Aluminum Fluoride Interactions with Troponin C

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ABSTRACT The increasing interest in the metal ion aluminum fluoride and beryllium fluoride complexes as phosphate analogs in the myosin ATPase reaction and in muscle fiber studies prompted the examination of their interactions with the regulatory system of troponin and tropomyosin. In this work, the effects of these metal ion analogs on the spectral properties of the Ca^{2+}-binding subunit of troponin, troponin C (TnC), were examined. In contrast to beryllium fluoride which did not change the spectral properties of TnC, aluminum fluoride binding induced an increase in both the α-helicity and the tyrosine fluoresence of TnC and exposed a hydrophobic region on this protein for fluorescent probe binding. Aluminum fluoride also reduced the Ca^{2+} and/or Mg^{2+}-induced changes on TnC. These results indicate a direct interaction of aluminum fluoride with TnC and merit consideration in designing muscle fiber experiments with this phosphate analog.

INTRODUCTION

Aluminum and beryllium fluoride complexes are metal ions which have been used as phosphate analogs (Chabre, 1990) to characterize, among others, myosin-bound intermediates generated during the cross-bridge cycle (Phan and Reisler, 1992; Werber et al., 1992; Chase et al., 1993). They have been shown to bind to the active site of myosin subfragment 1 and form stable ternary complexes which resemble the predominant steady-state $M^{**}\cdot ADP\cdot P_i$ species of the ATPase pathway (Chase and Kushmerick, 1990; Maruta et al., 1991; Beck et al., 1992; Phan and Reisler, 1992; Werber et al., 1992).

The use of these phosphate analogs for studying intermediates of the myosin ATPase pathway in muscle fibers and myofibrils requires that the perturbations observed are due to effects of analogs on myosin alone. However, these metal ions may interact with other components of the cross-bridge system as well. The binding of beryllium fluoride and aluminum fluoride to actin has been demonstrated in a previous study (Combeau and Carlier, 1988). This binding is very slow (Combeau and Carlier, 1988) and, although beryllium fluoride has been reported to perturb the structure of actin filaments (Orlova and Egelman, 1992), it appears to have little effect on the myosin ATPase-activating function of actin (Muhlrad et al., unpublished results). Another protein that might interact with these metal ions is troponin C (TnC), a member of the homologous Ca^{2+}-binding protein family. TnC is the Ca^{2+}-binding subunit of troponin which together with two other troponin subunits, the inhibitory subunit (TnI) and the tropomyosin binding subunit (TnT), and tropomyosin forms the regulatory complex associated with the F-actin thin filaments of striated (skeletal and cardiac) muscle (Leavis and Gergerly, 1984). Ca^{2+} binding to TnC is thought to produce structural changes that are transmitted through TnI to TM and actin (Spudich and Watt, 1971; Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973; Wakabayashi et al., 1975). These changes alter the TM-actin complex to allow for activated actomyosin ATPase and contraction (Chalovich et al., 1981).

TnC has four well defined metal-binding sites, two low-affinity sites which bind Ca^{2+} specifically ($K = 3 \times 10^5$ M^{-1}), and two high-affinity sites which bind both Ca^{2+} and Mg^{2+} ($K_{Ca} = 2 \times 10^7$ M^{-1}, $K_{Mg} = 10^3$ M^{-1}) (Potter and Gergerly, 1975). TnC also binds monovalent cations (Mrakovcic et al., 1979). The abundance of metal-binding sites on TnC makes it a possible target for the binding of aluminum and beryllium fluorides.

The goal of the present study has been to examine the effects of aluminum and beryllium fluoride complexes on the spectral properties of TnC. Our results show that in contrast to beryllium fluoride, which does not have any effect on TnC, aluminum fluoride binding induces an increase in both the α-helicity and the tyrosine fluorescence of TnC and exposes hydrophobic groups on this protein. This shows the binding of aluminum fluoride to TnC and suggests that this analog may have a complex effect on muscle fiber properties.

MATERIALS AND METHODS

Reagents

$BeCl_2$ (which comes in 1% HCl), $AlCl_3$, NaF, EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). 1,1′-bi-1,4-Anilinolnaphthalene-5, 5′-disulfonic acid dipotassium salt (bis-ANS) was purchased from Molecular Probes Inc. (Junction City, OR). All other reagents were of analytical grade. Beryllium and its salts may be carcinogenic and should be handled with care.

Proteins

TnC and troponin (Tn) were prepared as described previously (Potter, 1982; Reisler et al., 1980). The concentrations of TnC and Tn were determined

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Abbreviations used: Tn, troponin; TnC, the Ca^{2+}-binding subunit of troponin; TnI, the inhibitory subunit of troponin; TnT, the tropomyosin-binding subunit of troponin; bis-ANS, 1,1′-bi-1,4-anilinonaphthalene-5,5′-disulfonate; ANS, 8-anilino-1-naphthalene sulfonate.

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spectrophotometrically by using the extinction coefficients for TnC at 280 nm, $E^{1\%} = 1.41\ cm^{-1}$; and for Tn at 278 nm, $E^{1\%} = 4.5\ cm^{-1}$.

Circular dichroism (CD)

CD measurements were made at room temperature in a JASCO J-600 spectropolarimeter and employing a 1.0-mm-path cell. TnC (0.3 mg/ml) was dissolved in 10 mM NaCl and 50 mM Tris-HCl, pH 7.5 (buffer A). For each spectrum, four scans were averaged. Differential absorption data were converted to mean residue ellipticity [Θ] (°·cm²/dmol) assuming the value of 113 for mean residue molecular weight of amino acids. The α-helical content was derived from the program Protein Secondary Structure Estimation Program (Japan Spectroscopic Co., Japan) and also calculated as described by Greenfield and Fasman (1969). The CD data for each wavelength, λ, were expressed as mean residue weight ellipticity.

$$[\Theta]_\lambda = \frac{\Theta_\lambda M}{10\, lc}\ deg \cdot cm^2 \cdot dmol^{-1}$$

Θ_λ is the measured ellipticity in degrees, l is the optical path length in centimeters, c is the concentration of TnC in grams cm^{-3}, M is taken as the mean residue weight, 113.

Fluorescence measurements

The tyrosine fluorescence of TnC was measured at 25°C in a Spex Fluorolog spectrophotometer (Spex Industries, Inc., Edison, NJ) at excitation and emission wavelengths of 280 nm and 315 nm. TnC (0.8 mg/ml) was dissolved in buffer A. The emission spectrum of TnC was measured in the presence of EGTA (1 mM), $CaCl_2$ (3 mM), $MgCl_2$ (2 mM), or $AlCl_3$ (4 mM) mixed with NaF (16 mM). The concentration of aluminum fluoride chosen for these experiments was the same as that used in some muscle fiber experiments (Chase and Kushmerick, 1990). For each emission spectrum, four scans were averaged. To calculate the changes in tyrosine fluorescence in the presence of ligands, the tyrosine fluorescence intensity of TnC in buffer A + EGTA (1 mM) was normalized to 100%.

Aluminum fluoride titration of TnC

The dissociation constant of aluminum fluoride from TnC in the presence and absence of Mg^{2+} was determined by titrating TnC with varying concentrations of aluminum fluoride. Briefly, the tyrosine fluorescence of TnC (0.8 mg/ml) in buffer A was measured in the presence of EGTA (1 mM). $MgCl_2$ (or water) was then added, followed by the addition of NaF (16 mM). Small aliquots of $AlCl_3$ were then added. All fluorescence intensities were corrected for dilution effects and normalized to the intensity of TnC in buffer A + EGTA.

Emission spectra of bis-ANS

The fluorescence of bis-ANS was measured at an excitation wavelength of 394 nm and that of emission ranged from 420 to 650 nm. The emission spectrum of bis-ANS (30 μM) was measured in buffer A in the presence of TnC (20 μM) and EGTA (2 mM). $CaCl_2$ (4 mM), $MgCl_2$ (2 mM), and $AlCl_3$ (4 mM) mixed with NaF (16 mM) were then added to the TnC and EGTA solutions.

RESULTS

Effects of aluminum fluoride and beryllium fluoride complexes on the CD of TnC

The binding of Ca^{2+}, Mg^{2+}, and even Na^+ or K^+ to TnC induces characteristic changes in its CD signal. Thus, to examine whether aluminum and beryllium fluorides bind to TnC, the CD of TnC was measured in the presence of different ligands (Fig. 1). The binding of Ca^{2+} to TnC, as reported by earlier studies (Kawasaki and Van Eerd, 1972; Murray and Kay, 1972; Van Eerd and Kawasaki, 1972), induced a large change in the conformation of TnC (curve *d*). A smaller change in TnC was observed upon $MgCl_2$ addition (curve *c*). The addition of 4 mM aluminum fluoride in the presence of EGTA, caused a conformational change in TnC, as evidenced by changes in the CD in the 200- to 240-nm region (Fig. 1, curve *b*). This change, even though smaller than those induced by Ca^{2+} and Mg^{2+} was nonetheless significant. Beryllium fluoride did not have any effect on the CD of TnC (not shown).

Aluminum fluoride caused a rapid (within mixing time) 11 (±5) % increase in α-helicity of TnC, as compared with 37 (±5) % change induced by Ca^{2+} and 24 (±5) % by Mg^{2+} (Table 1). The increase in α-helicity of TnC observed upon binding was not due to the change in ionic strength of the solution; no effect was observed when equivalent amount of KCl (40 mM) was added to the protein (Table 1). Also, neither $AlCl_3$ nor NaF alone could elicit similar changes in the CD of TnC. Thus, the spectral effects reported in this study depended on the presence of aluminum fluoride species in TnC solutions. At pH 7.5 and at millimolar concentrations of fluoride, several aluminum fluoride complexes exist (Martin, 1982). The predominant species, predicted from equilibria calculations, is AlF_4^- (Martin, 1982). This prediction was supported by nuclear magnetic resonance (NMR) studies on aluminum fluoride binding to smooth muscle myosin (Murata et al., 1991). In the TnC system, however, the specific aluminum fluoride ions which affect the protein structure were not determined in this work.

It is interesting to note that when more than one ligand was added, the order of addition was important. Indeed, alumi-

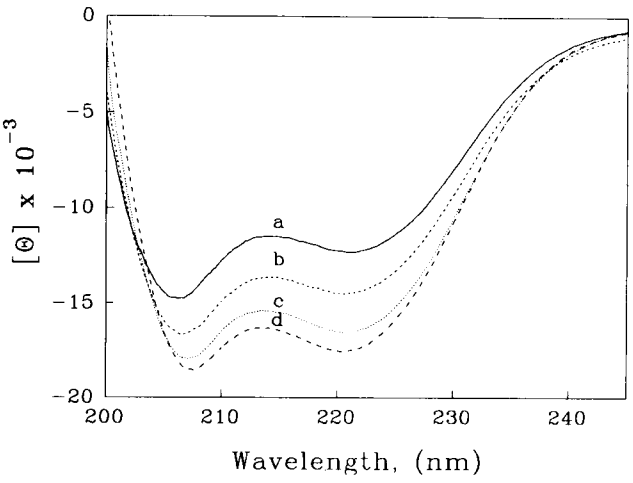

FIGURE 1 Effects of different ligands on the CD of TnC. CD measurements were carried out at 25°C on solutions containing 0.3 mg/ml of TnC in 10 mM NaCl, 50 mM Tris-HCl at pH 7.5 (buffer A). The protein samples contained also 1 mM EGTA (curve *a*); 1 mM EGTA + 3 mM $CaCl_2$ (curve *d*); 1 mM EGTA + 4 mM $AlCl_3$ + 16 mM NaF (curve *b*); or 1 mM EGTA + 2 mM $MgCl_2$ (curve *c*). The pathlength was 0.1 cm. Each spectrum is the average of four scans.

TABLE 1 Effects of different ligands on the α-helicity of TnC

Ligand	% Change in α-helix
None	—
$CaCl_2$	37 ± 5
AlF_x[a]	11 ± 5
$MgCl_2$	24 ± 5
BeF_x[b]	0 ± 2
AlF_x + $CaCl_2$	27 ± 8
$CaCl_2$ + AlF_x	35 ± 2
AlF_x + $MgCl_2$	20 ± 2
$MgCl_2$ + AlF_x	19 ± 5
KCl	0 ± 2

The α-helical content of TnC was determined from the fitting program Protein Secondary Structure Estimation (Japan Spectroscopic Co., Japan) or calculated as described by Greenfield and Fasman (1969). The percentage change in α-helicity induced by ligands is related to the α-helicity of TnC in buffer A + 2 mM EGTA. All ligands were added to solutions of TnC in buffer A + 2 mM EGTA.

[a] Aluminum fluoride complexes.

[b] Beryllium fluoride complexes.

num fluoride added before Ca^{2+} impeded the action of Ca^{2+}, as the change in α-helicity was reduced from 37 (± 5.5) to 27 (± 8) %. Yet, Ca^{2+}, when added before aluminum fluoride, still induced the maximum change. The change in α-helicity induced by Mg^{2+} and aluminum fluoride was about the same regardless of the order of additions (19 or 20%, Table 1). It is higher than the change induced by aluminum fluoride alone but lower than the change induced by Mg^{2+} alone, suggesting that aluminum fluoride binding affects the high-affinity divalent sites.

Effects of aluminum fluoride and beryllium fluoride on the tyrosine fluorescence of TnC

The tyrosine and phenylalanine chromophores on TnC can be utilized to localize some of the conformational changes indicated by the CD measurements. To this end the tyrosine fluorescence of TnC was measured in the presence of various ligands (Fig. 2). As reported by previous studies (Van Eerd and Kawasaki, 1972; Kawasaki and Van Eerd, 1972; Potter et al., 1976), Ca^{2+} enhanced the tyrosine fluorescence of TnC by 55 (± 9) % (Table 2). The binding of Mg^{2+} increased the tyrosine fluorescence by 14 (± 5) %. Beryllium fluoride did not have any effect on the tyrosine fluorescence of TnC (not shown). A large and rapid (within mixing time) increase of 41 (± 9) % was observed upon aluminum fluoride addition to TnC. This effect was reduced to 30 (± 5) % when Mg^{2+} was added before aluminum fluoride suggesting again that aluminum fluoride binding affects the Mg^{2+} binding sites in the COOH-terminal part of TnC. It should be noted that both the fluorescence and CD changes induced in TnC by aluminum fluoride were reversible and rapidly abolished (within mixing time) by EGTA.

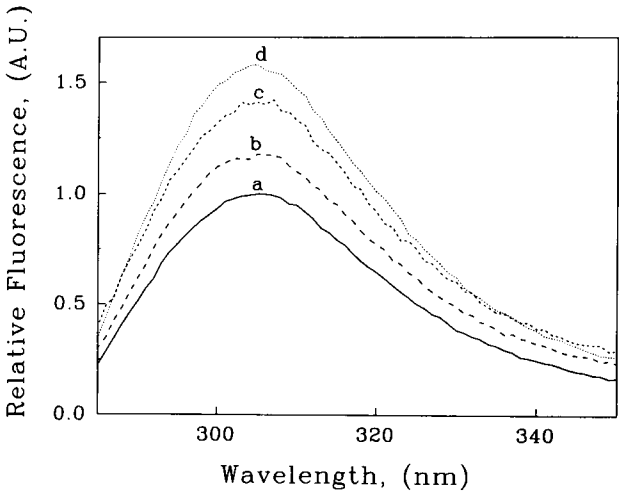

FIGURE 2 Effects of different ligands on the tyrosine fluorescence of TnC. Fluorescence measurements were carried out at 25°C on solutions containing 0.8 mg/ml of TnC in buffer A in the presence of 1 mM EGTA (curve *a*); 1 mM EGTA + 3 mM $CaCl_2$ (curve *d*); 1 mM EGTA + 4 mM $AlCl_3$ + 16 mM NaF (curve *c*); or 1 mM EGTA + 2 mM $MgCl_2$ (curve *b*). Each spectrum is the average of four scans. The excitation wavelength was 280 nm.

Aluminum fluoride titration of TnC in the presence and absence of Mg^{2+}

To further test the hypothesis that aluminum fluoride binding affects the Mg^{2+} binding sites, TnC was titrated with aluminum fluoride in the presence and absence of 2 mM Mg^{2+}. The addition of aluminum fluoride to TnC induced a 41% enhancement of the tyrosine fluorescence with a transition midpoint at $\approx 2 \times 10^{-3}$ M aluminum fluoride (Fig. 3). The presence of 2 mM Mg^{2+} resulted in a shift of the transition midpoint which could not be determined due to technical difficulties (high $AlCl_3$ concentration induced protein aggregation). The heterogeneity of the titration curve in the presence of 2 mM Mg^{2+} suggests that more than one type of binding site is involved. The high concentrations of aluminum needed for significant changes in TnC fluorescence, as compared with the concentrations which cause force inhibition in skinned muscle fibers (Chase and Kushmerick, 1990) suggest that the primary effect of aluminum fluoride is at the active site of myosin. Nevertheless, the above results show that aluminum fluoride may independently affect TnC as well.

TABLE 2 Effects of different ligands on the tyrosine fluorescence of TnC

Ligand	% Change in fluorescence
None	—
$CaCl_2$	55 ± 9
AlF_x	41 ± 9
$MgCl_2$	14 ± 5
BeF_x	0.6 ± 1
$MgCl_2$ + AlF_x	30 ± 5
AlF_x + $MgCl_2$	41 ± 5

Tyrosine fluorescence of TnC (0.8 mg/ml) was measured in 10 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 2 mM EGTA in the presence and absence of ligands. To obtain the percentage changes in fluorescence in the presence of ligands (corrected for dilution effect); the tyrosine fluorescence of TnC in buffer A + 2 mM EGTA was normalized to 100%.

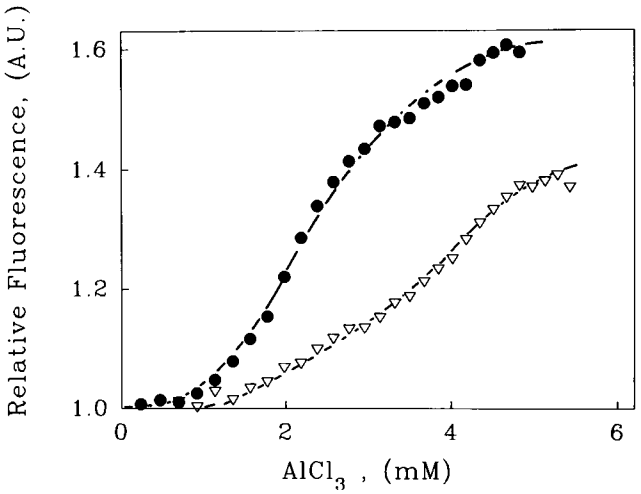

FIGURE 3 Aluminum fluoride titration of TnC in the presence (▽) and absence (●) of 2 mM $MgCl_2$. Small volumes of $AlCl_3$ were added directly to cuvettes containing 0.8 mg/ml of TnC in buffer A + 1 mM EGTA and 16 mM NaF. The ordinate represents the ratio of the tyrosine fluorescence at each added $AlCl_3$ concentration (after a correction for dilution) to that of $AlCl_3$ free protein. The excitation and emission wavelengths were 280 nm and 315 nm, respectively.

Aluminum fluoride binding exposes a hydrophobic region on TnC

Ca^{2+} induces a conformational change in TnC that exposes hydrophobic sites on the surface of the molecule (Tanaka and Hidaka, 1981). The exposure of hydrophobic sites results in an increase in the affinity of TnC for hydrophobic probes (Cachia et al., 1986a). Several hydrophobic probes have been used to demonstrate this effect in TnC (Tanaka and Hidaka, 1981) and calmodulin, a ubiquitous Ca^{2+}-regulatory protein homologous to TnC (Johnson and Wittenauer, 1983; Laporte et al., 1980; Tanaka and Hidaka, 1980). Fig. 4 shows the emission spectra of a hydrophobic probe, bis-ANS, which is an analog of the frequently used ANS (8-anilino-1-naphthalene sulfonate) (Johnson and Wittenauer, 1983; Laporte et al., 1980). As reported by earlier studies (Laporte et al., 1980), the exposure of hydrophobic regions is manifested at two levels: by a blue shift in the emission maximum and an increase in the fluorescence intensity of the probe. In the presence of EGTA, TnC enhanced the fluorescence intensity of bis-ANS by 60% (Fig. 4 *B*, curve *d*). The same enhancement has been observed upon calmodulin binding of ANS (Tanaka and Hidaka, 1980). As reported by previous studies (Johnson and Wittenauer, 1983; Tanaka and Hidaka, 1981) calcium binding to TnC results in the exposure of a hydrophobic site, which is evidenced by more than a 16-fold increase in the fluorescence of bis-ANS and a shift in λ_{max} from 520 nm to 490 nm. Mg^{2+} alone did not enhance bis-ANS binding to TnC (Fig. 4 *B*, curve *e*), supporting the idea that the Ca^{2+}-induced exposure of hydrophobic regions is mediated through the Ca^{2+}-specific sites (Johnson and Wittenauer, 1983). Although to a much lesser extent, aluminum fluoride binding also exposed hydrophobic groups on TnC, as revealed by a shift in λ_{max} from 520 to 510 nm and a 40%

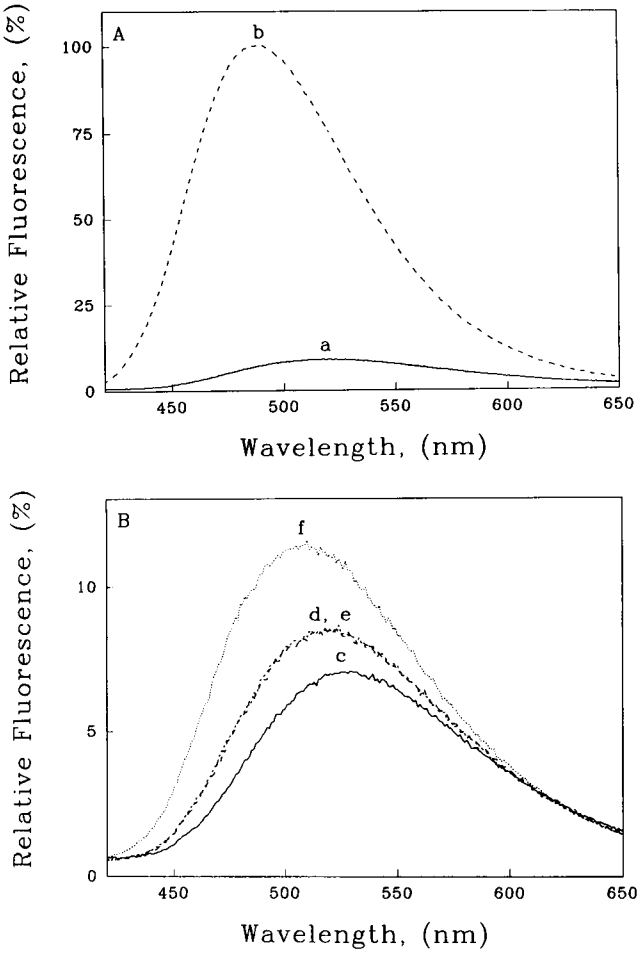

FIGURE 4 Effects of different ligands on the fluorescence of bis-ANS in the presence of TnC. The emission spectrum of bis-ANS (30 μM) was measured with the excitation wavelength set at 394 nm. Each emission spectrum is the average of four scans. All emission spectra are plotted relative to the maximum intensity of bis-ANS obtained in the presence of TnC (20 μM) and $CaCl_2$ (3 mM). (*A*) curve *a*, Emission spectrum of bis-ANS (30 μM) in buffer A in the presence of TnC (20 μM) and 2 mM EGTA; curve *b*, emission spectrum of bis-ANS in the presence of TnC (20 μM), EGTA (2 mM) and $CaCl_2$ (3 mM). (*B*) curve *c*, emission spectrum of free bis-ANS in buffer A; curve *d*, emission spectrum of bis-ANS in the presence of TnC (20 μM) and EGTA (2 mM); curve *e*, same as curve *d* plus $MgCl_2$ (2 mM); curve *f*, emission spectrum of bis-ANS in the presence of TnC, EGTA, $AlCl_3$ (4 mM), and NaF (16 mM) (curve *c*). Note the difference in the scale between parts *A* and *B*.

enhancement in bis-ANS fluorescence intensity. Interestingly, in the presence of 4 mM aluminum fluoride the Ca^{2+}-induced binding of bis-ANS to TnC was reduced by 20%, suggesting that aluminum fluoride affects or competes for the Ca^{2+}-specific sites. This reduced hydrophobic exposure may be important because the increase in hydrophobic character of TnC upon Ca^{2+} binding has been associated with an increase in its affinity for TnI (Grabarek et al., 1981; Dalgarno et al., 1982; Gariepy and Hodges, 1983; Leavis and Gergerly, 1984; Cachia et al., 1986b; Zot and Potter 1987). The actual effect of aluminum fluoride on the binding of TnC to TnI in the presence of Ca^{2+} has been examined more directly by monitoring the change in TnI tryptophan fluorescence.

Titrations of 100 nM TnI with TnC showed that the pretreatment of TnC with aluminum fluoride reduced the binding affinity of TnC for TnI by about twofold (data not shown).

The extension of the hydrophobic probe approach to the intact Tn appears as a simple way to test for specific effects of Ca on TnC in the intact Tn. However, the results of such experiments can not be easily linked to the observations on isolated TnC. First, Tn binds bis-ANS well even in the absence of Ca^{2+} and enhances manyfold the fluorescence of the probe. Ca^{2+} causes a 34% quenching of bis-ANS emission on the Tn, whereas aluminum fluoride quenches the signal by 17%. In the presence of aluminum fluoride the quenching by Ca^{2+} amounts to only a 21% decrease in the fluorescence of Tn-bis-ANS. Although no conclusive interpretation can be given for these results it is worth noting that in analogy to isolated TnC, aluminum fluoride inhibits the effect of Ca^{2+} also in the intact Tn.

DISCUSSION

The increasing interest in beryllium and aluminum fluorides as analogs of P_i in the myosin ATPase reaction and the possible use of these compounds for mechanical studies on muscle fibers (Chase and Kushmerick, 1990; Chase et al., 1993) merit a consideration of their interactions with the regulatory system of Tn and tropomyosin. Indeed, a frequently used P_i analog, vanadate, has been recently shown to release TnI from permeabilized porcine ventricular muscle (Strauss et al., 1992).

According to the three spectroscopic approaches used in this work, aluminum fluoride binds to and affects the structure of TnC, whereas beryllium fluoride has no effect on this protein. In the first method, the CD measurements report on relatively large changes in α-helical content of TnC upon binding of Ca^{2+} or Mg^{2+}. These changes have been linked to the occupancy of the high-affinity Ca^{2+}/Mg^{2+} sites III and IV in the COOH-terminal part of TnC by either Ca^{2+} or Mg^{2+} (Grabarek et al., 1992). The fact that aluminum fluoride increases the α-helicity of TnC and decreases the Mg^{2+}-induced change in CD suggests that aluminum fluoride binds to or affects the Ca^{2+}/Mg^{2+} sites.

The second assay of cation interactions with TnC involved tyrosine fluorescence measurements. Earlier work showed that the fluorescence changes noted upon binding of Ca^{2+} to TnC reflect a local perturbation of Tyr-109 on rabbit skeletal TnC. The binding of Ca^{2+} to the adjacent carboxylate groups in the high-affinity site III was viewed as the source of that perturbation (Leavis and Lehrer, 1978; Nagy and Gergerly, 1979). The effect of aluminum fluoride on tyrosine fluorescence is rather large, much closer to that elicited by Ca^{2+} than by Mg^{2+}. Yet, as shown above, despite strong local perturbation of Tyr-109 environment, aluminum fluoride has only a small impact on the overall secondary structure of TnC.

The third method of probing the interactions of cations with TnC depended on the enhanced binding of a hydrophobic fluorescent reagent to TnC in the presence of Ca^{2+}. The large increase in the binding of ANS and bis-ANS to TnC in the presence of Ca^{2+} has been interpreted as due to the exposure of a hydrophobic site in the NH_2-terminal part of TnC (Cachia et al., 1986a). The interaction of this site with TnI is expected to increase the affinity of TnC for TnI. Inasmuch as the enhancement of bis-ANS fluorescence is specific to Ca^{2+} and is not produced by Mg^{2+} (Fig. 4 *B*, curve *e*), it probably monitors the binding of Ca^{2+} to the Ca^{2+}-specific sites I and II on TnC. As in the previous tests, aluminum fluoride but not beryllium fluoride induced a partial shift in the spectral properties of bis-ANS. Thus, to a limited extent, aluminum fluoride perturbs the hydrophobic site on TnC, most likely through binding to or affecting the Ca^{2+}-specific sites on this protein. Aluminum fluoride inhibits also the Ca^{2+}-induced exposure of the hydrophobic site on TnC and lowers the affinity of TnC for TnI.

It is possible that in the intact Tn, in the absence of Ca^{2+}, bis-ANS binds primarily to a hydrophobic site on TnI. The binding of Ca^{2+} to TnC and the consequent pairing of hydrophobic sites on TnI and TnC may then reduce the availability of a hydrophobic region on TnI to bis-ANS resulting in the observed fluorescence quenching.

The spectral effects of aluminum fluoride on TnC reported in this work demonstrate a direct interaction of this phosphate analog with TnC and a consequent inhibition of Ca^{2+} effect on the protein. Although these results may be of general interest for investigations of other Ca^{2+}-binding proteins, they merit special attention in the context of mechanical studies on muscle fibers. Caution with respect to aluminum fluoride-induced changes in Tn will be particularly well placed in experiments conducted at submaximum Ca^{2+} concentrations and when monitoring the transition from relaxation to activation in muscle fibers.

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