THE EFFECTS OF TERBIUM AND LANTHANUM ON THE BIOLOGICAL ACTIVITY OF SOME REPRESENTATIVE MUSCLE PROTEIN SYSTEMS

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Received 2 July 1980

1. Introduction

Calcium plays a significant regulatory role in many biological processes, often by interaction with proteins. Substitution of Ca^{2+} in these metal–protein complexes, by members of the rare-earth or lanthanide series results in derivatives which are useful probes of the chemical and structural nature of Ca^{2+} binding sites (reviewed [1]).

However, these ions have been used in physicochemical studies such as fluorescence, without close attention to biological replaceability. As pointed out [1] substitution of Ca^{2+} by Ln^{3+} can either inhibit, activate or have essentially no effect on a particular system.

Since Ln^{3+} substitution is becoming very popular in the field of muscle biochemistry we have studied the effects of La^{3+} and Tb^{3+} on the ATPase activity of the myosin S-1 enzyme system alone, and in combination with F-actin. We show that these ions have a pronounced inhibitory effect on this enzymic reaction, central to muscle contraction.

These studies suggest that a bio-assay should be employed to ascertain the degree of preservation of the intact, native, active structure, before using current sophisticated physical techniques. Interpretation of the resulting data might be much simpler and more relevant to the biological system under study.

Abbreviations: ATPase, adenosinetriphosphatase; F- and G-actin, fibrous and globular forms of the protein actin; myosin S-1, subfragment 1 produced by chymotryptic digestion of myosin; Pipes, piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid); P_i, phosphate; TN-C and TN-I, Ca^{2+}-binding and inhibitory subunits, respectively, of the troponin complex; TM, tropomyosin; Ln^{3+}, lanthanide; La^{3+}, lanthanum; Tb^{3+}, terbium

2. Materials and methods

2.1. *Protein preparations*

Skeletal muscle myosin was isolated from rabbit back muscle by the dilution–precipitation method essentially as in [2]. S-1 was prepared from this myosin by chymotryptic digestion in 0.12 M NaCl, 20 mM sodium phosphate (pH 7.0), 1 mM EDTA and 1 mM DTT [3]. The digestion was stopped by the addition of phenylmethane sulfonyl fluoride (100 mM in ethanol) to 10^{-4} M final conc. The protein was stored in 50% glycerol solution in the freezer. [S-1] was determined by absorbance using $A^{1\ mg/ml}_{280\ nm}$ = 0.8 cm^{-1} [4]. When required, aliquots of S-1 were removed, dialyzed overnight at 4°C against 20 mM Pipes at pH 6.8, clarified by centrifugation and the concentration determined.

Actin was prepared from an acetone powder of rabbit skeletal muscle as in [5]. The final dialysis of G-actin was carried out against 2 mM Tris–HCl (pH 7.5), 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$. The protein was lyophilized from this solution and the powder stored in a dessicator at −20°C until required. Concentrations were estimated using $A^{1\ mg/ml}_{280\ nm}$ = 1.1 [6]. To regenerate active G-actin, 10–20 mg lyophilized powder was dissolved in 1–2 ml and dialyzed against 3 changes of 200 vol. G-actin buffer. The protein solution was clarified by centrifugation and the concentration determined.

Troponin subunits and tropomyosin were prepared by routine procedures from this laboratory.

2.2. *ATPase measurements*

ATPase assays were done at pH 6.8 and 37°C for 10 min. For the Ca^{2+}-ATPase, conditions routinely used were 0.4 M KCl, 20 mM Pipes, 10 mM $CaCl_2$,

1 mM DTT, 2.5 mM ATP. 60–100 μg S-1 was used. The actin-activated ATPase was measured in 4 mM KCl, 20 mM Pipes, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM ATP. 35–60 μg S-1 was used. Total assay volume in both cases was 0.5 ml. The reaction was stopped by addition of 0.5 ml 15% trichloroacetic acid. After clarification by centrifugation, the liberated P_i was estimated by a micro-colorimetric procedure on 0.1 ml aliquots [7]. To prepare the S-1–actin complex the 2 proteins were mixed in a 1:1 (w/w) ratio in 20 mM Pipes, 20 mM KCl (pH 6.8) and allowed to stand for 2 h at room temperature before use in the assay. Lanthanides were added as their soluble chlorides in the amounts indicated.

3. Results and discussion

3.1. *Ca^{2+}-ATPase*

The results of the Ca^{2+}-ATPase study on S-1 are shown in table 1. The initial activity of S-1, (0.86 μmol P_i . mg^{-1} . min^{-1}) is lower than the range quoted in [8] (1.2–2.0 μmol P_i . mg^{-1} . min^{-1}) but is quite acceptable considering that in [8] the study was done near the optimum pH 8.0, while this study was done at pH 6.8, to retain the solubility of the lanthanides. Note the dramatic inhibition of the ATPase produced by the presence in the assay of relatively low concentrations of La^{3+} and Tb^{3+}. These observations agree well with studies on the Ca^{2+}-activated ATPase from canine cardiac sarcoplasmic reticulum, where Ln^{3+} inhibited potently at >10 μM [9]. Inhibition by Mg^{2+} is also demonstrated in these results but a comparable inhibitory effect is produced only at ~1 order of magnitude increase in metal concentration.

Table 1
Ca^{2+}-ATPase of myosin S-1

Metal	[Metal] (M)	Activity[a] (μmol P_i . mg^{-1} . min^{-1})	% inhib.
–	–	0.864 ± 0.100	–
$TbCl_3$	5.0×10^{-5}	0.605	30
	1.3×10^{-4}	0.337	61
	2.6×10^{-4}	0.112	87
$LaCl_3$	1.2×10^{-4}	0.580	33
	3.0×10^{-4}	0.346	60
	6.0×10^{-4}	0.138	84
$MgCl_2$	2.0×10^{-4}	0.432	50
	5.0×10^{-4}	0.268	69
	1.0×10^{-3}	0.181	79
	2.0×10^{-3}	0.060	93

[a] The assay was done as in the text

The reaction mixture in all cases contained 10 mM $CaCl_2$ in addition to the different metals added in the table. These are the averaged results of 3 determinations

3.2. *Actin-activated ATPase*

The results from the study of the Mg^{2+}-activated ATPase of the actin–S-1 complex are listed in table 2. We conclude that La^{3+} and Tb^{3+} induce extensive inhibition of this enzyme assay as well. As much as 2 mM Ca^{2+} has a minor effect, producing 15% inhibition.

We cannot say whether Ln^{3+} are acting in a similar manner in this more complicated system. This effect could be exerted either on S-1 or on the actin, or conceivably on the complex of both. Addition of >4 mol Ln^{3+} to solutions of G-actin, caused G-actin to aggregate to a form containing little or no F-actin [10]. This would undoubtedly affect the interaction with myosin S-1 and consequently the enzymic activity.

Since the development of luminescence from Tb^{3+} has been introduced as a sensitive probe of Ca^{2+}-binding sites in proteins, TM–TN and in this case to TN-C [11,12], we decided to examine the Mg^{2+}–actin-activated ATPase of S-1 in the presence of members of the regulatory system and in addition to monitor the effect of Ln^{3+} ions. The results are presented in table 3.

A quantity of TN-I was chosen (30 μg) to inhibit partially the enzyme system. Both Tb^{3+} and La^{3+} were able to exert a further inhibitory effect on the reac-

Table 2
Actin-activated ATPase of myosin S-1

Metal	[Metal] (M)	Activity[a] (μmol P_i . mg^{-1} . min^{-1})	% inhib.
–	–	0.264 ± 0.062	–
$TbCl_3$	1.3×10^{-4}	0.146	45
	2.6×10^{-4}	0.146	45
$LaCl_3$	3.0×10^{-4}	0.146	45
	6.0×10^{-4}	0.106	60
$CaCl_2$	1.0×10^{-4}	0.264	0
	2.0×10^{-3}	0.222	15

[a] The activity is reported in terms of [myosin S-1]

The assay was done as in the text. The reaction mixture in all cases contained 0.010 M $MgCl_2$ in addition to the different metals noted in the table. The activities are the average results of 3 determinations

Table 3
Actin-activated ATPase activity of myosin S-1 in the presence of the members of the regulatory system

Metal	[Metal] (M)	Protein	Activity[a] (μmol P_i . mg^{-1} . min^{-1})	% inhib.
–	–	–	0.264 ± 0.062	–
–	–	TN-I (30 μg)	0.102	63
$TbCl_3$	3.0×10^{-4}	TN-I (30 μg)	0.014	95
$LaCl_3$	3.0×10^{-4}	TN-I (30 μg)	0.030	89
–		TN-I (20 μg)		
		TM (30 μg)	0.148	44
–		TN-I (20 μg)		
		TM (30 μg)		
		TN-C (45 μg)	0.264	–
$TbCl_3$	1.3×10^{-4}	(TN-I TM TN-C)	0.204	23
$LaCl_3$	3.0×10^{-4}	(TN-I TM TN-C)	0.174	34
b		(TN-I TM TN-C)	0.264	–
$TbCl_3$[b]	1.3×10^{-4}	(TN-I TM TN-C)	0.238	10
$LaCl_3$[b]	3.0×10^{-4}	(TN-I TM TN-C)	0.212	20
	6.0×10^{-4}	(TN-I TM TN-C)	0.158	40
b		(TN-I TN-C)	0.264	–
$TbCl_3$	1.3×10^{-4}	(TN-I TN-C)	0.238	10
	2.6×10^{-4}	(TN-I TN-C)	0.158	40
$LaCl_3$[b]	3.0×10^{-4}	(TN-I TN-C)	0.220	17
	6.0×10^{-4}	(TN-I TN-C)	0.106	60

[a] The activity is reported in terms of [myosin S-1]
[b] The assay contained 5×10^{-4} mol $CaCl_2$

The assay was done as in the text. The reaction in all cases contained 0.010 M $MgCl_2$. The results are the average of 3 determinations

tion. When TN-C is incorporated into the TN-I inhibited system, complete restoration of activity is noted. Since no metal chelating agent is present in these assays there will be enough Ca^{2+} present in the reaction mixture to allow the TN-C to exert its biological influence, by neutralizing the inhibitory effect of the TN-I. It should be noted that in the two cases where Tb^{3+} and La^{3+} are added, complete restoration of activity is not obtained. These experiments were repeated in the presence of excess Ca^{2+} and essentially similar results were obtained.

It is difficult to say precisely at what level Ln^{3+} will operate in this much more complicated system. Substitution of Ca^{2+} by Ln^{3+} in TN-C is likely to produce little effect on this protein since available evidence suggests a primarily structural role for the metal ion [13]. On the other hand, since the ATPase activity of myosin S-1 and actin–S-1 are dramatically influenced by the presence of the lanthanides, we conclude that Ca^{2+} may be near the active site or participate in the mechanism of action of this enzyme.

This investigation demonstrates quite clearly that whenever Ca^{2+} is substituted by Ln^{3+} in a particular protein system it is advantageous to carry out some enzymic reaction wherever possible to ascertain whether or not the replacement has altered the native form of the protein. If there has been any modification this should be considered when any physicochemical measurements are planned.

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