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Different Polymeric Forms of Actin Detected by the Fluorescent Probe Terbium Ion†

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ABSTRACT: The interaction of actin with Tb^{3+} was studied by following the fluorescence emitted at 545 nm when the protein was excited at 285 nm in the presence of Tb^{3+}. It was shown that, at low ionic strength, each actin monomer binds, at saturation, six Tb^{3+} with an association constant of 0.8 μM^{-1}. In the presence of 0.1 M KCl the association constant decreases to 0.15 and 0.24 μM^{-1} at subcritical and overcritical actin concentrations, respectively; the number of the binding sites remains six. When polymeric actin is formed by the addition of 2 mM $MgCl_2$, the association constant drops to 0.008 μM^{-1} and the number of the binding sites to four. The lower number of the Tb^{3+} binding sites (four) in the actin polymerized by $MgCl_2$ as compared to the number of binding sites (six) of the actin polymerized by KCl is taken as evidence of a looser structure of this latter polymer. We have also shown that Tb^{3+} does not replace $^{45}Ca^{2+}$ at the single, "high-affinity" site of G-actin. Removal of this Ca^{2+}, in the presence of Tb^{3+}, destroys the typical G- and F-actin structures.

G-actin is polymerized by many agents: monovalent cations at high concentration, divalent cations (Kasai et al., 1962), polyamines (Oriol-Audit, 1978), and lanthanides (Barden & Dos Remedios, 1980). The nature of the polymer formed depends on the nature as well as on the concentration of the polymerizing agent. One millimolar Ca^{2+} and Mg^{2+} form double-stranded F-actin. When the concentration of the divalent cation is increased to 5 mM, paracrystals are formed. Ni^{2+} produces F-actin filaments which tend to break into short fragments. Zn^{2+} forms globular aggregates which coexist with the filaments (Strzelecka-Golaszewka et al., 1978). G-actin, in the presence of gadolinium ions, forms microcrystalline aggregates which, by addition of 0.1 M KCl, are converted into tubular structures (Dos Remedios & Dickens, 1978). In this work the study of the interaction between actin and Tb^{3+} reveals that the polymers formed by treating G-actin with either 0.1 M KCl or 2 mM $MgCl_2$ behave differently with respect to Tb^{3+} ions. These differences could be explained by a more compact structure of the polymer formed in the presence of Mg^{2+}. Studies are in progress to inquire whether the conformational differences of the two polymers are accompanied by functional differences.

Materials and Methods

G-actin from rabbit muscle was prepared according to Spudich & Watt (1971) and stored as a concentrated solution (5 mg/mL) at 0 °C in 0.2 mM ATP,[1] 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol, 0.5 mM NaN_3, and 2 mM Tris-HCl buffer, pH 8.2. Immediately before use the actin stock solution, diluted with an equal volume of 30 mM imidazole hydrochloride buffer, pH 7.0, was treated for 2 min at 2 °C with 1:15 (v/v) wet 100–200-mesh Dowex 1-X8 Cl form resin to remove free ATP. The solution was then separated from the resin by centrifugation and treated, by the same procedure, with 1:15 (v/v) wet 100–200-mesh Dowex 50-X2 K form resin to remove free or weakly bound Ca^{2+}, the resin being equilibrated in all the cases with 15 mM imidazole hydrochloride buffer, pH 7.0. Finally, the protein solution was adjusted to the desired concentration with 15 mM imidazole hydrochloride buffer, pH 7.0. By this procedure no appreciable loss in protein concentration or biological activity was observed. G-actin retained fully the capability to polymerize and to inhibit DNase I.

K-F-actin and Mg-F-actin were obtained by addition of 0.1 M KCl and 2 mM $MgCl_2$, respectively, to the G-actin treated with the ion-exchange resins. The formation of the polymer was followed until constant viscosity was reached. Alternatively, when low concentrations of actin were employed, the state of aggregation of the protein was ascertained by 90° light scattering measurements (incident light 580 nm, scattered light 580 nm). $TbCl_3$ was from Ventron-Gmbh, Karlsruhe, Germany; $^{45}CaCl_2$, carrier free, was from Amersham, England. Twice distilled water was used in all the operations, the first distillation being performed in the presence of $KMnO_4$.

Actin concentration was measured from the absorbance at 290 nm, the absorbance of 1 mg of pure actin/mL (light path 1 cm) being taken to be 0.62 (Gordon et al., 1976). Alternatively, the method of Lowry et al. (1951) was used. The molar concentration of G-actin was calculated on the basis of a molecular weight of 42 000 (Collins & Elzinga, 1975).

Calcium concentration was measured with a Perkin-Elmer atomic absorption spectrophotometer, Model 603, after deproteinization of the solution with 5% $HClO_4$. [$^{45}Ca^{2+}$]G-actin

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; DNase, deoxyribonuclease.

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was prepared by incubating overnight at 2 °C a stock solution of G-actin with adequate amounts of carrier-free $^{45}CaCl_2$. Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer after addition of 10 mL of Bray's solution (Bray, 1960) to 0.2-mL samples. Sonication of F-actin was performed with a KG 100 sonication apparatus (Kerry's Ultrasonic Ltd) equipped with an ice-cold water thermostating bath.

Fluorescence measurements were performed with a Perkin-Elmer MPF3 spectrofluorometer equipped with a Linseis recorder. The cell holder was thermostated at 20 ± 0.2 °C. A cutoff filter for removal of the scattered light ($\lambda < 430$ nm) was inserted in front of the photomultiplier. The exciting wavelength was 285 nm; excitation and emission band-passes of 6 nm were chosen. The 530–560-nm region of the emission spectra was recorded, and any background emission due to the long-wavelength tail of intrinsic protein luminescence was subtracted (corrected fluorescence). Because of the tendency of actin to denature under extended UV irradiation, only one fluorescence measurement per sample was made.

For analysis of the data on the titration of actin by Tb^{3+}, a rearranged form of the Scatchard equation (Scatchard, 1949) was used

$$\frac{[Tb]_b/[actin]_0}{[Tb]_f} = nK - \frac{[Tb]_b}{[actin]_0}K \qquad (1)$$

The subscripts b, f, and 0 stand for bound, free, and total, respectively; K stands for the association constant between Tb^{3+} and actin; n stands for the number of actin binding sites.

On substitution of $[Tb]_b = n[actin]_0 L/L_\infty$ and $[Tb]_f = [Tb]_0 - [Tb]_b$, where L represents the corrected fluorescence intensity for a given value of $[Tb]_0$ and L_∞ the corrected fluorescence intensity at saturation, eq 1 can be rewritten

$$K - nK[actin]_0\frac{L}{L_\infty}\frac{1}{[Tb]_0} = \frac{L}{L_\infty - L}\frac{1}{[Tb]_0} \qquad (2)$$

and by plotting $L/[(L_\infty - L)[Tb]_0]$ vs. $L/(L_\infty[Tb]_0)$ the intercept on the ordinate represents K and the intercept on the abscissa represents $1/(n[actin]_0)$.

For analysis of the data on the competition of Tb^{3+} and Ca^{2+} for actin, a rearranged form of the equation of Lo Scalzo & Reed (1976) was used

$$\frac{n-\nu}{\nu}[Tb]_{fi} = \frac{1}{K_{Tb}} + [Ca]_{fi}\frac{K_{Ca}}{K_{Tb}} \qquad (3)$$

$\nu = [Tb]_{bi}/[actin]_0$; K_{Tb} and K_{Ca} are the association constants for Tb^{3+} and Ca^{2+}, respectively. $[Tb]_{fi}$ is calculated from

$$[Tb]_{fi} = [Tb]_0 - \frac{L_i}{L}[Tb]_b \qquad (4)$$

L is the fluorescence intensity of the sample without Ca^{2+} to which corresponds the bound terbium ion concentration $[Tb]_b$, calculated from the mass action law, knowing n and K_{Tb}. L_i is the fluorescence intensity of the same sample after the addition of $[Ca]_0$. Plotting $[(n-\nu)/\nu][Tb]_f$ vs. $[Ca]_f$ and taking $[Ca]_f \simeq [Ca]_0$, the intercept on the ordinate represents $1/K_{Tb}$ and the intercept on the abscissa represents $-1/K_{Ca}$.

Results

A Fluorescent Actin–Tb^{3+} Complex. The addition of 65 μM $TbCl_3$ to 2.5 μM G-actin generates two fluorescent bands, with maxima at 490 and 545 nm, respectively, when the solution is excited at 285 nm (Figure 1). Spectra were also recorded at higher and lower protein concentrations to ensure that the observed excitation spectrum was not affected by the absorbance of actin. Very similar spectra were obtained in all cases. No fluorescence was detected in the absence of protein.

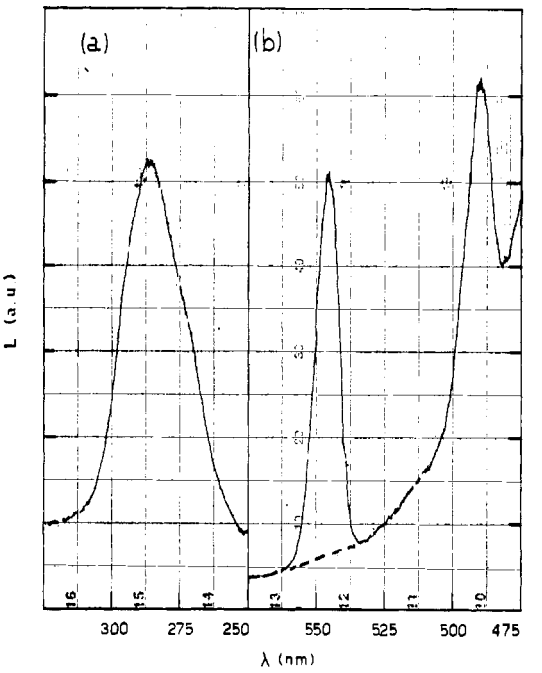

FIGURE 1: Excitation and emission spectra of the G-actin–Tb^{3+} complex. Excitation (a) and emission (b) spectra of a solution containing 2.5 μM Dowex 1, Dowex 50 treated G-actin, 65 μM $TbCl_3$, and 15 mM imidazole hydrochloride buffer, pH 7.0. The temperature was 20 °C. The excitation spectrum was obtained by detecting the 545-nm emitted light; the emission spectrum was obtained by exciting at 285 nm. Instrumental conditions are those described under Materials and Methods.

Table I: Association Constants and Number of Tb^{3+} Binding Sites in the Tb^{3+}–Actin Complex [a]

actin concn (μM)	added salts	K (μM^{-1})	n
0.75 [b]	none	0.800	6.06
1.0 [c]	0.1 M KCl	0.150	6.06
7.4 [d]	0.1 M KCl	0.240	6.18
7.4 [e]	2 mM $MgCl_2$	0.008	4.25

[a] Data are taken from Figure 2. [b] Figure 2a. [c] Figure 2b. [d] Figure 2c. [e] Figure 2d.

The fluorescence is very likely due to a Förster-type energy-transfer process from the nearby aromatic side chains of the protein. The energy transfer populates the $^5D_4$ ion electronic level and enhances strongly the $^5D_4 \rightarrow {}^7F_5$ radiative decay (Brittain et al., 1976; Horrocks & Sudnick 1979). The 545-nm emission band was selected for our investigation because of the lesser interference with the intrinsic protein fluorescence.

Titration of Actin with Tb^{3+} and Back-Titration with Ca^{2+}. The reaction of G-actin with Tb^{3+} was studied by following the increase of the fluorescence as a function of the amount of $TbCl_3$ added to the solution (Figure 2a). Analysis of the data by means of a rearranged (see Materials and Methods) Scatchard equation (Scatchard, 1949) shows that every actin monomeric unit binds 6.06 Tb^{3+} ions with an association constant of 0.8 μM^{-1} (Table I). A difficulty in the interpretation of the data arises because, in the presence of Tb^{3+}, G-actin, even at the low concentrations employed in these experiments, forms microcrystalline aggregates (Barden & Dos Remedios, 1980). The formation of the aggregates is revealed by an increase in light scattering intensity at 580 nm, which

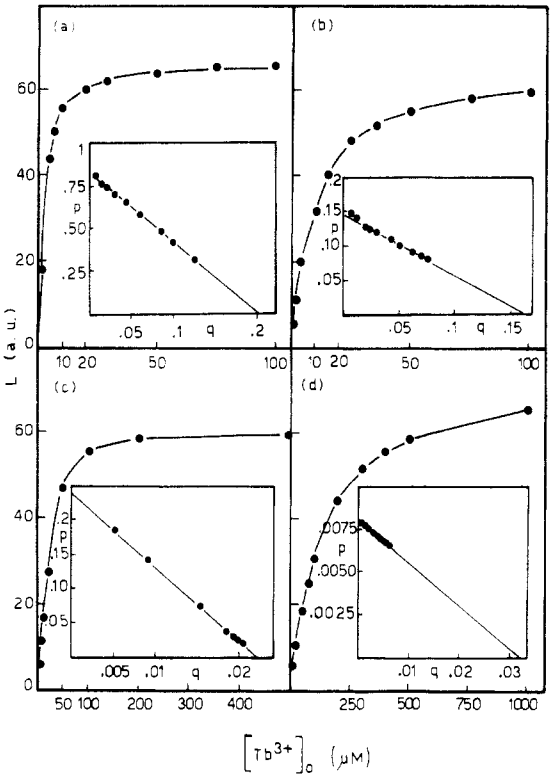

FIGURE 2: Association constants and number of Tb^{3+} binding sites in Tb^{3+}–actin complexes. The mixtures contained 15 mM imidazole hydrochloride buffer, pH 7.0, $TbCl_3$ to give the final concentrations reported in the figure, and (a) 0.75 μM G-actin, (b) 1.0 μM G-actin plus 0.1 M KCl, (c) 7.4 μM G-actin plus 0.1 M KCl, and (d) 7.4 μM G-actin plus 2 mM $MgCl_2$. The temperature was 20 °C. Fluorescence was measured at 545 nm (excitation was performed at 285 nm). In all the experiments actin was treated with Dowex 1 and Dowex 50 as described under Materials and Methods. When it was needed, actin was preincubated 90 min at 20 °C in the presence of 0.1 M KCl (experiments b and c) and $MgCl_2$ (experiment d) before the addition of $TbCl_3$. The addition of $TbCl_3$ was always made by mixing equal volumes of the actin and $TbCl_3$ solutions, the latter containing, when it was needed, either 0.1 M KCl or 2 mM $MgCl_2$ in imidazole buffer. In the inserts $p = L/[(L_\infty - L)[Tb]_0]$ is plotted against $q = L/(L_\infty[Tb]_0)$.

follows the addition of $TbCl_3$ to the actin solution. The light scattering intensity increases linearly between 0 and 100 μM $TbCl_3$, the increase being 200%, at 100 μM $TbCl_3$, over the value obtained in the absence of $TbCl_3$. The Scatchard plot, however, is linear over all the range of the concentrations of $TbCl_3$ explored. This means that the association constant for Tb^{3+} is independent of the saturation fraction of the Tb^{3+} binding sites as well as of the extent of formation of the microcrystalline aggregates. It is thus very likely that the same association constant for Tb^{3+}, 0.8 μM^{-1}, holds, not only for actin in the microcrystalline aggregates but also for G-actin.

Ca^{2+} was found to compete with Tb^{3+} for actin (Figure 3a) as shown by the decrease of the fluorescence of the solution when Ca^{2+} is added to 0.75 μM actin and 7.5 μM $TbCl_3$. Analysis of the data by means of a rearranged form of the equation of Lo Scalzo & Reed (1976) (see Materials and Methods) shows that the experimental points fall on a straight line (Figure 3b). From the intercept on the abscissa ($-1/K_{Ca}$) and association constant $K_{Ca} = 0.2 \times 10^3$ M^{-1} for the weakly bound Ca^{2+} is obtained.

"High-Affinity" Calcium Site of G-actin Is Not Available to Tb^{3+}. In the preceding section we have shown that, in the microcrystalline aggregates, every actin monomer binds six Tb^{3+} ions per molecule and that Ca^{2+} competes for these binding sites with an association constant of 0.2×10^3 M^{-1}. The next question was whether Tb^{3+} could displace Ca^{2+} from

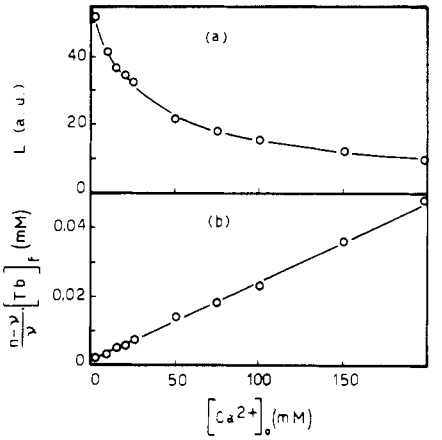

FIGURE 3: Competition between Ca^{2+} and Tb^{3+} for monomeric actin. The samples (2 mL) were prepared by mixing 1 mL of an actin–Tb^{3+} stock solution with 1 mL of 15 mM imidazole hydrochloride buffer, containing $CaCl_2$ at the suitable concentrations. The stock solution was prepared by mixing equal volumes of two 15 mM imidazole hydrochloride buffer solutions containing respectively 3 μM Dowex 1, Dowex 50 treated G-actin and 30 μM $TbCl_3$. The pH was 7.0, and the temperature was 20 °C. (a) Fluorescence was recorded at 545 nm (excitation was performed at 285 nm). (b) Analysis of the data of (a) was performed by making use of a rearranged form of the equation of Lo Scalzo & Reed (1976) as described under Materials and Methods.

the high-affinity site. Two sets of experiments were designed to answer this question.

It was first shown (Figure 4a) that 75 μM Tb^{3+} does not enhance the release of $^{45}Ca^{2+}$ from the high-affinity site of actin. From the radioactivity and fluorescence intensity measurements of the same experiment, it was also clear that G-actin binds simultaneously both $^{45}Ca^{2+}$ at the high-affinity site and Tb^{3+} at the other sites.

In a second set of experiments the effect of Tb^{3+} was studied on the sonication-allowed $^{45}Ca^{2+}$ exchange between F-actin and the medium. As it is shown in Figure 4b, 62.5 μM Tb^{3+} does not enhance the release of Ca^{2+} from sonicated F-actin, in comparison with a control sample without Tb^{3+}. The addition of 1 mM Tb^{3+}, on the contrary, decreases substantially the amount of $^{45}Ca^{2+}$ bound to the actin. The release of Ca^{2+} is accompanied, however, by the appearance of turbidity due to protein aggregation. This phenomenon is only displayed by sonicated samples. The release of Ca^{2+} from the high-affinity site is thus more likely caused by the obliteration of the site itself than by substitution of Ca^{2+} with Tb^{3+}.

Interaction of Tb^{3+} with Monomeric and Polymeric Actin in the Presence of either KCl or $MgCl_2$. Evidence has been reported of conformational differences between G-actin at low ionic strength and monomeric actin at subcritical concentration and high ionic strength (Rich & Estes, 1976). We have sought to study these phenomena by studying the fluorescence of the actin–Tb^{3+} complex at low (0.4 μM) and high (8 μM) actin concentrations in the presence of different concentrations of either $MgCl_2$ or KCl.

As is shown in Figure 5, at 0.4 μM actin, the fluorescence decreases with increases in the $MgCl_2$ and KCl concentrations. Comparison of the effect of the two cations makes it clear that the decrease of fluorescence in the presence of $MgCl_2$ is not due solely to the increase of the ionic strength of the medium but also to specific interactions with the binding sites for Tb^{3+}. Analogous results were obtained with Ca^{2+} (Figure 3). (The addition to 0.4 μM actin of either KCl or $MgCl_2$, at the concentrations indicated in Figure 5, does not increase the intensity of the light scattering of the protein solution. Thus, before the addition of $TbCl_3$, actin is still in the monomeric

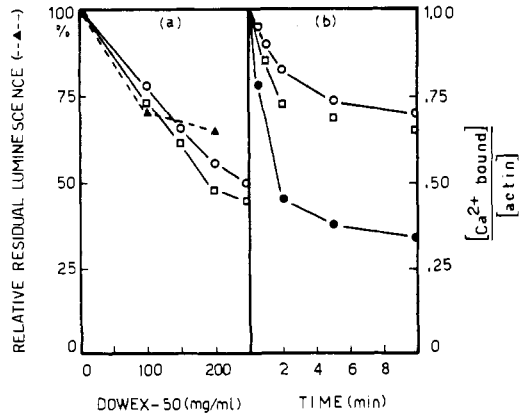

FIGURE 4: Effect of addition of Tb^{3+} on binding of Ca^{2+} to the high-affinity site of G-actin. (a) A solution containing 10 μM Dowex 1 treated G-actin, 24 μM $^{45}Ca^{2+}$ (total concentration) (specific radioactivity 12000 cpm/nmol), and 15 mM imidazole hydrochloride buffer, pH 7.0, was mixed with an equal volume of a solution containing either 150 μM $TbCl_3$ in 15 mM imidazole hydrochloride buffer, pH 7.0 (○), or buffer alone (□). After 60 min of incubation at 2 °C separate 1-mL aliquots of the sample and of the control solutions were mixed for 2 min at 2 °C with increasing amounts of Dowex 50 and separated from the resin by a short centrifugation as described under Materials and Methods. The intensity of the fluorescence (▲) and the radioactivity to the protein ratio (○, □) were determined in the supernatant solutions. (b) A solution containing 30 μM G-actin (Dowex 1, Dowex 50 treated), 30 μM $^{45}Ca^{2+}$ (actin bound) (specific radioactivity 12000 cpm/nmol), and 15 mM imidazole hydrochloride buffer, pH 7.0, was polymerized by the addition of 0.1 M KCl. An aliquot of the above solution was mixed with an equal volume of either 0.1 M KCl in 15 mM imidazole hydrochloride buffer, pH 7.0 (□), or 125 μM (○) or 2 mM (●) $TbCl_3$ in the same solution. After 60 min of incubation at 20 °C to restore the G ⇌ F equilibrium, samples were ice cooled and sonicated at 2 °C. At the times indicated in the figure F-actin was recovered by filtration of 0.2-mL samples through 0.45 μM pore size Millipore filters (Grazi & Magri, 1981) and the radioactivity determined by counting the filters directly in 10 mL of Bray's solution. The amount of protein on the filter was determined as the difference between total protein and the protein found in the filtrate.

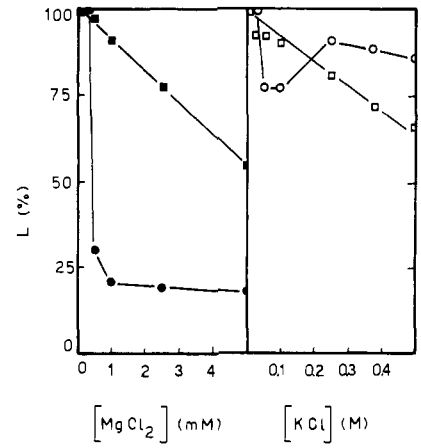

FIGURE 5: Fluorescence intensities of the actin–Tb^{3+} complexes as a function of Mg^{2+} and K^+ concentrations. The samples contained 15 mM imidazole hydrochloride buffer, pH 7.0, either $MgCl_2$ or KCl, at the concentrations indicated in the figure, and either 0.4 μM G-actin plus 20 μM $TbCl_3$ (squares) or 8 μM G-actin plus 50 μM $TbCl_3$ (circles). The temperature was 20 °C. In all the cases Dowex 1 and Dowex 50 treated actin was incubated 90 min at 20 °C in the imidazole buffer containing either $MgCl_2$ or KCl at $^{10}/_9$ of the concentrations indicated in the figure. To the incubation mixtures (1.8 mL) was then added, with stirring, 0.2 mL of either 0.2 or 0.5 mM $TbCl_3$ in the imidazole buffer, and the incubation was continued for additional 20 min. The fluorescence intensity of the samples was then determined as described under Materials and Methods.

state.) At 8 μM actin, in the presence of KCl, the fluorescence intensity decreases steeply between 0 and 0.05 M KCl, rises again between 0.1 and 0.25 M KCl (this behavior, although confirmed in all the experiments, remains unexplained), and then slowly decreases between 0.3 and 0.5 M KCl.

With $MgCl_2$ a sudden drop of the luminescence intensity was observed at 0.5 mM $MgCl_2$ followed by a smooth decrease between 1 and 5 mM $MgCl_2$. The fluorescence data were further analyzed according to the rearranged Scatchard equation (Figure 2 and Table I). It was then found that the association constant of the actin–Tb^{3+} complex decreases from 0.8 μM^{-1} at low ionic strength to 0.15 and 0.24 μM^{-1} in 0.1 M KCl at 0.4 and 8 μM actin, respectively. In 2 mM $MgCl_2$ the association constant was further decreased to 0.008 μM^{-1}. The number of Tb^{3+} binding sites, unaffected by the addition of KCl, was decreased from six to four by the addition of 2 mM $MgCl_2$.

Discussion

By studying the fluorescent actin–Tb^{3+} complex, we have shown that actin possesses six Tb^{3+} binding sites with an association constant of 0.8 μM^{-1}. Mg^{2+} and Ca^{2+} compete with Tb^{3+}, and from this competition an association constant of 0.2×10^3 M^{-1} for the actin–Ca^{2+} complex was calculated. The same number of Tb^{3+} binding sites was also found, by an independent method, by Dos Remedios et al. (1980).

An association constant of 6×10^3 M^{-1} was calculated by Strezelecka-Golaszewka et al. (1978) for the calcium binding sites responsible for the polymeriztion of actin. This figure differs too much from that obtained in our study (0.2×10^3 M^{-1}) to be explained by the different methods employed in the two cases. It is more likely that the two figures refer to two different classes of sites and that the Tb^{3+} binding sites are not involved in the divalent cation promoted actin polymerization. This veiw is also strengthened by the consideration that microcrystalline aggregates and not F-actin are formed from G-actin in the presence of high Tb^{3+} concentrations (Dos Remedios & Dickens, 1978).

We are unable to confirm the claim of Barden & Dos Remedios (1979, 1980) that lanthanides replace Ca^{2+} at the high-affinity site. We have shown that, as long as G-actin maintains its properties (i.e., can be polymerized into F-actin), Ca^{2+} remains bound to the high-affinity site. Furthermore, when [$^{45}Ca^{2+}$]F-actin is sonicated, $^{45}Ca^{2+}$ is exchanged with the medium without denaturation of the protein, unless 1 mM Tb^{3+} is present. In the latter case $^{45}Ca^{2+}$ is released and the solution becomes turbid, probably because of the formation of the tubular structures described by Dos Remedios et al. (1980).

We conclude, therefore, that the substitution of Tb^{3+} for Ca^{2+} at the high-affinity site is incompatible with the typical G- and F-actin structures. Ca^{2+} is displaced, but the typical G-actin structure is lost. Our interpretation is further supported by the finding (Barden & Dos Remedios, 1980) that the tubular structures, at difference with F-actin, contain ATP and not ADP. Evidently the monomer–monomer interactions in the tubular structures are different from those of F-actin and do not allow the dephosphorylation of ATP to take place. The major contribution of our study, however, seems to be the finding that F-actin possesses a different number of Tb^{3+} binding sites depending of whether the polymer has been obtained in the presence of either KCl (six sites) or $MgCl_2$ (four sites).

We show that the association constant for Tb^{3+} is decreased 4 to 5 times in the presence of 0.1 M KCl. The formation of the polymer, however, does not induce per se a significant change either in the association constant or in the number of

the binding sites. Since the monomer to monomer interactions in the polymer involve a large fraction of the total surface of the monomer, it seems unlikely that polymerization may occur without decreasing the number of the binding sites unless a very loose polymeric structure is formed as seems to be the case for actin polymerized in the presence of KCl. If this is true, polymerization induced by $MgCl_2$ must have led to a much more compact polymeric structure characterized by a further (20-fold) decrease of the binding constant for Tb^{3+}. This view is supported by the very recent finding of Crawford et al. (1980), who isolated, from guinea pig polymorphonuclear leukocytes, two different polymeric forms of actin which they called Mg-actin and K-actin. The structural differences of the two polymers could be the basis of functional differences. This possibility is now under study.

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Proton Nuclear Magnetic Resonance Evidence for the Absence of a Stable Hydrogen Bond between the Active Site Aspartate and Histidine Residues of Native Subtilisins and for Its Presence in Thiolsubtilisins†

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ABSTRACT: The very low field proton nuclear magnetic resonance (^1H NMR) found in aqueous solutions of serine proteases and their zymogens is characteristic of the hydrogen bond between the imidazolium and aspartate groups of the catalytic triad: Ser-His-Asp [Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71*, 507–511]. According to ^1H correlation NMR spectroscopic studies performed in 80/20 (v/v) $H_2O/^2H_2O$, no such resonance is found in native subtilisins (even at −2 °C and pH 6.0), but it is present in thiolsubtilisins and in the phenylboronic acid derivatives of the serine enzymes. The resonance was not visible in the mercuric or carboxamidomethyl derivatives of the thiol enzymes or in the phenylboronic acid–serine enzyme complex if the serine enzyme was first acylated with phenylmethanesulfonyl fluoride. The histidine at the catalytic site of thiolsubtilisin carries a positive charge between pH 5.6 and 8.4, in accord with previous data in favor of a mercaptide–imidazolium ion pair at the catalytic site. The charge distribution (− + −) at the active site of thiolsubtilisin and in the phenylboronic acid derivatives of the serine enzymes resembles that in the tetrahedral transition state formed between a serine enzyme and its substrate. Therefore, the stable hydrogen bond (found in the thiol enzyme and in the phenylboronic acid derivative of the serine enzyme) should be more important during catalysis than in the substrate-free enzyme.

X-ray diffraction studies on serine proteases, such as chymotrypsin, elastase, trypsin, and subtilisin, revealed the presence of an aspartate carboxyl group in the vicinity of the imidazole of the active site histidine residue [see Kraut (1977) for a review]. Blow et al. (1969) proposed that the triad of residues (aspartate, histidine, serine) at the catalytic center constitutes a "charge relay" that can conduct the electron pair from Asp to His to Ser, thereby enhancing the nucleophilicity of the catalytic serine oxygen. Such a relay of charge was questioned on chemical grounds (Polgár & Bender, 1969; Polgár, 1972; Rogers & Bruice, 1974). It was suggested by Polgár (1972) that the proton that is bound to the imidazole

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