Physicochemical Studies on the Interaction of the Calcium-Binding Protein (Troponin C) with the Inhibitory Protein (Troponin I) and Calcium Ions†

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ABSTRACT: A complex of troponin C and inhibitory protein in 1:1 mole ratio has been prepared and subjected to physicochemical analysis using sedimentation velocity, circular dichroism, biological, and fluorescence techniques. It has been demonstrated that the sedimentation constant is greater than that of either constituent and the value increases in the presence of Ca^{2+}. Circular dichroism measurements indicated that the interaction of troponin C with inhibitory protein produces a slight conformational change. These studies also showed that the Ca^{2+}-induced conformational change still occurred in the complexed state, and the binding of Ca^{2+} was enhanced. Circular dichroism melting experiments revealed that the structure, in the presence of Ca^{2+}, was more resistant to thermal denaturation than that produced in the absence of Ca^{2+}. Biological studies indicated no effect by the complex, on the Mg^{2+}-activated ATPase of synthetic actomyosin, suggesting that the effect of the inhibitory protein had been neutralized. Flourescence measurements with 8-anilino-1-naphthalenesulfonic acid revealed that the dye was bound in a more hydrophobic environment in the complex, than with either troponin C or inhibitory protein alone. The intensity of fluorescence, as well as the binding of the probe, increased in the presence of Ca^{2+}, implying that a conformational change had been induced in the protein molecule, thus creating a more nonpolar binding environment for the dye. The possible significance of a Ca^{2+}-induced conformational change in the troponin C–inhibitory protein complex to the relaxing mechanism of skeletal muscle is discussed briefly.

The active unit of troponin from skeletal muscle is a complex consisting of three components present in equimolar amounts, each responsible for a different function (Murray and Kay, 1971; Drabikowski *et al.*, 1971; Wilkinson *et al.*, 1972). The inhibitory protein TN-I[1] inhibits the Mg^{2+}-activated ATPase of synthetic actomyosin independently of the concentration of free Ca^{2+}. This inhibition is removed in the presence of Ca^{2+} by the second component, TN-C which binds Ca^{2+} strongly. The third component, TN-T has a high affinity for tropomyosin and perhaps also for F-actin (Drabkiowski *et al.*, 1973).

It has been shown that TN-C binds Ca^{2+} very strongly (Fuchs, 1971), with a binding constant of about 10^6 M^{-1} (Ebashi *et al.*, 1968; Hartshorne and Pyun, 1971). Upon binding Ca^{2+}, TN-C undergoes a dramatic conformational change with no alteration in molecular weight (Murray and Kay, 1972; Van Eerd and Kawasaki, 1972). It has been suggested that the Ca^{2+}-binding sites on TN-C are certain carboxyl groups of aspartic and glutamic acid residues, and that the conformational change is induced by charge neutralization, allowing the molecule to adopt a different conformation (McCubbin and Kay, 1973). As this conformational change occurs over a physiological range of concentration of Ca^{2+} ions, it may well be involved in the ability of TN-C to neutralize the restraining effects imposed on the actomyosin system by TN-I.

This study was initiated with a view to establishing whether or not the Ca^{2+}-induced conformational change in TN-C would still operate in the presence of TN-I. In particular, TN-C and TN-I were combined in 1:1 molar ratio, thereby allowing an interaction to occur with formation of the complex TN-IC. Sedimentation velocity measurements were undertaken to demonstrate that complex formation had indeed occurred. Circular dichroism studies were employed to ascertain if the protein–protein interaction had produced any conformational changes and also if the system was still responsive to the presence of Ca^{2+}. Circular dichroism melt experiments were utilized to measure the stability of the complex to thermal denaturation in the absence and presence of Ca^{2+}. Parallel biological activity studies indicated the complex ineffective on the Mg^{2+}-activated ATPase activity of synthetic actomyosin. Fluorescence studies, employing the hydrophobic probe ANS, were also designed to monitor conformational changes upon formation of TN-IC, and those induced by the presence of Ca^{2+}. Results from all these techniques indicated formation of a complex between TN-C and TN-I, and the conformation stability of this complex was sensitive to Ca^{2+} ions.

Materials and Methods

Isolation and Purification of TN-C and TN-I. TN-C and TN-I were isolated and purified from rabbit skeletal muscle as described previously (McCubbin and Kay, 1973; Mani *et al.*, 1973). The purity of TN-C and TN-I was checked by gel electrophoresis in the presence of sodium dodecyl sulfate as outlined by Murray and Kay (1972). Both TN-C and TN-I were homogeneous by this criterion as shown in earlier studies (Murray and Kay, 1972; Mani *et al.*, 1973).

Biological Activity Studies. Assays of ATPase activity were carried out as described earlier (McCubbin and Kay, 1973). However, in the present study synthetic actomyosin (SAM) prepared by mixing myosin and actin in the weight ratio of 4:1 (Shigekawa and Tonomura, 1972) was used instead of desensitized actomyosin.

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonic acid; ATPase, adenosine triphosphatase; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SAM, synthetic actomyosin; TN-I, inhibitory protein; TN-C, calcium-binding protein; TN-IC, protein complex of TN-I and TN-C; *Q*, quantum yield.

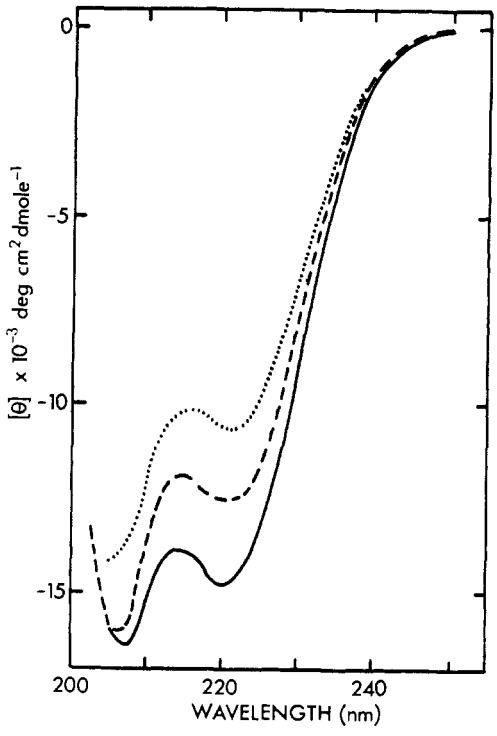

FIGURE 1: Far ultraviolet circular dichroism spectra of TN-IC protein complex in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (- - - -) and in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA, 5×10^{-4} M free Ca^{2+} at pH 8.0 (—). Theoretical circular dichroism spectrum for TN-IC in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (· · · ·).

Protein Concentrations. For TN-C, protein concentrations were routinely measured by ultraviolet absorption, employing an $E^{1\%}_{1\text{ cm},277.5\text{ nm}}$ of 2.3 (Murray and Kay, 1972). In the case of TN-I, the protein concentration was determined by the Lowry method (Lowry *et al.*, 1951). The TN-IC protein complex was formed by mixing TN-I and TN-C in 1:1 ratio. Prior to mixing, the proteins were dialyzed against 0.5 M KCl–1 mM EGTA–50 mM Tris-HCl buffer (pH 8.0).

Circular Dichroism. The circular dichroism measurements were made on a Cary Model 6001 circular dichroism attachment to a Cary 60 recording spectropolarimeter in accordance with previously described methodology (Oikawa *et al.*, 1968). The ellipticity melt experiments were all carried out at 220 nm in a water-jacketed cell compartment connected to a Lauda thermoregulator. The protein solution temperature was increased in a stepwise manner from 5 to 70°. The steps in temperature were not equally spaced and in the transition region, they were very close together. These melt studies therefore should not be taken as true equilibrium melts. The fractional helix, f_H, as a function of temperature, was calculated from the relationship: $f_H = ([\theta]^{t°}_{220\text{ nm}} - 4400°)/([\theta]^{5°}_{220\text{ nm}} - 4400°)$ where 4400° was taken as the ellipticity value for a random-coil structure (Greenfield and Fasman, 1969) and 5° was the lowest temperature at which the ellipticity could be measured conveniently.

Fluorescence Measurements. These were made in a Turner Model 210 recording spectrofluorometer, in which the sample compartment was water jacketed and a constant temperature of 20° was maintained by a Lauda thermoregulator. Tryptophan fluorescence of the TN-IC protein complex was observed at 355 nm after exciation at 294 nm. The OD_{280} value of the protein complex was between 0.1 and 0.2. The ammonium salt of ANS was used in the dye binding studies.

Ca^{2+} Concentrations. These were adjusted by means of a Ca^{2+} buffer consisting of Ca^{2+} and 1 mM EGTA in 0.5 M KCl–50 mM Tris-HCl buffer (pH 8.0). In the calculation of free or available Ca^{2+} ion concentration, a binding constant for EGTA of 2×10^7 M^{-1} was assumed (Chaberek and Martell, 1959).

Ultracentrifugation. Sedimentation velocity experiments were carried out at 60,000 rpm and 20° in a Beckman Spinco Model E ultracentrifuge, equipped with a photoelectric scanner, multiplex accessory, and high-intensity light source. Double-sector charcoal-filled Epon cells with wide aperture window holders were used.

Results and Discussion

Sedimentation Velocity Studies on the Complex of Tn-I and TN-C. Sedimentation velocity measurements were carried out in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) and in the same buffer containing a known amount of Ca^{2+}. TN-C, at a concentration of 3.5 mg/ml, had sedimentation constants of 1.65 and 2.0 S, in the absence and presence of Ca^{2+}, respectively, in excellent agreement with the published values of Murray and Kay (1972), when correction is made for the concentration dependence. TN-I had a sedimentation constant of approximately 1.9 S at a concentration of 1 mg/ml, which was invariant of Ca^{2+} concentration.

When TN-I mixed with TN-C in 1:1 mol ratio in the absence of Ca^{2+}, was centrifuged, an interesting sedimentation pattern was observed. The trace of the boundary was quite symmetrical and it was, concluded therefore, that a single species was present. The sedimentation constant was 2.35 S at a concentration of 1.3 mg/ml and in the presence of 5×10^{-4} M free Ca^{2+} this value rose to approximately 2.9 S at the same protein concentration. The faster sedimenting boundary is indicative of complex formation between TN-I and TN-C under these conditions. Drabikowski *et al.* (1973) have suggested that TN-I disaggregates when it is solubilized by TN-C, and the magnitude of the sedimentation constants obtained here, both in the absence and presence of Ca^{2+}, would support this hypothesis. It would also appear that the increase in the value of the sedimentation constant in the presence of Ca^{2+} (*cf.* native TN-C) is indicative of a conformational change induced by this bivalent cation.

Biological Activity Studies. The TN-IC protein complex had no significant effect on the ATPase activity of SAM suggesting that the inhibitory effect of TN-I on the ATPase activity of actomyosin, has been neutralized by the presence of TN-C. Wilkinson *et al.* (1972) and Drabikowski *et al.* (1973) have also reported that TN-I and TN-C are sufficient for reconstituting the activity of intact troponin. Control experiments showed TN-I and TN-C to be biologically active, *i.e.*, TN-I inhibited the Mg^{2+}-stimulated ATPase activity of SAM and TN-C was able to overcome the effect of TN-I, in the presence of Ca^{2+}. Thus the observed effect of TN-IC on the ATPase activity of SAM is due to complex formation between TN-I and TN-C.

Circular Dichroism Results. Figure 1 shows typical far-ultraviolet circular dichroism spectra for TN-IC. Measurements were made in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) and in the same buffer containing a known amount of Ca^{2+}. This figure also includes a theoretical curve for TN-IC, in the absence of Ca^{2+}, calculated from the ellipticity values of TN-I and TN-C alone. In the presence of 1 mM EGTA, *i.e.*, the virtual absence of any free Ca^{2+}, $[\theta]_{220\text{ nm}}$ is $-12{,}500 \pm 300$ (deg cm^2) dmol^{-1}; while the addition of Ca^{2+}

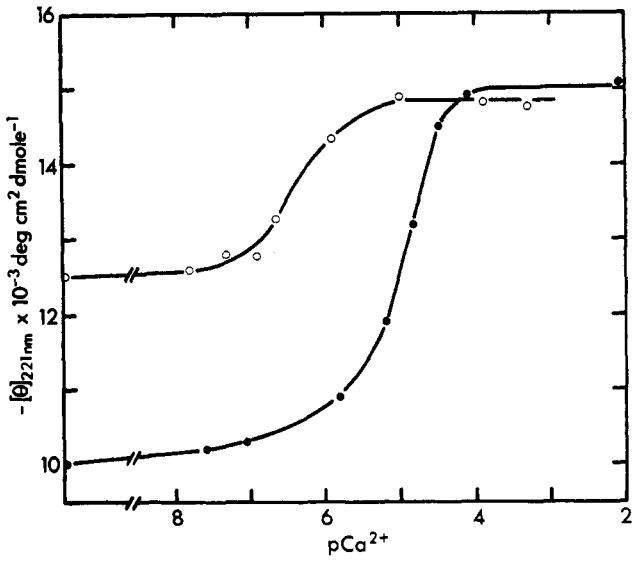

FIGURE 2: The change in ellipticity at 220 nm as a function of the free concentration of Ca^{2+} ions for TN-C (●-●-●) and TN-IC protein complex (O-O-O). Solvent was 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) to which was added known aliquots of $CaCl_2$.

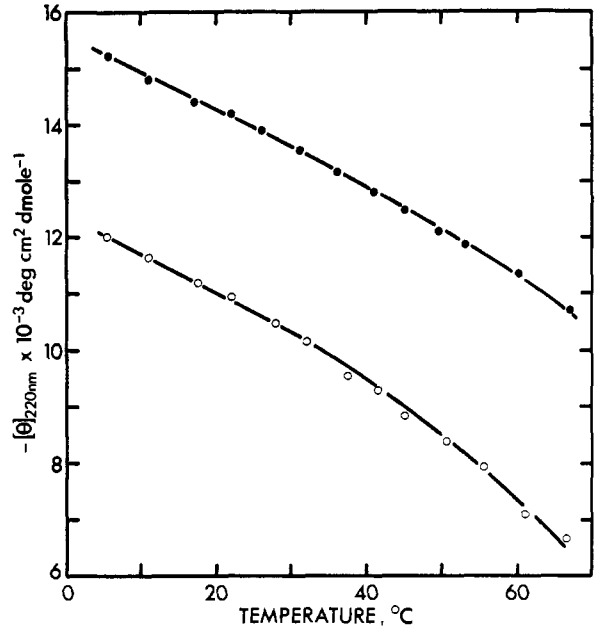

FIGURE 3: The change in ellipticity at 220 nm as a function of temperature for TN-C in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (O-O-O) and in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA–5×10^{-4} M free Ca^{2+} at pH 8.0 (●-●-●).

to a final value of 5×10^{-4} M available or free Ca^{2+}, causes an increase to $-14{,}800 \pm 300$ (deg cm^2) dmol^{-1}. These figures compare with the calculated values for $[\theta]_{220\ nm}$ in a 1:1 mol complex of TN-I and TN-C, in the absence and presence of Ca^{2+} of $-10{,}500 \pm 300$ and $-13{,}500 \pm 300$ (deg cm^2) dmol^{-1}. The experimental values obtained were not time dependent as identical results were obtained from freshly prepared samples, or ones allowed to stand at room temperature for 2 hr.

It should be noted that the experimentally observed ellipticity values for TN-IC in the absence of Ca^{2+} are greater than the calculated values, suggesting that the interaction has induced some type of conformational change. A plausible explanation may be the following. TN-C possesses a considerable net negative charge at pH 8.0 so the interaction with the basic protein TN-I, which presumably is of an electrostatic nature, will involve a charge reduction. This removal of negative charge facilitates the conformational change. Such a mechanism has already been proposed to explain the higher ellipticity values for carboxyl-modified TN-C (McCubbin and Kay, 1973).

Addition of Ca^{2+} to the system to a free Ca^{2+} concentration of approximately 5×10^{-4} M produces a further increase in the negative ellipticity values, as is demonstrated in Figure 1. The important conclusion to be drawn from this latter observation is that the Ca^{2+}-induced conformational change, which occurs in native TN-C, can apparently still occur when TN-C is complexed with TN-I. The interaction of Ca^{2+} with TN-IC was studied by circular dichroism in greater detail. The change in $[\theta]_{220\ nm}$ as a function of the free Ca^{2+} concentration is shown in Figure 2. The half-maximal value of the ellipticity change occurs at a pCa^{2+} value of 6.4. Figure 2 also presents data obtained for TN-C under similar conditions. The half-maximal value occurs at a pCa^{2+} value of 5.0. These yielded binding constants of 2.5×10^6 and 10^5 M^{-1}, respectively. These data imply that when TN-C is complexed with TN-I the binding of Ca^{2+} is stronger than in the case with TN-C alone. This is in good agreement with the suggestion of Potter and Gergely (1972), that TN-I has an enhancing effect on the binding of Ca^{2+} by TN-C.

Earlier observations on the Ca^{2+} induced conformational change in TN-C had demonstrated that the molecule becomes much more compact and rigid in the presence of Ca^{2+}, without any change in molecular weight (Murray and Kay, 1972; Van Eerd and Kawasaki, 1972). This suggests that one role of Ca^{2+} might be to stabilize the protein molecule. To test the validity of this hypothesis it was decided to study the thermal stability of TN-C and TN-IC in the absence and presence of Ca^{2+}, by carrying out circular dichroism melt experiments.

When the ellipticity at 220 nm of a dilute solution (1–1.5 mg/ml) of TN-C is measured over the temperature range 5–70° in the absence and presence of Ca^{2+}, the ellipticity melt profiles shown in Figure 3 are obtained. No significant turbidity developed in the protein solutions over this temperature range. In the absence of Ca^{2+}, the ellipticity values decrease smoothly and almost linearly to approximately 55°. From here to 70° the ellipticity values decrease more quickly. No sharp transition point can be discerned as has been demonstrated in the melting of paramyosin (Halsey and Harrington, 1973). One can only state that in the region 55–60° TN-C, in the absence of Ca^{2+}, begins to melt more readily.

In the presence of sufficient free Ca^{2+} to elicite the maximum conformational change (5×10^{-4} M), the melt profile is more linear, suggesting no apparent point or region at which the structure starts to break down, the process being a slow gradual loss in ellipticity. These data tend to support the idea that the secondary structure of TN-C is more stable in the presence of Ca^{2+}.

The melt data in both cases have been used to calculate fraction helix at each temperature as described in the Materials and Methods section. These results are shown in Figure 4. It is apparent from this figure that the rate of loss of secondary structure in TN-C in the presence of Ca^{2+} is considerably less than in its absence. Figure 4 also includes the melt profile for TN-I in the absence of Ca^{2+}. (Earlier observations (Mani *et al.*, 1973) showed that the CD spectrum of TN-I was unaffected by Ca^{2+}.) The present data are suggestive of a characteristic S-shaped curve with the extrema lying out with

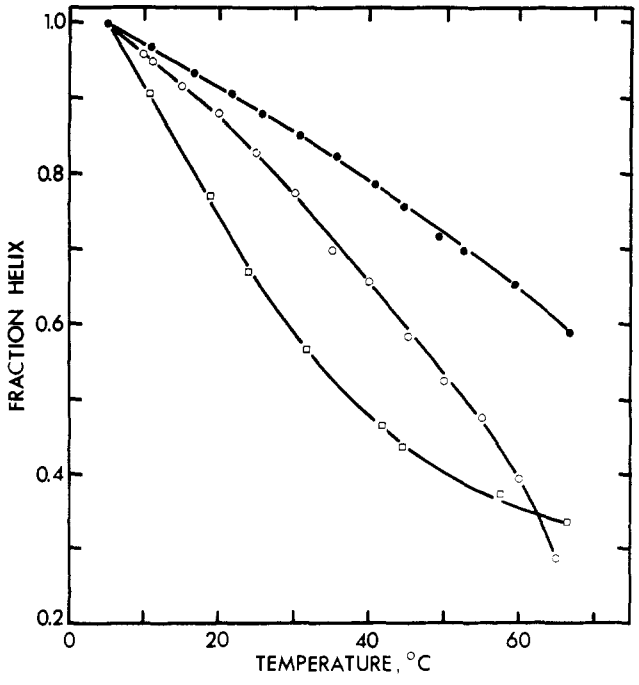

FIGURE 4: Thermal denaturation of TN-C in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA at pH 8.0 (O-O-O) and in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA–5 × 10^{-4} M free Ca^{2+} at pH 8.0 (●-●-●). Also included is TN-I in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (□-□-□). In all cases fraction helix, f_H, as calculated under Materials and Methods, is plotted *vs.* temperature.

the temperature range 5–70° implying a rather stable structure. Schaub and Perry (1971) have demonstrated that solutions of TN-I can be heated to 100° for periods up to 2 hr with little detrimental effect on its inhibitory action.

It seemed opportune at this point to study the melting

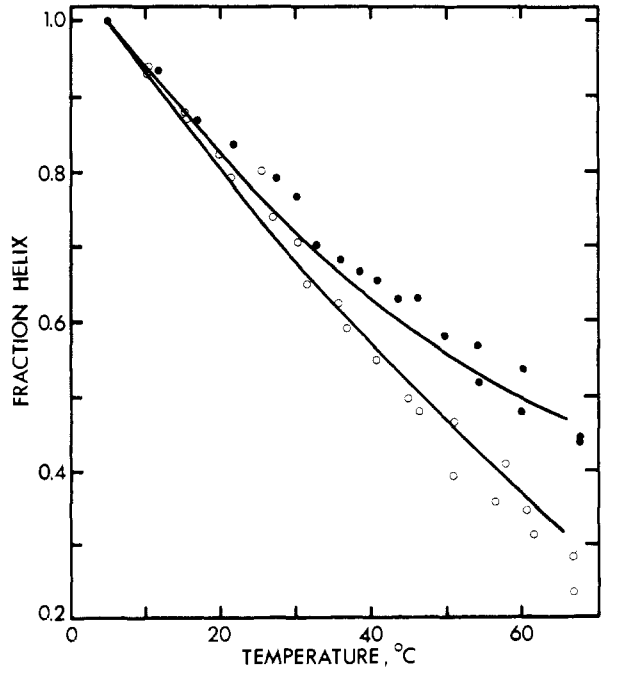

FIGURE 5: Thermal denaturation of TN-IC in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (○○○) and in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA–5 × 10^{-4} M free Ca^{2+} at pH 8.0 (●●●). Fraction helix plotted *vs.* temperature. Solid lines represent an averaged fraction helix at each temperature computed from the values for TN-C and TN-I alone in the absence and presence of Ca^{2+}.

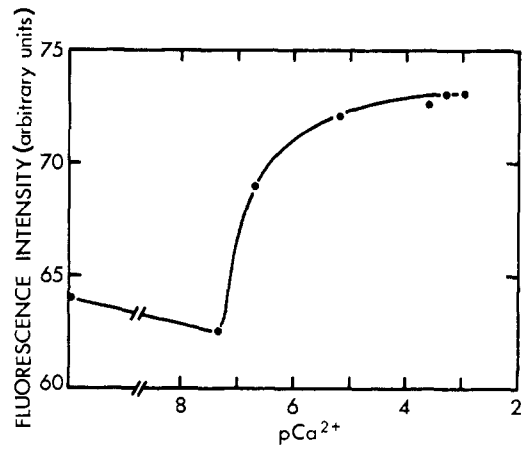

FIGURE 6: The change in the fluorescence intensity of TN-IC–ANS complex as a function of the free Ca^{2+} concentration, pCa^{2+}. The protein concentration is 0.50 mg/ml and the excitation was at 294 nm. The intensity of fluorescence is uncorrected and given in arbitrary units. Solvent was 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) to which was added known aliquots of $CaCl_2$.

characteristics of the 1:1 mol complex TN-IC in the absence and presence of Ca^{2+}. The melt data that were obtained were used to calculate fraction helix at each temperature as was done for TN-C and TN-I alone. These results are shown in Figure 5. The solid lines represent theoretical melting curves calculated by averaging the fraction helix of TN-C and TN-I at each temperature, both in the absence and presence of Ca^{2+}. The experimentally observed points fit these curves rather well. There is no actual transition temperature, rather a fairly smooth loss in helix content over the temperature range used. The data obtained in the presence of Ca^{2+} imply a slower rate of loss of secondary structure in the presence of this bivalent cation. The conformational change in TN-IC, induced and stabilized by Ca^{2+}, apparently renders the molecule less susceptible to thermal denaturation.

In all circular dichroism melt experiments reported in this study, good reversibility of the heating effect was observed. Solutions which had been heated to approximately 70° when recooled to 27° gave CD spectra identical within 3–5% of those originally observed.

Fluorescence Studies. The tryptophan fluorescence properties of TN-IC were monitored in the absence and presence of Ca^{2+}. In both instances the data obtained were essentially identical. This implies that the environment around the tryptophan moieties in TN-I is essentially unchanged upon complex formation. It was therefore decided to try an alternative fluorescence technique.

ANS is almost nonfluorescent in water (Q = 0.004) but it becomes highly fluorescent when adsorbed to certain proteins (Weber and Laurence, 1954). Several workers have since used this dye as a sensitive probe for studying changes of macromolecular conformation with muscle proteins (Duke *et al.*, 1966; Cheung and Morales, 1969). The rationale in the present study was to employ ANS as a hydrophobic probe to follow possible conformational changes in the TN-IC complex upon binding Ca^{2+}.

In the presence of ANS, the emission maxima for the proteins TN-C and TN-I occurred at 505 and 495 nm, respectively, when they were excited at 380 nm. However in the case of the complex, the TN-IC–ANS fluorescence was blue shifted to 485 nm, suggesting that the ANS-binding site is relatively more hydrophobic in nature (Stryer, 1965). Addition of Ca^{2+} to TN-IC in the presence of ANS produced an approximately

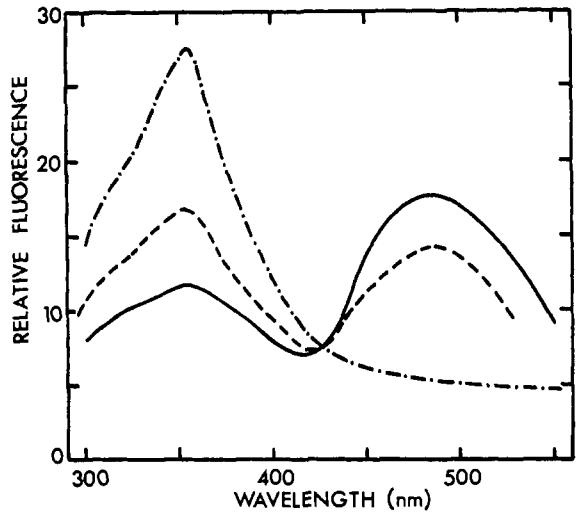

FIGURE 7: Fluorescence emission spectra of TN-IC–ANS complex. TN-IC concentration, 0.35 mg/ml, and excitation was at 294 nm. The symbols represent: TN-IC alone in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0) (–·–·–), TN-IC plus 3×10^{-5} M ANS (- - -), and TN-IC plus 5×10^{-5} M ANS (——).

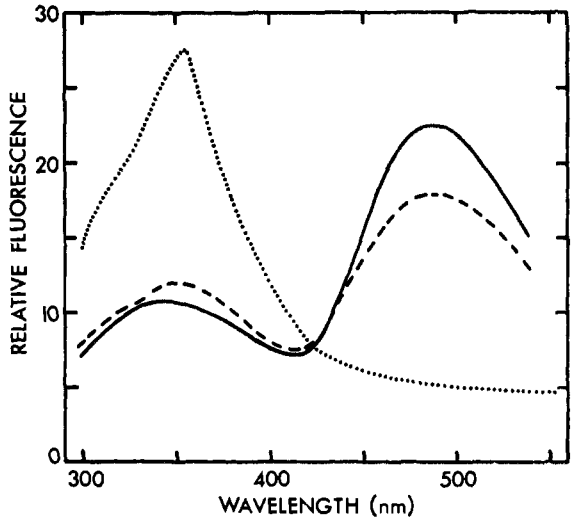

FIGURE 8: Fluorescence emission spectra of TN-IC–ANS complex in 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0). TN-IC concentration, 0.35 mg/ml, and excitation was at 294 nm. The symbols represent: TN-IC alone (· · · ·), TN-IC plus 5×10^{-5} M ANS in the absence of Ca^{2+} (- - - -), and TN-IC plus 5×10^{-5} M ANS in the presence of 5×10^{-4} M free Ca^{2+} (—).

15% increase in fluorescence intensity (Figure 6). However, addition of Ca^{2+} did not cause an apparent shift in the emission maximum from 485 nm. The binding constant for Ca^{2+}, as evaluated from the half-maximal value of the transition curve (Figure 6), was approximately 5×10^6 M^{-1}, which is in good agreement with the value of 2.5×10^6 M^{-1} obtained from circular dichroism measurements.

The emission spectra of TN-IC and TN-IC–ANS in the absence and presence of Ca^{2+} excited at 294 nm, a wavelength at which both TN-IC and ANS absorb, are displayed in Figures 7 and 8. In the absence of ANS, the fluorescence peak at 355 nm arises from excitation of the tryptophan residues. Addition of ANS produced quenching of the tryptophan fluorescence and the extent of this quenching became greater as the level of ANS was increased (Figure 7). At the same time, the protein–ANS fluorescence at 485 nm was found to increase as the ANS level was increased. For any particular concentration of protein and dye, the amount of quenching of the tryptophan fluorescence and the increase in the protein–ANS fluorescence at 485 nm were greater in the presence of Ca^{2+}, implying that the dye binds more strongly to TN-IC in the presence of Ca^{2+} (Figure 8).

In view of the above observation the binding of ANS to TN-IC was studied in the absence and presence of Ca^{2+} (Figure 9). The intrinsic dissociation constant, K, was determined from the relationship given by Cheung and Morales (1969): $F/F_\infty = D/(D + K)$, where F is the observed fluorescence at a certain dye concentration D and F_∞ is the maximum possible fluorescence in the presence of excess dye. K values of $3.4 \pm 0.2 \times 10^{-5}$ M and $1.1 \pm 0.1 \times 10^{-5}$ M were obtained in the absence and presence of Ca^{2+}, respectively, suggesting that the ANS dye indeed binds more strongly to TN-IC in the presence of Ca^{2+}.

In summary, the present investigation has clearly demonstrated that the Ca^{2+}-induced conformational change in TN-C is operative in the presence of TN-I. TN-C binds Ca^{2+} more strongly when complexed with TN-I and the thermal stability of the TN-IC protein complex is enhanced by the presence of Ca^{2+}. Fluorescence measurements, in the presence of ANS, have demonstrated that the formation of TN-IC from its individual protein constituents results in a more nonpolar environment in the neighborhood of the dye-binding site. Stronger dye binding to TN-IC in the presence of Ca^{2+} is also of significance since this implies a structural alteration in the ANS binding site on TN-IC in the presence of Ca^{2+}. These observations are biologically important, since any consideration of the mechanism of Ca^{2+} control of the relaxing system in muscle must acknowledge that the active troponin molecule is a complex of three components, each responsible for different functions. Consequently a structural alteration or effect produced by any one member of the complex, to be physiologically relevant, must be operative in the presence of the other members. With TN-IC these conditions have been achieved partially. Studies presently underway in our laboratory will include the contribution of TN-T in this system.

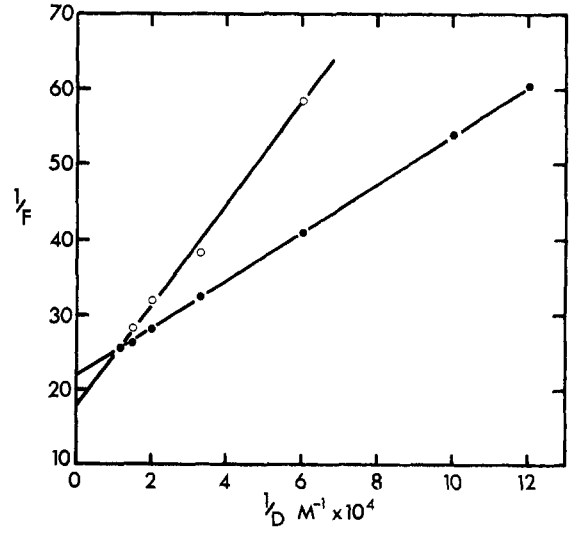

FIGURE 9: Binding of ANS to TN-IC. Concentration of TN-IC was 0.35 mg/ml (fixed), ANS concentration was varied between 8×10^{-6} and 1×10^{-4} M at 20°. Excitation was at 380 nm and fluorescence was observed at 485 nm. The symbols represent: TN-IC plus ANS in the absence of Ca^{2+} (O-O-O) and TN-IC in the presence of 5×10^{-4} M free Ca^{2+} (●-●-●). Solvent system used was 0.5 M KCl–50 mM Tris-HCl–1 mM EGTA (pH 8.0).

Acknowledgments

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References

Chaberek, S., and Martell, A. E. (1959) Organic Sequestering Agents, New York, N. Y., Wiley.

Cheung, H. C., and Morales, M. F. (1969), *Biochemistry 8*, 2177.

Drabikowski, W., Dabrowska, R., and Barylko, B. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 148.

Drabikowski, W., Dabrowska, R., and Barylko, B. (1973), *Acta Biochim. Polon. 20*, 181.

Duke, J. A., McKay, R., and Botts, J. (1966), *Biochim. Biophys. Acta 126*, 600.

Ebashi, S., Kodama, A., and Ebashi, F. (1968), *J. Biochem. (Tokyo) 64*, 465.

Fuchs, F. (1971), *Biochim. Biophys. Acta 245*, 221.

Greenfield, N. J., and Fasman, G. D. (1969), *Biochemistry 8*, 4108.

Halsey, J. F., and Harrington, W. F. (1973), *Biochemistry 12*, 693.

Hartshorne, D. J., and Pyun, H. Y. (1971), *Biochim. Biophys. Acta 229*, 698.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 256.

Mani, R. S., McCubbin, W. D., and Kay, C. M. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29*, 243.

McCubbin, W. D., and Kay, C. M. (1973), *Biochemistry 12*, 4232.

Murray, A. C., and Kay, C. M. (1971), *Biochem. Biophys. Res. Commun. 44*, 237.

Murray, A. C., and Kay, C. M. (1972), *Biochemistry 11*, 2622.

Oikawa, K., Kay, C. M., and McCubbin, W. D. (1968), *Biochim. Biophys. Acta 168*, 164.

Potter, J., and Gergely, J. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 501.

Schaub, M. C., and Perry, S. V. (1971), *Biochem. J. 123*, 367.

Shigekawa, M., and Tonomura, Y. (1972), *J. Biochem. (Tokyo) 72*, 957.

Stryer, L. (1965), *J. Mol. Biol. 13*, 482.

Van Eerd, J., and Kawasaki, Y. (1972), *Biochem. Biophys. Res. Commun. 47*, 859.

Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, 31.

Wilkinson, J. M., Perry, S. V., Cole, H. A., and Trayer, I. P. (1972), *Biochem. J. 127*, 215.

The Disulfide Bonds of α_1-Acid Glycoprotein†

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ABSTRACT: For the elucidation of the positions of the disulfide bonds of human plasma α_1-acid glycoprotein, the amino acid sequences of the cystine-containing peptides isolated from an enzymatic digest of this globulin were determined and compared with the earlier established, linear amino acid sequence of the protein. For this purpose the protein was first digested with pepsin at pH 2.0 and then with trypsin and chymotrypsin at pH 6.5. The resulting digest was fractionated by gel filtration through Sephadex G-25 and the obtained glycopeptide fraction was further resolved by passage through a Sephadex G-75 column. The cystine-containing glycopeptides and peptides which were then isolated in homogeneous form by chromatography on Dowex 50W-X2 were subsequently oxidized with performic acid. The two cysteic acid containing peptides resulting from each of the cystine peptides were separated from each other by the same ion exchange chromatographic procedure and their amino acid compositions and amino acid sequences were determined. The obtained data indicated that α_1-acid glycoprotein possesses two disulfide bonds and that one of these disulfide bonds links residue 5 to residue 147, while the other connects residue 72 with residue 164 of the protein.

Recently, the linear amino acid sequence of human plasma α_1-acid glycoprotein was elucidated and found to consist of 181 residues and to contain four half-cystine residues (Schmid *et al.*, 1973; Ikenaka *et al.*, 1972). In the present report we wish to describe the location of the two disulfide bonds of this protein.

Materials and Methods

α_1-Acid Glycoprotein. This globulin (for review, see Jeanloz, 1972) was isolated from Cohn fraction VI of pooled normal human plasma by a procedure described earlier (Bürgi and Schmid, 1971) and its apparent homogeneity was established by several criteria of purity (Jeanloz, 1972; Bürgi and Schmid, 1971; Ikenaka *et al.*, 1966).

Enzymatic Digestions. The peptic (a), tryptic (b), and chymotryptic (c) digestions of the protein were performed under the conditions similar to those described by Spackman *et al.* (1960): (a) pH 2.0, 24°, protein concentration 1%, enzyme–protein ratio 1:500 and 17 hr, (b) pH 6.5, 24°, protein concentration 1.3%, enzyme–protein ratio 1:250 and 4 hr, and

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