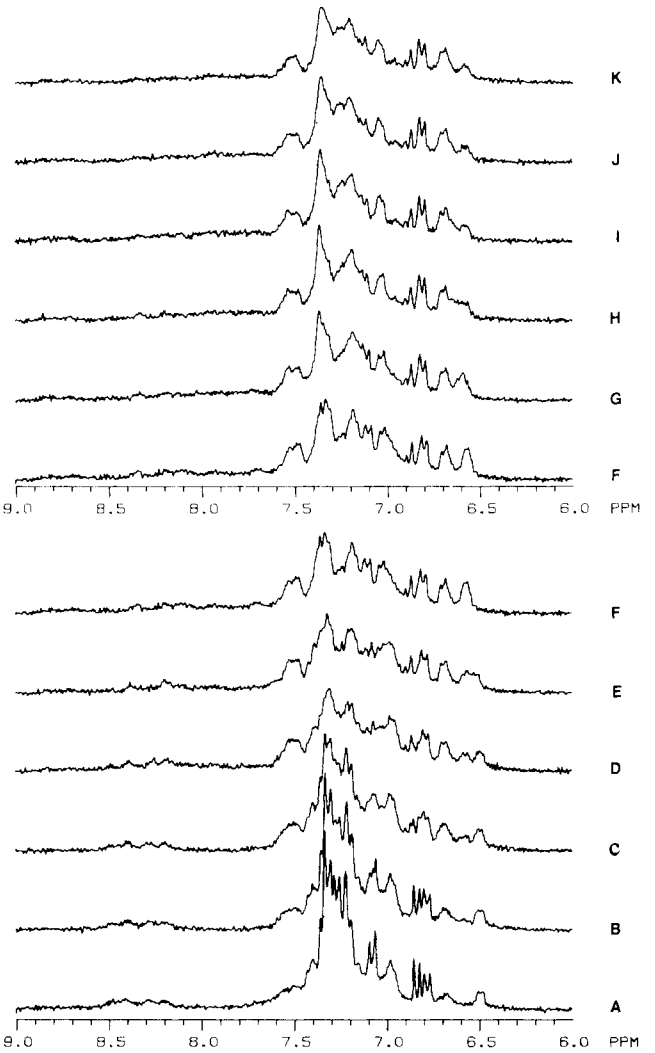

FIGURE 6: Aromatic region of the 270-MHz ^1H NMR spectrum of pike TN-C shown as a function of added calcium. 1.2 mM pike TN-C–0.1 mM NaCl–10 mM Pipes, pH 6.91; 250 scans/spectrum. The ratios of added calcium to protein were (A) 0.0, (B) 0.48, (C) 0.95, (D) 1.43, (E) 1.91, (F) 2.37, (G) 2.86, (H) 3.33, (I) 3.80, (J) 4.77, and (K) 5.70.

Rabbit TN-C shows the well-known 50% increase in fluorescence intensity that has been well documented (Lehrer & Leavis, 1974). The fluorescence of pike TN-C, on the other hand, is quenched by about 10% by the action of this cation. This lack of fluorescence enhancement is an obvious reflection of differences in the microenvironment around tyrosine residues as a result of Ca^{2+} binding to the two proteins.

(*f*) *^1H NMR Studies.* The aromatic region of the 270-MHz ^1H NMR spectrum of pike TN-C is shown in Figure 6 as a function of added Ca^{2+}. All of the spectral features reported

Table III: Chemical Shifts of Tyrosine Residues in Pike TN-C

		δ_{AH}	δ_{A^-}
	Apo Pike TN-C		
narrow	$pK_a = 10.64 \pm 0.03$	6.833 ± 0.003	6.567 ± 0.003
broad	$pK_a = 11.09 \pm 0.04$	6.779 ± 0.003	6.510 ± 0.005
	Ca^{2+}-Pike TN-C		
downfield	$pK_a = 10.67 \pm 0.02$	6.886 ± 0.002	6.638 ± 0.002
upfield	$pK_a = 10.93 \pm 0.04$	6.805 ± 0.004	6.532 ± 0.005

are very similar to those reported for rabbit skeletal TN-C by Seamon et al. (1977) with the exception of the tyrosine residues. The spectrum of apo pike-TN-C shown in Figure 6A results mostly from the phenylalanine residues, including the unique upfield-shifted phenylalanine residues seen for the calcium-binding proteins between 6.4 and 6.7 ppm (Levine et al., 1977; Seamon et al., 1977; Hincke et al., 1981). Other resolvable nonphenylalanyl resonances include the slowly exchanging NH resonances between 8.0 and 8.5 ppm, a tyrosine meta (2,6) protons doublet resonance centered at 7.1 ppm, and two tyrosine ortho (3,5) protons doublet resonances centered at 6.80 and 6.83 ppm. The broader upfield doublet resonance has a line width similar to that observed for tyrosines in other calcium-binding proteins such as rabbit skeletal and cardiac TN-C, and this tyrosine resonance is relatively insensitive to calcium binding as are the two tyrosines of rabbit skeletal TN-C and tyrosine-111 of bovine cardiac TN-C. The second tyrosine (3,5) protons doublet resonance at 6.83 ppm in apo-pike-TN-C is uniquely narrow, suggesting a relatively mobile tyrosine residue.

As the apo pike-TN-C is titrated with calcium, dramatic changes occur in the ^1H NMR spectrum (see Figure 6B–K). The changes that occur happen in two distinct phases, 0–2 mol of calcium/TN-C (Figure 6A–F) and 2–4 mol of calcium/TN-C (Figure 6F–K). As the first 2 mol of calcium is added, the overall spectral envelope is altered. Specifically, the narrower tyrosine (3,5) ortho protons doublet resonance and the tyrosine (2,6) meta protons doublet resonance are broadened and disappear and are replaced by new resonances at 6.89 and 7.10 ppm, respectively, which are not further affected by the binding of the second 2 mol of calcium. The broader tyrosine (3,5) ortho protons doublet resonance is shifted slightly to 6.81 ppm upon binding the first 2 mol of calcium and then does not shift further. The upfield-shifted phenylalanine resonance at 6.5 ppm [labeled D by Seamon et al. (1977)] disappears and is replaced by a new resonance at 6.6 ppm. These changes, which occur as the first 2 mol of calcium are added to the protein, all occur in the NMR slow exchange limit. Taking the change in the resonance position of the narrower tyrosine (3,5) ortho protons doublet resonance as an example ($\Delta\delta = 0.06$ ppm), this implies that the rate constant for release of calcium from the two tight sites of the protein is $k_{off} < 1 \times 10^{-3}$ s^{-1}.

As the last two calciums are added to the protein, further changes occur in the spectrum. An example is the gradual shift in the position of the resonance intensity in the unfield phenylalanine region from 6.6 to 6.7 ppm (see Figure 6G–J). These changes occur in the NMR fast exchange limit; that is, for these weaker sites $k_{off} > 6 \times 10^{-3}$ s^{-1}. No changes occur in the spectrum beyond 4 mol of calcium/TN-C.

The protein was titrated in both the apo and calcium-saturated form. The initial and final chemical shifts and the pK_as for the tyrosine residues are listed in Table III. Figure 7 shows the data for the titration of the tyrosine (3,5) ortho protons doublet resonance at 6.81 ppm in calcium-saturated TN-C. All tyrosine titration curves were similar, ideal one-proton titration curves. The pK_as of the narrower and broader tyrosine (3,5) ortho protons doublet resonances in apo-TN-C were 10.64 and 11.09, respectively, which change to 10.67 and 10.93, respectively, in the calcium-saturated protein.

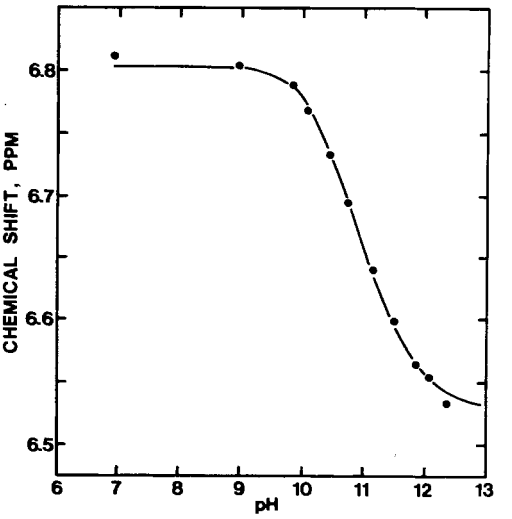

FIGURE 7: Observed ^1H NMR chemical shift of the tyrosine ortho (3,5) protons doublet resonance at 6.81 ppm at pH 7 in calcium-saturated pike TN-C plotted as a function of pH. The solid curve is for an ideal one-proton titration curve with a pK_a of 10.93 ± 0.04.

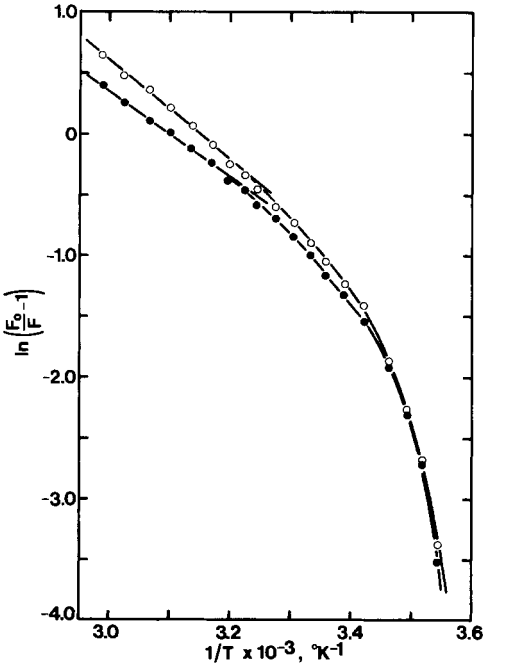

FIGURE 8: Arrhenius plot of the fluorescence temperature dependence for apo pike TN-C (●) and apo rabbit skeletal TN-C (○). Solvent is 100 mM NaCl–25 mM Pipes, pH 7.35. Excitation was at 276 nm and fluorescence was observed at 304 nm. The protein concentration was 3.2×10^{-6} M. F_0 is the fluorescence at the lowest temperature studied, and F is the fluorescence at a temperature above this.

(*g*) *Thermal Denaturation Studies.* Since the rabbit is a warm-blooded mammal and the pike a cold-blooded fish, it was felt that useful structural information might be gleaned by following the thermal denaturation of pike and rabbit TN-C by fluorescence techniques, in the absence and presence of Ca^{2+}. These thermal studies were carried out in 100 mM NaCl–25 mM Pipes, pH 7.35, and cover the temperature range from 5 to 65 °C. The thermal unfolding profiles for the apoproteins are presented in Figure 8. At temperatures below 25 °C or so, the profiles for the two proteins are very similar. Above the ambient temperature, the curves diverge

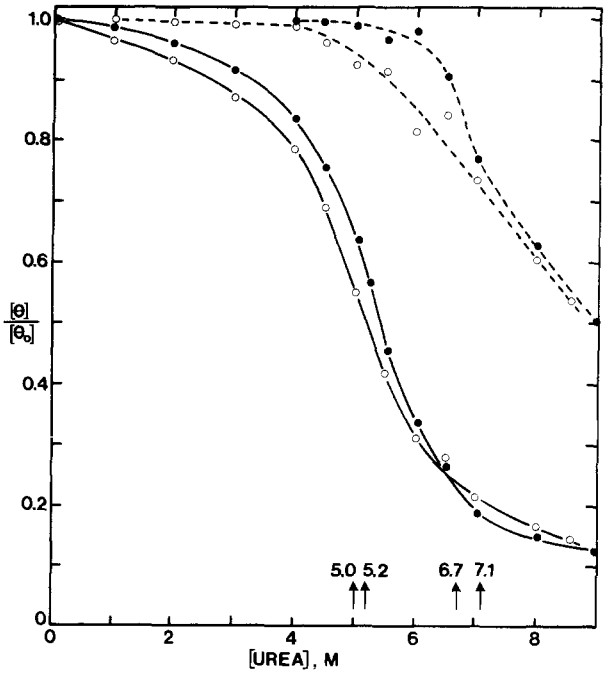

FIGURE 9: Variation of the relative CD intensity for pike (●) and rabbit (○) TN-C in the absence (—) and presence (---) of Ca^{2+}, as a function of the urea concentration. The protein concentration was 0.45 mg/mL. The temperature was 27 °C. $[\theta]$ is the ellipticity at 221 nm at any particular concentration of urea. $[\theta]_0$ is the value in the absence of urea. The arrows refer to the midpoints of the transitions.

and it is apparent that pike TN-C is more heat sensitive than its rabbit counterpart. This is reflected in the values of activation energy for quenching as calculated from the limiting slopes of the denaturation profiles at high temperature. For rabbit TN-C, E_a = 8.1 kcal mol^{-1}, while for pike TN-C, E_a = 6.9 kcal mol^{-1}. In the presence of sufficient Ca^{2+} to saturate all binding sites E_a for rabbit TN-C is 6.9 kcal mol^{-1}, while for pike TN-C, E_a is 6.0 kcal mol^{-1}.

(h) Urea Denaturation. Urea-induced unfolding of the two molecules was monitored by far-UV CD measurements of ellipticity at 221 nm, reflecting the average backbone conformation in the absence and presence of Ca^{2+}. Figure 9 shows the decrease of the CD signal for pike and rabbit TN-C upon unfolding by increasing concentrations of urea. Apo pike TN-C appears to be marginally more stable than apo rabbit TN-C, the transition midpoint occurring at 5.2 M urea for the former, compared with a value of 5.0 M for the latter. In the presence of Ca^{2+} the difference is exaggerated; the midpoints are now 7.1 and 6.7 M urea, respectively. The remarkable stabilizing effect of Ca^{2+} on both proteins is noteworthy. The ellipticities of samples exposed to 8 M urea and then dialyzed extensively against the starting buffer are within experimental error of the original values, suggesting that urea denaturation of TN-C is reversible.

Since the processes are apparently reversible the denaturation curves may be subjected to quantitative analysis to extract thermodynamic parameters related to the stability of the native proteins. The data treatment utilized is that of Greene & Pace (1974) wherein if a two-state unfolding mechanism is assumed, the equilibrium constant, K, and the free energy of denaturation, ΔG_{app}, can be determined from the experimental data by using the equation

$$K = e^{-\Delta G_{app}/(RT)} = ([\theta]_N - [\theta])/([\theta] - [\theta]_D) \quad (1)$$

where $[\theta]$ is the observed ellipticity at any particular urea concentration and $[\theta]_N$ and $[\theta]_D$ are the ellipticities of the native and denatured states. It is found that ΔG for the unfolding of both TN-C's varies in a linear fashion with the urea concentration as depicted in Figure 10, and these data are fit to eq 2 as proposed by Greene & Pace (1974):

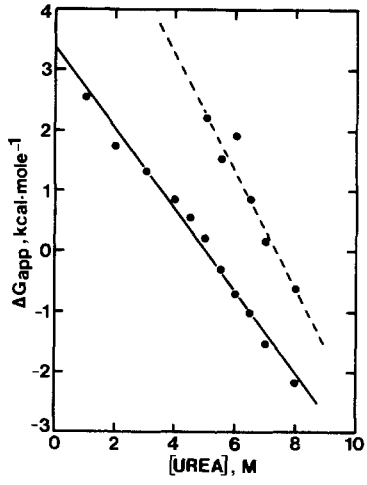

FIGURE 10: Variation of the free energy of unfolding, ΔG_{app}, for pike TN-C in the absence (—) and presence (---) of Ca^{2+}. ΔG_{app} is calculated from eq 1 and is plotted here as a function of urea concentration. The lines are least-squares fits to the data by using eq 2. The temperature was 27 °C. The solvent was 100 mM NaCl–25 mM Pipes, pH 6.81.

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m[\text{urea}] \quad (2)$$

For apo pike TN-C, m = 0.67 kcal L mol^{-2} and $\Delta G_{app}^{H_2O}$ = 3.3 kcal mol^{-1}, while for rabbit TN-C, m = 0.58 kcal L mol^{-2} and $\Delta G_{app}^{H_2O}$ = 2.7 kcal mol^{-1}. In the presence of Ca^{2+}, for pike TN-C, m = 0.97 kcal L mol^{-2} and $\Delta G_{app}^{H_2O}$ = 7.1 kcal mol^{-1}. For rabbit TN-C, m = 0.68 kcal L mol^{-2} and $\Delta G_{app}^{H_2O}$ = 4.5 kcal mol^{-1}.

Discussion

The data presented above show that Tn-C isolated from a very primitive fish source (pike) is very similar in many of its properties to the widely studied protein from rabbit skeletal white muscle. There are, however, some intrinsic differences in properties that may be relevant to the biological function of this primitive fish muscle.

Previous studies on the isolation and characterization of TN-C from the Pacific dogfish (Malencik et al., 1975), as well as two types of reptiles and one fish, the hake (Dmaille et al., 1974), indicated that the protein as isolated from these diverse sources was similar in many respects to the protein from rabbit muscle. Differences in amounts of Ca^{2+} bound and amino acid composition were noted. The interest in pike as a source of TN-C is that it is a very primitive fish and therefore would be worthy of study from an evolutionary point of view.

The far-UV CD Ca^{2+} titrations on rabbit skeletal TN-C of Hincke et al. (1978) demonstrated that at pH 6.94 about 70% of the ellipticity change noted at 221 nm is associated with binding Ca^{2+} to high-affinity sites with an apparent binding constant, K_b, of 1.8 × 10^7 M^{-1} and that about 20% is due to Ca^{2+} binding at sites with lower affinity, viz., K_b = 4.48 × 10^5 M^{-1}. The remaining 10% or thereabouts is affected by nonphysiological Ca^{2+} binding to very weak sites, K_b = 2.3 × 10^3 M^{-1}.

Pike TN-C was titrated with Ca^{2+} in 100 mM NaCl–50 mM Mops–1 mM EDTA at pH 7.2 according to the CD methodology of Hincke et al. (1978) (data not shown). It was found that about 95% of the induced conformational change occurs over a decade of Ca^{2+} ion concentration, viz., from

pCa^{2+} 8 to 7. This extremely sharp transition is more reminiscent of data obtained for bovine cardiac TN-C (Hincke et al., 1978). The binding constant calculated for this transition is $\approx 5 \times 10^7$ M^{-1}. This result implies either a lack of low-affinity binding sites in pike TN-C or that CD, in this instance, cannot monitor this binding. The ^1H NMR results presented herein suggest that 4 mol of calcium is capable of binding to the protein.

The magnitude of the Ca^{2+}-induced change in $[\theta]_{221nm}$ noted at pH 7.2 for pike TN-C is −5600° for 55% of the initial value for the apoprotein. If the data is plotted as the change in α-helical content as a function of moles of Ca^{2+} bound, it is apparent that most of the change accompanies the binding of 1 mol of Ca^{2+} to the protein. This is completely analogous to rabbit TN-C where most of the structural change occurs in region III (Nagy et al., 1975, 1978; Collins et al., 1977).

The pH titration of $[\theta]_{221nm}$ for apo pike TN-C again shows a similar pattern to that noted for rabbit TN-C, but the apparent pK of the ionizable groups controlling this transition seem to be somewhat lower, viz., 5.9 vs. a value of 6.2–6.3 for the rabbit protein. It is still highly probable that there are abnormal carboxyl groups but that the environment around them has been modified somewhat by either extra carboxyl residues present or a change in the disposition of key hydrophobic functions or some combination of both.

The Ca^{2+}-induced absorption difference spectrum of pike TN-C shows dramatic differences from that of its rabbit counterpart, namely, the reduced signal corresponding to perturbation of tyrosine residues and the considerably enhanced phenylalanine signal. On the premise that the relatively small positive difference spectral change, taken together with the small CD spectral difference, suggests an alteration in the electrical charge balance in the environment of the tyrosine residues in rabbit skeletal TN-C (Donovan, 1969), then this charge alteration is clearly modified and reduced for pike TN-C. Again, this may relfect the presence in the molecule of extra carboxyl functions, altered hydrophobic regions, or some additional justapositioning not yet understood. The considerably enhanced peak to peak heights noted below 270 nm for pike TN-C imply the involvement of considerably more than the three to five phenylalanine residues believed to be transferred from solvent water to a hydrophobic medium in rabbit skeletal TN-C (Nagy et al., 1978). Thus, the environment of some or all of the phenylalanine residues is different in the two proteins as far as Ca^{2+}-induced folding is concerned.

The Ca^{2+}-induced absorption difference spectrum of rabbit TN-C in the region 250–215 nm shows positive peaks near 234 nm, probably representing a contribution from the transfer of tyrosine residues to a more nonpolar environment, while those near 219 nm represent an analogous situation for phenylalanine residues. Although molar extinction coefficients are known for the transfer of these residues (Donovan, 1973), no attempt has been made to calculate the actual numbers of each type of residue which are buried. The data in the inset of Figure 4 show that the tyrosine contribution, as measured at 234 nm, is apparently complete after binding of 2 mol of Ca^{2+}/mol of protein, whereas the signal from phenylalanine continues to rise throughout the titration. This might be a reflection of metal binding to low-affinity sites in pike TN-C.

Although this study is designed primarily to compare pike TN-C with rabbit skeletal TN-C, for a more complete picture the solvent perturbation experiments have been extended to include bovine cardiac TN-C and calmodulin. It has been demonstrated that apo rabbit skeletal TN-C shows a single class of tyrosines with a pK_a value of 10.4 (Seamon et al., 1977). This figure is somewhat higher than the usually accepted values of 9.5–10.0 for a fully exposed tyrosine residue in a protein matrix (Nozaki & Tanford, 1967), but as Klee (1977) has pointed out, this elevated pK_a value could be the result of hydrogen bonding of tyrosine residues located on the surface of the protein to nearby carboxyl groups. It is apparent that Me_2SO (molecular diameter 4.0 Å) does not have unrestricted access to both tyrosine chromophores. Furthermore, in the presence of Ca^{2+}, this access by solvent becomes more restricted. This is in accordance with absorption difference results presented here.

The three tyrosine residues in apo bovine cardiac TN-C also exhibit a single pK_a value (10.5) (Hincke et al., 1981). The solvent perturbation results show that 20% Me_2SO completely solvates the phenolic group of all three tyrosine residues, whereas in the presence of Ca^{2+}, one residue become inaccessible. This is in agreement with absorption difference spectral results (Hincke et al., 1978) and a recent NMR study (Hincke et al., 1981), wherein it was shown that in Ca^{2+}-saturated bovine cardiac TN-C, tyrosine-150, located in binding region IV, is buried in the hydrophobic core of the protein.

On the basis of the pK_a values for the two tyrosines in apocalmodulin, 10.4 and 11.9, it is apparent that one residue may be considered buried, and as Seamon et al. (1977) showed, this is tyrosine-138 in binding site IV. This is confirmed by the solvent perturbation data, where restricted approach of solvent to only one residue is noted. This accessibility is slightly decreased in the presence of Ca^{2+}.

The ^1H NMR results described here indicate that in apo pike TN-C the two tyrosine residues have different pK_a values, viz., 10.6 and 11.1. The tyrosine residue with a pK_a of 10.6 has a very narrow resonance, suggesting that it is mobile. The residue with a pK_a of 11.1 is broader and more characteristic of a tyrosine bound to a protein of M_r 18 000. This tyrosine has very similar chemical shifts, pK_as, and calcium sensitivity to those of tyrosine-111 of bovine cardiac TN-C or the two indistinguishable (by NMR) tyrosines (10 and 109) of rabbit skeletal TN-C. Upon calcium binding, the ^1H NMR shows that the environment of the narrower tyrosine resonance is changed, including a change in line width to one more characteristic of a less mobile residue. In confirmation of this fact, Me_2SO has fractional access to only one residue, which is considerably reduced in the Ca^{2+}-saturated form. Neither tyrosine changes the pK_a much upon calcium binding.

The reactivity of the single thiol group in rabbit TN-C (cysteine-98) is dependent on the Ca^{2+} concentration (Potter et al., 1976). Cox et al. (1981) believe that the variability in reactivity of cysteine-98 noted in different laboratories is possibly due to carbamoylation of the thiol group, due to prolonged exposure to high concentrations of urea. For rabbit and pike TN-C samples prepared as described in this study, essentially one thiol group per molecule is found by DTNB titration in the absence of Ca^{2+}. The reactivity in the Ca^{2+}-saturated form, although somewhat reduced, is quite comparable, suggesting a similarity in environment for the thiol group in each protein.

The intrinsic tyrosine fluorescence of rabbit skeletal TN-C has been extensively studied (Lehrer & Leavis, 1974; Leavis & Lehrer, 1978). These workers showed that the fluorescence enhancement associated with Ca^{2+} binding to the protein results from a dequenching mechanism involving the neutralization of vicinal carboxyl groups by the metal ion rather than an indirect affect associated with the observed conformational

changes. In addition, the relative contribution of each of the two tyrosines to the overall fluorescence was computed in the absence and presence of Ca^{2+}. It was found that the ratio of the fluorescence quantum yield of tyrosine-109 to that of tyrosine 10 was about 2 in the absence of Ca^{2+} and increased to more than 3 when Ca^{2+} was bound. The reduction in relative quantum yield of the tyrosine fluorescence for pike TN-C is indicative of a reduced degree of solvent exposure to a partially buried tyrosyl chromophore in this protein (Cowgill, 1968), which agrees with the various other data presented, e.g., the reduced tyrosine contribution to the absorption difference spectrum and the fractional exposure of one tyrosine residue as measured by solvent perturbation spectroscopy using 20% Me_2SO. The effect of Ca^{2+} binding, a 10% quenching rather than the 50% enhancement noted for rabbit TN-C, suggests a remarkable difference in the microenvironment of one of the tyrosine residues of the pike TN-C system. What factors might give rise to this considerable difference in fluorescence behavior? The pK_a value of the groups controlling the backbone conformational change in pike TN-C appears to be 5.9, suggesting that they are abnormal carboxyl groups, as in rabbit TN-C. The lack of dequenching noted upon addition of Ca^{2+} is probably a reflection of the partially buried nature of one of the tyrosine residues coupled with an environment that includes several extra negative charges acting in concert to shield the fluorophore. Even when some of the carboxyls are neutralized by Ca^{2+} binding, this shielding effect still operates and the normal fluorescence enhancement is no longer observed.

If one invokes sequence homology between pike and rabbit TN-C, then one tyrosine residue should be analogous to tyrosine-109 in rabbit TN-C and be responsive to Ca^{2+} binding. The other would be located near the N terminus and should display essentially similar fluorescence properties irrespective of the presence or absence of Ca^{2+}, as suggested by Leavis & Lehrer (1978).

Unfolding of pike and rabbit TN-C has been accomplished both by thermal means and by urea addition. Following the thermal unfolding by monitoring fluorescence changes has indicated differences in the activation energy for quenching at high temperatures implying subtle structural differences between the two protein systems. One wonders if this is a reflection of the difference in evolutionary level between the warm-blooded rabbit and the cold-blooded pike. The urea-induced unfolding of pike and rabbit TN-C has been followed by CD in the absence and presence of Ca^{2+}. The denaturation curves indicate a high degree of structural resistance, particularly so in the Ca^{2+}-saturated form, to the action of this denaturant, as indicated by the high values for the midpoints of the transitions. That the transitions show weak cooperativity, particularly in the absence of Ca^{2+}, is indicated by the small values of m (calculated by eq 2). One possible answer to this is that the systems are showing a deviation from the assumed two-state mechanism (Pace, 1975).

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