FLUORESCENCE AND CONFORMATIONAL CHANGES CAUSED BY PROTON BINDING TO TROPONIN C[1]

Sherwin S. Lehrer[2] and Paul C. Leavis[3]

Department of Muscle Research
Boston Biomedical Research Institute
and Department of Neurology
Harvard Medical School
Boston, Mass.

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Summary: The binding of H^+ to troponin C induces a large conformational change and an enhancement of the tyrosyl fluorescence. Carboxyl groups with abnormal pK' values of 6.0 appear to be controlling these changes.

Recent studies on troponin C, the calcium binding subunit of the troponin complex (1,2) have shown a change in secondary structure (3-6), an enhancement of tyrosyl fluorescence (6), a reduction of spin label mobility (7,8), an enhancement of the fluorescence of a dansyl label (8) and a decreased reactivity of the single sulfhydryl group (7,8) upon binding Ca^{+2} with high affinity ($K_{Ca} = 10^7 - 10^8$). Many small molecule chelates and proteins utilize carboxylate groups to coordinate Ca^{+2} (9). Carboxylates can also quench tyrosyl fluorescence if they are bonded to, or located near tyrosyl side chains (10,11). If this quenching mechanism is operating in the case of troponin C, the Ca^{+2} induced fluorescence enhancement would be explained by the removal of carboxylates from the vicinity of tyrosyl side chains. This could occur whether or not these carboxylates are directly involved in the binding of Ca^{+2}, since the large conformational change caused by the Ca^{+2} binding could affect a carboxylate-tyrosyl interaction far from the binding site. In order to obtain evidence for the involvement of carboxylates in the Ca^{+2} induced fluorescence enhancement and in an attempt to distinguish

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[2]Address correspondence to S.S.L., Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford Street, Boston, Mass., 02114.
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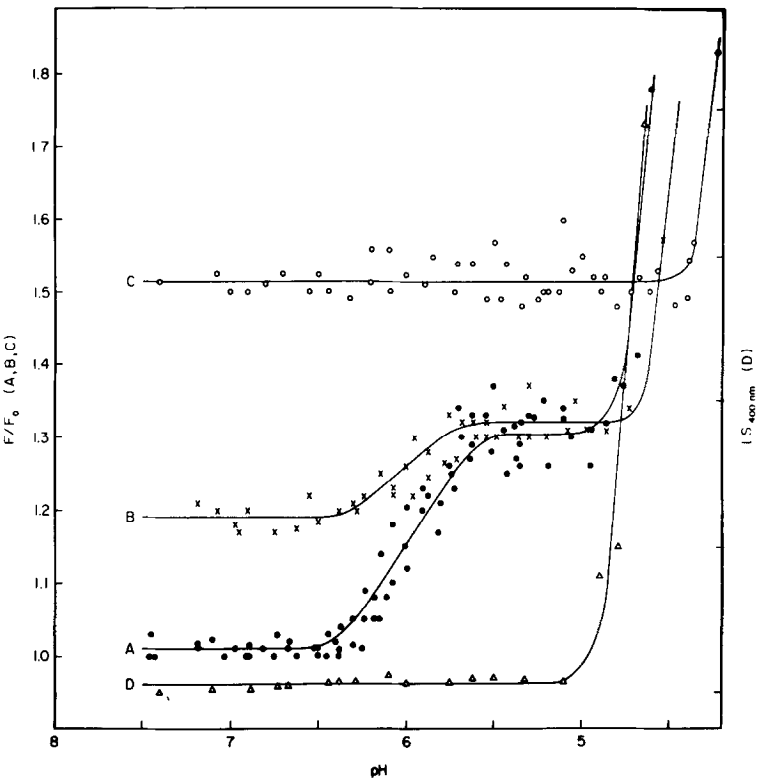

Figure 1. Changes in the relative tyrosyl fluorescence and light scattering of troponin C in the acid pH region. A) F/F_o in buffer (no Ca^{+2} or Mg^{+2}); B) F/F_o in buffer + 2 mM $MgCl_2$; C) F/F_o in buffer plus 0.5 mM $CaCl_2$ (pCa = 7); D) right angle light scattering of troponin C at 0.18 mg/ml in buffer at 400 nm. Buffer is 50 mM KCl, 2 mM EGTA and 10 mM tris-acetate initially at pH 7.0-7.5. The fluorescence data represent the average of a few titrations at concentrations of troponin C between 0.07 and 0.2 mg/ml. Reversibility was checked by adding 1N NaOH. T = 24±1°C, λ_{ex} = 275 nm, λ_{fl} = 305 or 310 nm. See (2) for troponin C preparation and (20) for fluorescence methods.

between the two enhancement mechanisms we decided to study the consequences of H^+ binding to troponin C. A fluorescence enhancement would be expected when these carboxylates become protonated by analogy with model compound studies, in particular, the study of poly(Glu Tyr) (12,13). Our results indicate that the protonation of acid groups of pK' = 6.0 does result in an enhancement of fluorescence. As in the case of the Ca^{+2} enhancement, however, a large change in secondary structure accompanies the protonation. It was therefore not possible to conclusively distinguish between the two enhancement mechanisms.

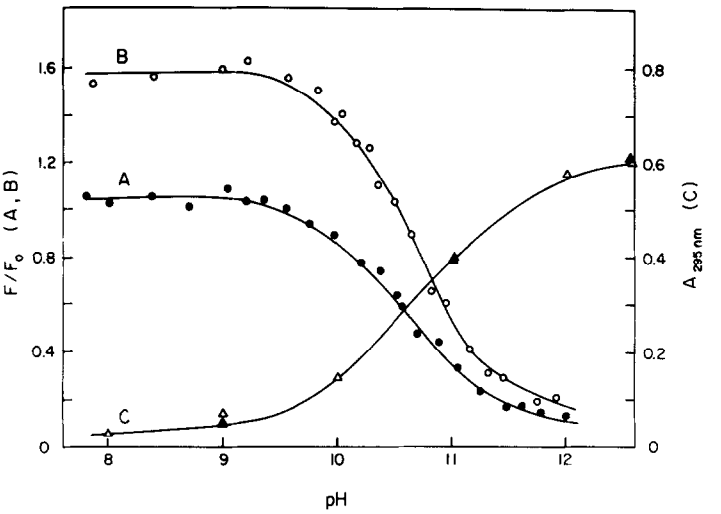

Figure 2. Changes in the relative tyrosyl fluorescence and tyrosyl absorption of troponin C in the alkaline pH region. A) fluorescence in buffer (no Ca^{+2}, no Mg^{+2}); B) fluorescence in buffer + 2.5 mM $CaCl_2$; C) absorption at 295 nm ± Ca^{+2}. Buffer is 2 mM EGTA and 10 mM phosphate initially at pH 7.8. Reversibility was checked with small additions of 1N HCl. Troponin C concentration was 1.8 mg/ml for absorption and 0.18 mg/ml for fluorescence. λ_{ex} = 284 nm, λ_{fl} = 310 nm.

Results

Fluorescence pH Titrations: The fluorescence spectrum of troponin C, a protein that contains 2 tyrosines and no tryptophan (2), showed a typical tyrosyl band (λ_{max} = 303nm). The tyrosyl quantum yield relative to L-tyrosine in H_2O at neutral pH in the absence of Ca^{+2} and Mg^{+2} was estimated to be R_{Tyr} = 0.40±0.02 at 24°C. A 30% fluorescence increase results from the protonation of groups with pK'_H = 6.0 (Fig. 1, curve A). Cooperative interactions appear to be involved since the transition occurs over a narrower pH range than would be the case for a single site or a class of independent sites. The light scattering contribution was negligible over the pH transition and only contributed to an apparent fluorescence increase below pH 4.8 as the pH approached the isoelectric point (ca pH 4.3 (14)) (Fig. 1, curve D).

The possibility that the fluorescence increase at low pH was actually caused by the binding of contaminating calcium released from the EGTA[4]

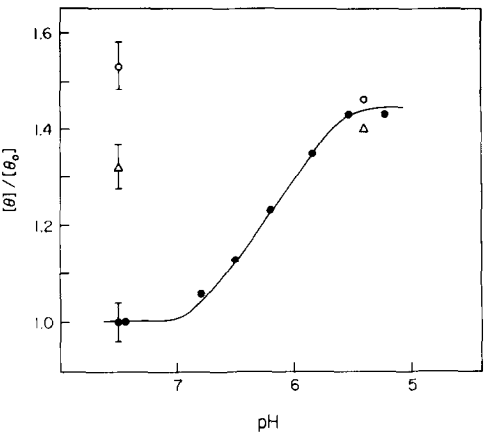

Figure 3. Changes in the relative ellipticity at 222 nm of troponin C in the acid pH region. The same solutions were used as in Fig. 1. (●), no Ca^{+2}, no Mg^{+2}; (Δ), 2 mM $MgCl_2$ in buffer of Fig. 1; (○), 0.1 mM $CaCl_2$ in buffer of Fig. 1. T = 24±1°C. Error bars apply to all points. See (20) for CD methods.

chelate had to be considered, since Ca^{+2} produces a large fluorescence enhancement (Fig. 1, curve C). This possibility was eliminated as follows: Troponin C was exhaustively dialyzed against multiple changes of 0.05M KCl, 10 mM Tris, pH 7.5 initially containing 2 mM EDTA and ending with 10^{-5}M EDTA. A fluorescence pH titration gave results identical to those shown in Fig. 1, curve A. After this titration and several checks of reversibility by the addition of NaOH and HCl, the solution was found to contain only 1.2 X 10^{-5}M Ca^{+2} by atomic absorption photometry. The binding of this amount of Ca^{+2} to 10^{-4}M troponin C would be expected to produce a maximum enhancement of 1.06 on the basis of the Ca^{+2} enhancement seen in Fig. 1, curve C. The observed enhancement of 1.30 must, therefore, be due to proton binding.

It does not appear that H^{+} can replace Ca^{+2} since, in the presence of Ca^{+2}, the pH titration of troponin C showed no change in fluorescence from pH 7.5 to 4.3. The corresponding titration in the presence of Mg^{+2} showed a slight increase to the same value at pH 5.5 as in the absence of Mg^{+2}. This suggests that protons have replaced bound Mg^{+2}.

[4]Abbreviations include: CD, circular dichroism; EGTA, ethyleneglycol-bis (β-amino ethylester); EDTA, ethylene-diamine tetra acetic acid.

Fluorescence and absorption titrations in the alkaline pH region show that the binding of Ca^{+2} does not affect the ionization of tyrosine. As can be seen in Fig. 2, as the pH increased, the fluorescence declined to 0 (corresponding to the nonfluorescent ionized tyrosine) and the absorption due to ionized tyrosine increased, both giving $pK'_H = 10.7 \pm 0.1$ whether or no Ca^{+2} was present. This also shows that both tyrosyls of troponin C titrate with the same pK'_H.

The Effect of pH on Circular Dichroism: The ellipticity at 222 nm increased over the same pH range as the fluorescence enhancement (Fig. 3). Ca^{+2} and Mg^{+2} produced slightly larger (53%) and smaller (32%) conformational changes respectively as compared to H^+ (44%) (±5% estimated error). These data show that the binding of protons as well as divalent metal ions (3-5) can produce qualitatively similar conformational changes.

Discussion

From these data it is clear that protonation of acid groups of $pK'_H = 6.0$ produces substantial increases in the tyrosyl fluorescence and the ellipticity at 222 nm of troponin C. Since the transition range in both cases is quite steep ($\measuredangle 1$ pH unit) it appears that there are cooperative interactions, due to the binding of H^+ by two or more groups. Although the single histidine of troponin C (2) can be protonated in this pH region several facts suggest that the pK' = 6 groups are carboxylates. The high pK' is not unusual for proteins since several proteins with carboxyl groups having abnormally high pK values ($\gtrsim 6$) have been reported (15,16). Such high pK values can be associated with carboxylates located in regions containing adjacent carboxylates, particularly if the environment is somewhat hydrophobic. Troponin C has a negative charge of about 30 at neutral pH with many such groupings of acid residues (17). Also, recent studies with carboxyl modified troponin C have shown a reduction in both the Ca^{+2} induced fluorescence enhancement and conformational change (18). In view of these observations we tentatively associate the $pK'_H = 6.0$ value with abnormally titrating carboxylates. It

seems probable that these carboxylates are also involved in Ca^{+2} binding. Recent studies have shown that of the 4 Ca^{+2} that bind to troponin C, 2 are bound in the ranges of pCa (8) that produce the conformational change (pCa = 7-8) (3). Glutamate and aspartate side chains are involved in the coordination of Ca^{+2} in a protein from carp (9) which, although it is quite different in size and function, has many sequence homologies with troponin C (17). Our data show that similar changes in secondary structure occur when Ca^{+2}, Mg^{+2} or H^{+} are bound. The slightly different values of ellipticity could be due to the effects of slightly different bonding and charge distribution in the binding site.

Changes in the tyrosyl fluorescence of proteins reflect changes in the proximity of vicinal quenching groups which include carboxylate and peptide carboxyl (19). The fluorescence enhancement of troponin C at low pH can be interpreted as being due to a decrease in the proximity of quenching groups caused by proton binding to carboxylates of $pK'_H = 6.0$. We cannot decide from our data if the groups with pK' = 6 are themselves the quenchers (direct mechanism) or whether they regulate the proximity of other quencher groups to tyrosyl (indirect mechanism) because of the large conformational change which occurs over the same pH range. If the direct mechanism applies cooperative binding by carboxylates near a tyrosyl would be required to explain the steepness of the transition. Since similar effects are produced when Ca^{+2} binds (6), the same ambiguities of interpretation exist. The actual mechanism may involve contributions from both. The slight variation in fluorescence in the presence of saturation amounts of Ca^{+2}, Mg^{+2} and H^{+} can be explained by either mechanism.

The high value of tyrosine quantum yield as compared to most globular proteins (11,19), the relatively normal pK'_H for tyrosyl ionization, and the lack of Ca^{+2} effect on the ionization profile of tyrosyl despite the large conformational change suggests that both tyrosyls are relatively exposed to solvent. These observations by themselves do not exclude the possibility

that the pK' = 6 carboxylates are located near a tyrosyl. The groups could be non-interacting in the ground state but have sufficient flexibility to allow a mutual collision to occur during the excited state lifetime.

It should be noted that the structure of the homologous carp muscle calcium binding protein has been studied by X-ray techniques on crystals grown in the presence of Ca^{+2}, but that it has not been possible to grow crystals of the calcium free form (9). It would be of interest to determine whether Ca^{+2}, Mg^{+2} and H^{+} can induce conformational changes of the type seen for troponin C; if so, the data presented here suggest that it may be possible to crystallize the carp protein in the absence of calcium at low pH.

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