THE EFFECT OF TERBIUM ON THE STRUCTURE OF ACTIN AND MYOSIN SUBFRAGMENT 1 AS MEASURED BY CIRCULAR DICHROISM

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Received 14 March 1981

1. Introduction

Substitution of Ca^{2+} by La^{3+} or Tb^{3+} produced a pronounced inhibitory effect on the ATPase activity of myosin S-1 alone, or in combination with F-actin [1]. To gain further insight into the possible mechanism of action of Ln^{3+} a CD investigation was carried out, wherein the effect of Tb^{3+} on the secondary structure of G- and F-actin, myosin S-1, as well as combinations of these proteins, was established. The results suggest that Tb^{3+} induces structural decreases in all these proteins and interactions between myosin S-1 and actin seem to be weakened in the presence of this lanthanide.

2. Materials and methods

2.1. *Protein preparations*

Myosin was isolated from rabbit back muscle by the dilution-precipitation method as in [2]. S-1 was prepared by chymotryptic digestion in the presence of EDTA and was isolated by chromatography on DEAE-cellulose [3]. The fractions corresponding to S-1(A_1) and S-1(A_2), confirmed by SDS-PAGE, were pooled, individually concentrated using immersible -CX-30 ultrafilters from Millipore, and stored in the freezer in 50% glycerol. When required, aliquots were dialyzed overnight at 4°C against 5 mM PIPES at pH 6.8, clarified by centrifugation and the concentration determined by absorbance using $A^{1\ mg/ml}_{280\ nm} = 0.8\ cm^{-1}$ [4].

Actin was prepared from an acetone powder of rabbit skeletal muscle as in [5]. The final dialysis of G-actin was against 2 mM Tris–HCl, pH 7.5, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$. The solution was clarified by centrifugation and frozen in liquid N_2 for storage in the freezer. Aliquots were removed, dialyzed overnight against 5 mM PIPES, pH 6.8, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$ and the concentration estimated, after clarification, by absorbance using $A^{1\ mg/ml}_{280\ nm} = 1.1$ [6]. Free nucleotide was removed from G-actin solutions by brief treatment with the ion-exchange resin Dowex 1 as in [7]. Protein concentration after this procedure was determined using $A^{1\ mg/ml}_{280\ nm} = 0.63\ cm^{-1}$ [8].

2.2. *CD measurements*

These measurements were effected at 27°C on a Cary 60 recording spectropolarimeter, with attached 6001 CD accessory as in [9]. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-camphorsulfonic acid. Constant N_2 flushing was employed. To convert ellipticity data into conformational parameters the procedures and equations of [10] were utilized. The reproducibility of all spectra was within ±3% for wavelengths greater than 205 nm.

3. Results and discussion

3.1. *G-actin*

The CD spectrum of G-actin in 5 mM PIPES, pH 6.8, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$

Abbreviations: ATPase, adenosinetriphosphatase; G- and F-actin, globular and fibrous forms of the protein actin; Ln^{3+}, lanthanide; La^{3+}, lanthanum; Tb^{3+}, terbium; myosin S-1(A_1) and (A_2), subfragment 1 produced by chymotryptic digestion of myosin containing light chains A_1 and A_2, respectively; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of socium dodecyl sulfate; DTT, dithiothreitol

Table 1
Conformational parameters for the various proteins studied

Protein	Concentration (μM)	$[Tb^{3+}]/[protein]$	$-[\theta]^{\circ}_{220\ nm}$	H	B	RC
G-actin	10.07	0.0	9700	0.293	0.268	0.439
		0.71	9540	0.294	0.246	0.460
		1.42	9510	0.291	0.262	0.447
		2.14	9510	0.299	0.229	0.472
		2.85	8070	0.258	0.195	0.547
S-1(A_1)	6.55	0.0	12 500	0.411	0.172	0.417
		0.20	12 300	0.406	0.159	0.435
		0.59	11 950	0.396	0.156	0.448
		0.99	11 560	0.389	0.125	0.486
S-1(A_2)	7.25	0.0	11 910	0.390	0.160	0.450
		0.45	11 500	0.385	0.141	0.474
F-actin	5.2	0.0[a]	10 510	0.334	0.234	0.432
		1.57	7400	0.242	0.146	0.612
		3.13	7610	0.254	0.144	0.602
		4.70	6200	0.211	0.124	0.665
		6.26	7950	0.263	0.135	0.602
G-actin-S-1(A_1)	6.24	0.0[a]	10 570	0.340	0.198	0.462
		0.75	10 820	0.344	0.206	0.449
		1.49	10 460	0.333	0.203	0.464
		2.23	9680	0.310	0.194	0.496
		2.97	9100	0.293	0.185	0.522
		3.71	8420	0.275	0.149	0.576
G-actin-S-1(A_1)[b]		0.0	10 730	0.336	0.227	0.437
G-actin-S-1(A_1)	3.48	0.0[a]	9600	0.316	0.160	0.524
		1.18	10 080	0.336	0.144	0.520
		2.35	9420	0.313	0.138	0.549
		3.53	9270	0.312	0.119	0.569
		4.70	8140	0.286	0.073	0.641
G-actin-S-1(A_1)[b]		0.0	10 840	0.345	0.196	0.459
F-actin-S-1(A_1)	5.71	0.0[a]	12 840	0.434	0.128	0.438
		1.35	9640	0.316	0.151	0.533
		2.68	9310	0.308	0.152	0.540
		4.00	8740	0.294	0.121	0.585
		5.30	8930	0.299	0.119	0.582
		6.61	9380	0.321	0.101	0.578

[a] In these cases the protein concentration is based on the amount of G-actin initially present in the solution. The solvents used for these measurements are described in the text. Tb^{3+} was added as an aqueous solution of $TbCl_3$

[b] A theoretical spectrum for the mixtures of G-actin and S-1(A_1)

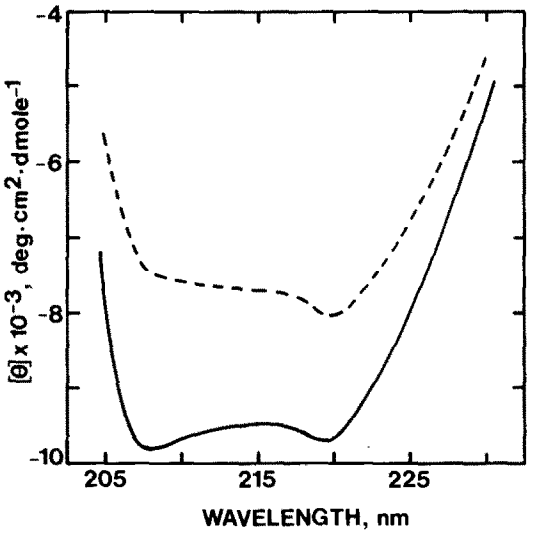

Fig.1. Far-UV CD spectra for Dowex 1-treated G-actin in (——) 5 mM PIPES, 0.2 mM $CaCl_2$, 0.5 mM DTT, pH 6.8, and in the same solvent containing 2.85 mol Tb^{3+}/mol G-actin (– – –). Protein concentration 0.42 mg/ml (10 μM).

in the 200–250 nm region indicated a fair amount of order in the secondary structure. Analysis of the data suggested that G-actin in this solvent contains 30% α-helix, 23% β-pleated sheet structures and 47% random coil. These figures compare favorably with those in [11], but are lower than those in [12].

To facilitate the interpretation of the Tb^{3+} titration data it was decided to remove free ATP from solutions of G-actin. The CD parameters for this material were established in 5 mM PIPES, pH 6.8, 0.5 mM DTT, 0.2 mM $CaCl_2$ and are presented in fig.1 and table 1. Removal of the free ATP produces essentially no change in structure from the native G-actin.

The titration with Tb^{3+} is documented in table 1. Little effect in structure is produced until a metal to protein ratio of 2.85–1 is reached. If it is borne in mind that the single mol of bound ATP associated with G-actin will preferentially bind the first mol of Tb^{3+} [13,14], then this effective ratio becomes 1.85. Unfortunately it has not been possible, because of incipient cloudiness in the solutions, to go to higher Tb^{3+} concentrations in order to monitor the interesting actin 'tube' structures [14]. At the highest Tb^{3+}/protein ratio examined, the loss of structure was approx. 3% α-helix and 7% β-structure, or in terms of ellipticity, $[\theta]_{220\ nm}$ was reduced by some 1600°.

3.2. *F-actin*

For the purpose of this study F-actin was prepared from the Dowex 1-treated G-actin to minimize the content of free nucleotide in solution. The basic solvent was 5 mM PIPES, pH 6.8, 0.5 mM DTT, and polymerization was induced by addition of $MgCl_2$ to 2 mM and KCl to 50 mM. The CD parameters obtained for this F-actin in the absence and presence of Tb^{3+} are presented in table 1.

It is apparent that F-actin contains slightly more secondary structure than the corresponding G-actin. There is a dramatic decrease in apparent helix content from the native value of 33% to 24% with the first addition of Tb^{3+} to a mol ratio, based on G-actin of 1.57. Subsequent addition of metal ion to a ratio of 4.7 produces a further small decrease to about 24% α-helix. After the last addition of Tb^{3+} to a mol ratio of 6.2, a slight increase in helix content to about 26% was observed.

In the case of G-actin, the first mol of Ln^{3+} will be taken up by the single mol of bound ATP. In F-actin this nucleotide is known to be present as ADP [8] which may not interact as strongly with the Ln^{3+}, thus allowing the metal ion to be available for binding to actin, thereby producing the observed structural alteration at a seemingly lower ratio of Tb^{3+} to protein.

3.3. *Myosin S-1(A_1) and S-1(A_2)*

CD spectra, in the wavelength range 200–250 nm have been recorded for S-1(A_1) and S-1(A_2) in 5 mM PIPES, pH 6.8, in the absence or presence of 0.5 mM DTT and 0.2 mM $CaCl_2$. Typical conformational parameters obtained for these isoenzymes as well as their titration behavior with Tb^{3+} are presented in table 1.

Two points emerge from these data. Firstly, S-1(A_1) has a higher ellipticity and helical content than S-1(A_2), viz. 41% *vs* 39%, or about 600° difference in $[\theta]_{220\ nm}$. Secondly, S-1(A_2) is much more sensitive than S-1(A_1) to Tb^{3+} addition, e.g., it was not possible to exceed a Tb^{3+}/S-1(A_2) ratio of 0.5 before incipient cloudiness developed in the protein solution. At this point the helical content was essentially unchanged. On the other hand, it was possible to add Tb^{3+} up to a 1:1 mol ratio with S-1(A_1). At this level of Ln^{3+}, the protein had lost about 2% of its apparent helix content as well as 5% β-structure, or in terms of ellipticity $[\theta]_{220\ nm}$ was reduced by about −1000°. Since it was possible to add more

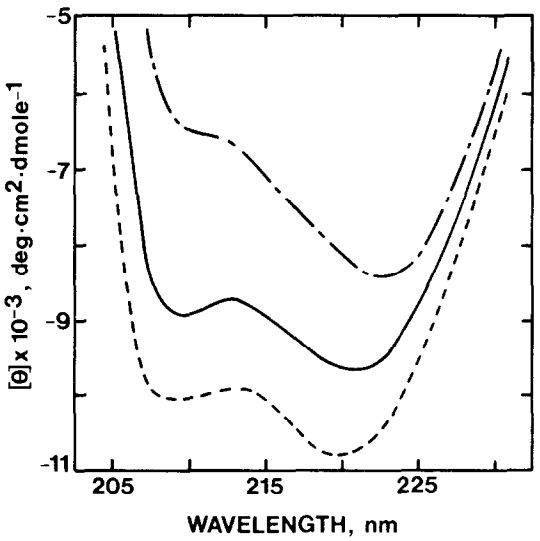

Fig.2. Far-UV CD spectra for mixtures of S-1(A_1) and G-actin 1:1 (molar ratio). ——, S-1(A_1)-G-actin observed spectrum; – – –, S-1(A_1)-G-actin theoretical spectrum; –·–, S-1(A_1)-G-actin plus Tb^{3+} (4.7 mol/mol G-actin). Solvent as in text. Protein concentration: S-1(A_1) 0.45 mg/ml (4.1 μM); G-actin 4.1 μM.

Tb^{3+} to solutions of S-1(A_1), it was decided to work exclusively with this apparently more stable isomer.

3.4. *S-1(A_1) G-actin*

Dowex 1-treated G-actin was mixed with S-1(A_1) in a 1:1 weight ratio, which corresponds to the protein ratio used in the previously described assay procedure [1], in 5 mM PIPES, 0.5 mM DTT, 5 μM $CaCl_2$ at pH 6.8. The CD parameters obtained in the native state, and upon titration with Tb^{3+}, are presented in table 1. For the samples mixed on a 1:1 (w/w) basis the experimental and theoretical spectra for the native cases are quite similar, indicating that if a complex has been formed, it is accompanied by little conformational change.

In the case of the sample prepared by combination of the proteins in a 1:1 ratio on a molar basis there is a very significant difference between the two spectra. As fig.2 shows, $[\theta]_{220\ nm}$ is about 1200° less for the observed spectrum suggesting that an appreciable conformational change follows complex formation. It is highly relevant in this connection to mention that in a recent NMR study of G-actin, S-1 mixtures in 1 mM phosphate buffer indicated that G-actin binds to S-1 thereby causing a large structure change in S-1, and this change seemed to be similar to that induced by binding F-actin [15].

The effect of adding Tb^{3+} appears to be 2-fold: a consistent slight increase in ellipticity at metal/protein ratios up to approx. 1.3, followed by a slow decrease in structure at higher metal ion concentrations.

3.5. *S-1(A_1) F-actin*

At the outset it should be stated that this is an extremely difficult system to study by CD. The property of F-actin to form fibers of course raises the problem of orientation artefacts arising when the protein solution is placed in the cuvet, and this effect seems to be enhanced when S-1(A_1) is present. The only way it was possible to achieve any degree of reproducibility was to adhere to the following protocol.

Dowex I-treated G-actin was polymerized at room temperature by the addition of $MgCl_2$ to 2 mM and KCl to 50 mM in 5 mM PIPES, 0.5 mM DTT, pH 6.8, and the resulting mixture was left for 1 h. Aliquots were removed, Tb^{3+} was added in varying amounts and the solutions allowed to stand undisturbed for another hour. The cuvet was then filled, introduced into the spectropolarimeter, allowed to stand 15 min, and the scan recorded. Although the CD results varied from batch to batch of S-1(A_1) F-actin there was quite good internal consistency within any particular series, viz. ±300° at 220 nm.

Typical CD parameters obtained in the absence and presence of Tb^{3+} are displayed in table 1. It is apparent that the behavior of the protein complex towards this Ln^{3+} is very similar to that of F-actin alone, viz., the loss of 11% or so α-helix is essentially complete after the addition of 1.35 mol Tb^{3+}/mol G-actin. Further titrations employing low levels of Tb^{3+} (data not shown) revealed that even after addition of 0.1 mol Tb^{3+}/mol G-actin, $[\theta]_{220\ nm}$ is reduced by some 1800°. It thus seems probably that an important mode of action of Tb^{3+} is to disrupt the F-actin-S-1(A_1) complex.

It is of interest to try and relate the observed biological effect of Tb^{3+} on the ATPase activity of S-1 alone, and in combination with F-actin, with the structural alterations noted from this CD study. It had previously been observed that Tb^{3+} greatly inactivated both the Ca^{2+}-ATPase and the actin-activated ATPase of S-1, suggesting that the key site of interaction with the Ln^{3+} might be located on the S-1 [1],

although important binding to actin could not be excluded.

The CD results clearly showed that structural changes accompany the interaction of G-actin with S-1(A_1), particularly in the sample prepared on a 1:1 mol ratio basis. This appeared to be true with F-actin-S-1(A_1) although here the effect was not so easy to define precisely. Addition of Tb^{3+} reduces the structural integrity of G- and F-actin slightly more so than S-1(A_1). The major effect for the F-actin-S-(A_1) complex seems to be a disruption of this complex at fairly low Tb^{3+} levels. The enzymic activity of S-1 is apparently particularly sensitive to even small structural changes as witnessed by the fact that a 2% drop in α-helix, with a 5% loss in β-pleated sheet structure for S-1(A_1), correlates with a 60–80% decrease in ATPase activity [1].

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