LABELING AND INHIBITION OF MYOSIN BY 'IN SITU-OXIDIZED' AND PRESYNTHESIZED Co(III) COMPLEXES

M. M. WERBER
Polymer Department, Weizmann Institute of Science, Rehovot, Israel

and

A. DANCHIN
Département de Biologie Moléculaire, Institut Pasteur, 25, Rue du Dr. Roux, Paris, France

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1. Introduction

The structure of the active site of myosin is being intensively investigated [1]. These studies have some bearing on the mechanism of energy conversion (from chemical to mechanical), which is performed by the contractile proteins in muscle. We have recently shown that myosin could be affinity labeled by the complex Co(III)–(phen)–ATP [2]. The same complex was found useful also in the labeling of other ATPases, such as for example, chloroplasts coupling factor 1 [3]. Various Co(III) complexes were previously used in other systems [4,5] and inert Co(III) complexes formed by H_2O_2 oxidation of the parent labile Co(II) complexes were successfully employed as affinity labels in nucleic acids [6] or enzymes, e.g., aspartokinase [7]. We now show that myosin can also be affinity labeled by the in situ oxidation product of Co(II)–ATP.

The structure of the previously used affinity label Co(III)–(phen)–ATP, has now been determined and shown to contain the labile superoxide ion [8]. It was interesting to see if its replacement in the complex by the inert cyanide ion would impair its efficiency as an affinity label. We report now results on the labeling and inhibitory abilities of this and 2 other Co(III) complexes.

2. Materials and methods

2.1. *Myosin*

Myosin was obtained from the back muscles of New Zealand rabbits and stored in 50% glycerol. Deglycerination was performed by dialysis against 0.6 M KCl-containing 5 mM phosphate buffer, pH 7.0 and the solution was freed from aggregates by 75 min centrifugation at 33 000 rev./min. The specific Ca-ATPase activity of the material thus obtained was between 0.6 and 0.7 μmol phosphate min^{-1}. mg^{-1} protein. The ATPase activity was determined in a pH-stat at pH 7.6 as previously described [2].

2.2. *Cobaltic complexes*

The cobaltic complexes were prepared by H_2O_2 oxidation of Co(II) in the presence of the various ligands, i.e., *O*-phenanthroline (phen), ATP and cyanide ion at a concentration of 10 mM [9]. In the case of Co(III)–(phen) and Co(III)–$(phen)_3$, the complexes could not be precipitated by ethanol at −20°C and they were therefore crude mixtures, whereas Co(III)–(phen)–ATP–(CN^-) was purified as previously described [2,9].

2.3. *Labeling and inhibition*

The in situ-labeling of myosin was performed at

0°C with 1 mM Co(II)–ATP, and with 1 mM Mg(II)–ATP in the control experiments, in the presence of several concentrations of H_2O at pH 7.0 (5 mM phosphate buffer). Inhibition of myosin Ca-ATPase by the various complexes was studied at 2.5 mM ATP at several inhibitor concentrations.

3. Results

3.1. *Labeling of myosin by oxidation of Co(II)–ATP in situ using H_2O_2*

The time-course of myosin labeling with a Co(III)–ATP complex formed in situ by oxidation with H_2O_2 at low and high ionic strengths is shown in fig.1. It is also seen that addition of dithiothreitol (DTT) to the Co(III)–ATP-labeled enzyme restored the ATPase activity to the level of the control (myosin which has been treated with the same H_2O_2 concentration in the presence of Mg(II)–ATP). Both the labeling and the control reactions (not shown) were much faster at low ionic strength. In order to maintain a good activity of the control, it was therefore found preferable to work at high ionic strength. Figure 2 shows the labeling pattern at high ionic strength using

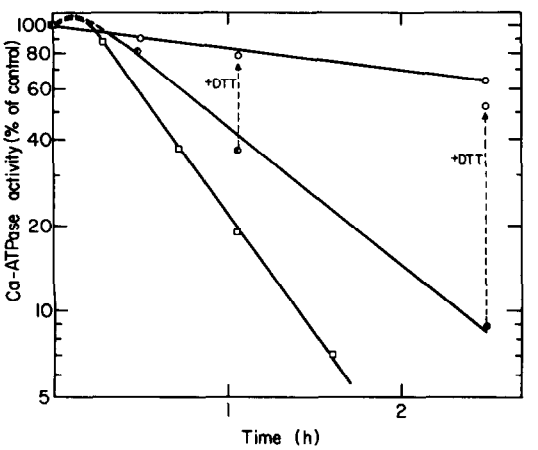

Fig.1. Time-course of myosin labeling with in situ-oxidized Co(III)–ATP. Myosin (4 mg/ml) was incubated at 0°C in the presence of either Mg(II)–ATP or Co(II)–ATP and three- or four-fold molar excess of H_2O_2. (○–○) Mg (II)–ATP (control), ionic strength – 0.60 M, (●–●) Co(II)–ATP, ionic strength – 0.60 M, (□–□) Co(II)–ATP, ionic strength – 0.15 M. Reactivation was achieved after 15 min incubation with 50 mM DTT at 0°C.

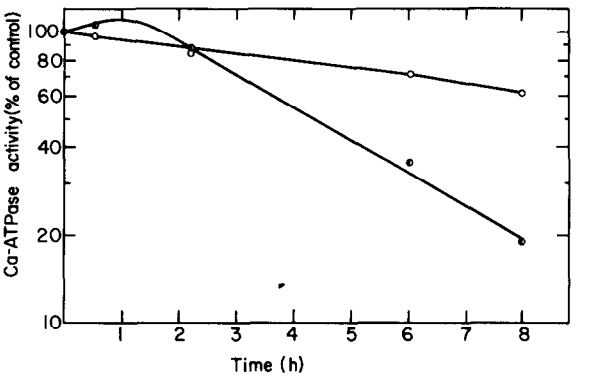

Fig.2. Time-course of myosin labeling with in situ-oxidized Co(III)–ATP at low H_2O_2 concentrations. Conditions as in fig.1, except that $[H_2O_2]/[Co(II)] = 0.7$. Ionic strength – 0.60 M. (○–○) Mg(II)–ATP (control), (●–●) Co(II)–ATP.

a 5-fold lower concentration of H_2O_2 than that used in fig.1. A biphasic labeling pattern is clearly observed in this experiment and is also apparent at the higher H_2O_2 concentration at both ionic strengths (fig.1). The rate constants for the inactivation reactions in the presence of the Co(III) complex – second phase of the labeling pattern – are summarized in table 1 and compared with the rate constants of the control reactions.

3.2. *Irreversible inhibition of myosin by pre-synthesized Co(III) complexes*

We attempted to inactivate myosin with various Co(III) complexes: Co(III)–phen, Co(III)–$(phen)_3$ and Co(III)–(phen)–ATP–(CN^-). Whereas we had previously observed [2] that 2 mM of Co(III)–(phen) ATP–(O_2^-) caused 90% inactivation of myosin Ca-ATPase in 3 h, there was hardly (<5%) any change

Table 1
Rate constants of inactivation of myosin by 'direct' affinity labeling with Co^{2+}, ATP and H_2O_2

Ionic strength (M)	0.15	0.60	0.60
$10^3 \times [H_2O_2]$ (M)	3.1	0.8	4.0
$k_{H_2O_2}$ (M^{-1} s^{-1})	0.054	0.020	0.012
$10^4 \times k_{corr}$ (s^{-1})	3.68	0.57	2.58
$k_{Co(III)-ATP}$ (M^{-1} s^{-1})	0.295	0.070	0.258

Table 2
Rates of inactivation of myosin by Co(III) complexes and apparent inhibition constants

Co(III) complex	Co(III)–(phen)	Co(III)–($phen_3$)	Co(III)–(phen)–ATP–(CN^-)	Co(III)–(phen)–ATP–(O_2^-)[b]
Half-lifetime of inactivation[a] (h)	7	14	∞[c]	1.5
Concentration (μM) causing 50% inhibition[d]	485	490	82	130

[a] In 'labeling' experiments with 2 mM of Co(III) complex
[b] From ref. [1]
[c] Less than 5% change in 24 h
[d] Ca-ATPase activity, using 2.5 mM ATP

with Co(III)–$(phen)_3$ or Co(III)–(phen)–ATP–(CN^-) during the same period (with the latter no change occurred even after 24 h). On the other hand, 30% of the Ca-ATPase activity was lost after 3 h incubation of myosin in the presence of 2 mM Co(III)–(phen). The half-lifetimes of inactivation of myosin by these Co(III) complexes are summarized in table 2.

Inhibition experiments using various concentrations of complexes in the assay medium for Ca-ATPase activity, showed that all Co(III) complexes tested in this and in a previous work [2] were rather good inhibitors. However, in all cases parabolic inhibitory patterns of $1/v$ versus [complex] were obtained (cf. ref. [2]). Therefore, only apparent inhibition constants – concentrations of Co(III) complex causing 50% inhibition – could be calculated (table 2).

4. Discussion

It is clearly shown in this work that the active site of myosin can be labeled by direct formation of the Co(III)–ATP complex in the active site of myosin. This site-directing effect is due to the fact that Co(III)–ATP most probably binds to myosin at the same site as Mg(II)–ATP. This is supported by the fact that Co(II)–ADP and Mg(II)–ADP have similar dissociation constants from heavy meromyosin [10]. The difficulty in this type of labeling is to find conditions under which the inactivation by H_2O_2 itself is reduced to a minimum. This seems to be the case at high ionic strength where the rate of inactivation by the Co(III) complex is 20-fold higher than that by H_2O_2 (table 1).

Two important features in these experiments, imply that the labeling occurs at the same site as with Co(III)–(phen)–ATP–(O_2^-) [2]:

(i) The biphasic labeling pattern, first enhancement of activity, then inactivation.

(ii) The reactivation by DTT.

These similarities in the labeling features, obtained by two different techniques, provide additional evidence for the anti-cooperative behavior of myosin active sites [2].

The inability of Co(III)–(phen)–ATP–(CN^-) to inactivate myosin in an irreversible manner definitively shows that in order to obtain affinity labeling, one of the ligands of the complex has to be rather easily exchangeable (by a protein ligand), such as O_2^- in Co(III)–(phen)–ATP–(O_2^-) [2,3,9]. Since neither ATP, phenanthroline nor CN^- can be exchanged, this complex cannot serve as an affinity label, although it is a good inhibitor (table 2). That CN^- is not exchanged is not surprising, since it forms with many metal ions, stable and inert complexes [11]. Moreover, we have also been able to make use of this property (that CN^- is a more stable ligand of Co(III) than O_2^-) in order to displace O_2^- from Co(III)–(phen)–ATP–(O_2^-) [8]. On the other hand, Co(III)–(phen), whose detailed structure was not determined, probably possesses at least one exchangeable ligand, possibly a superoxide ion, which enables it to attach itself to the protein. It does so, however, much more slowly than the ATP-containing complex, Co(III)–(phen)–ATP–(O_2^-), and

without a phase of activity enhancement. It is noteworthy that Co(III)–$(phen)_3$, is a much less potent inactivating reagent, probably because the cobaltic ion is surrounded by 3 non-exchangeable bidentate ligands. It is also possible that the inactivation mechanism operating in this case involves oxidation of the sulfhydryl groups of myosin, in a manner similar to that proposed for the Cu(II)–$(phen)_3$ complex in another system [12]. However, it seems that this mechanism does not play an important role in the case of ATP-containing Co(III) complexes since Co(III)–(phen)–ATP–(CN^-), for instance, cannot cause irreversible inactivation at all.

It may therefore be that Co(II)–(phen) and Co(III)–$(phen)_3$ are directed towards another site than that labeled by the presynthesized and in situ-oxidized Co(III)–ATP complexes.

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