The kinetics of bivalent metal ion dissociation from myosin subfragments

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Bivalent metal ions have multiple roles in subunit association and ATPase regulation in scallop adductor-muscle myosin. To help elucidate these functions, the rates of Ca^{2+} and Mg^{2+} dissociation from the non-specific high-affinity sites on the regulatory light chains were measured and compared with those of rabbit skeletal-muscle myosin subfragments. Ca^{2+} dissociation had a rate constant of about 0.7 s^{-1} in both species, as measured by the time course of the pH change on EDTA addition. Mg^{2+} dissociation had a rate constant of 0.05 s^{-1} , as monitored by its displacement with the paramagnetic Mn^{2+} ion. It is concluded that the exchange between Ca^{2+} and Mg^{2+} at the non-specific site, on excitation of both skeletal and adductor muscles, is too slow to contribute to the activation itself. The release of bivalent metal ions from the non-specific site is, however, the first step in release of the scallop regulatory light chain (Bennett & Bagshaw (1986) *Biochem. J.* 233, 179–186). In scallop myosin additional specific sites are present, which can bind Ca^{2+} rapidly, to effect activation of the ATPase. In the course of this work, Ca^{2+} dissociation from EGTA was studied as a model system. This gave rates of 1 s^{-1} and 0.3 s^{-1} at pH 7.0 and pH 8.0 respectively.

INTRODUCTION

Myosins possess four distinct classes of bivalent-metal-ion-binding site (Bagshaw, 1980). Under physiological conditions the ATPase site binds Mg^{2+} when the latter is complexed with a nucleotide (Bagshaw & Reed, 1976). All myosins that have been studied possess a high-affinity non-specific bivalent-metal-ion-binding site on each of their regulatory class of light chains (Bagshaw & Kendrick-Jones, 1979). In addition, some myosins, notably those from molluscs, have two Ca^{2+} -specific sites, which are directly involved in myosin-linked regulation of ATPase activity (Szent-Györgyi *et al.*, 1973; Wells & Bagshaw, 1985). The exact location of these sites is unclear, because each requires a portion of the heavy chain plus the two classes of light chain for its existence. However, removal of the regulatory light chain leaves a Ca^{2+} -binding site of reduced affinity on the remaining essential light chain-heavy chain complex, which is likely to be derived from the Ca^{2+} -specific site (Chantler & Szent-Györgyi, 1980). Finally, there are an undefined number of weak bivalent-metal-ion-binding sites, presumably on the myosin surface, that would be partially occupied by Mg^{2+} under physiological conditions (Bagshaw & Reed, 1976; Persechini & Rowe, 1984).

In the present paper we focus on the high-affinity non-specific sites of rabbit skeletal-muscle and scallop adductor-muscle myosins. Each site is located within the N-terminal domain of the regulatory light chains, which have amino acid sequences characteristic of the E-F hand structure of Ca^{2+} -binding proteins (Bagshaw & Kendrick-Jones, 1980). These sites bind Ca^{2+} some 10-fold tighter than Mg^{2+} , but it is not clear from this value whether Ca^{2+} would occupy the site during activation of muscle contraction. Previous studies, with rabbit skeletal-muscle myosin, implied that, even if Ca^{2+} could compete with

Mg^{2+} under physiological conditions, the exchange at the non-specific site would be much too slow to trigger contraction itself (Bagshaw & Reed, 1977). These studies relied on a change in protein fluorescence to monitor bivalent metal ion release. It could be argued, however, that this signal is indirect and that it may reflect a slow conformational change, following a more rapid dissociation of Mg^{2+} . Here we re-investigate this process, using alternative methods, and extend the study to scallop adductor-muscle myosin. In this species, removal of the bivalent metal ion from the regulatory light chain allows the latter to dissociate from the heavy chain (Szent-Györgyi *et al.*, 1973). We explore the mechanism of this process in the accompanying paper (Bennett & Bagshaw, 1986). The dissociation of bivalent metal ions from the non-specific site is the first step in the pathway.

In the course of this work we also investigated the dissociation of Ca^{2+} from EGTA as a model system. As suggested by Smith *et al.* (1984), Ca^{2+} dissociates rather slowly (about 0.3 s^{-1}), and this can introduce complications when CaEGTA is used to control the concentration of free Ca^{2+} *in vitro*.

EXPERIMENTAL

Materials

Scallops (*Pecten maximus*) were obtained from the Marine Biological Station (Millport, Isle of Cumbrae, Scotland, U.K.). Scallop HMM was obtained by tryptic digestion, and S1 was obtained by papain digestion of adductor-muscle myosin (Wells & Bagshaw, 1983). Rabbit HMM and S1 were prepared by chymotryptic digestion of skeletal-muscle myosin (Weeds & Taylor, 1975). Rabbit S1 was also obtained by papain digestion (Bagshaw & Reed, 1976). S1 preparations differ in their

Abbreviations used: Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid; HMM, heavy meromyosin; $\text{S1}^{+\text{RD}}$ and $\text{S1}^{-\text{RD}}$, subfragment 1 containing and lacking the regulatory domain respectively.

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Fig. 1. Dissociation of Ca^{2+} from EGTA at (a) pH 7.0 and (b) pH 8.0

The dissociation of Ca^{2+} from EGTA was monitored by proton uptake, on addition of EDTA, with a pH indicator. The reaction was monitored in a dual-wavelength stopped-flow spectrophotometer set up to record the differential transmission change at wavelengths appropriate to the indicator. (a) $5\ \mu\text{M}\text{-CaCl}_2/25\ \mu\text{M}\text{-EGTA}$ was mixed with $50\ \mu\text{M}\text{-EDTA}$ (reaction chamber concentrations) in a solvent containing $40\ \text{mM}\text{-NaCl}$, $100\ \mu\text{M}\text{-Mops}$ and $20\ \mu\text{M}\text{-Bromothymol Blue}$ at pH 7.0 at 20°C . The observed first-order rate constant was $1.0\ \text{s}^{-1}$. (b) As in (a), but the solvent contained $100\ \mu\text{M}\text{-Epps}$ and $25\ \mu\text{M}\text{-Phenol Red}$ at pH 8.0. The observed first-order rate constant was $0.31\ \text{s}^{-1}$. One division of the ordinate represents a change in transmission of 4.6%. The amplitude of the traces indicate that the pH increases by 0.1 during the reaction. In the absence of EGTA, Ca^{2+} binding to EDTA was too fast to measure, indicating that the second-order binding constant was greater than $10^7\ \text{M}^{-1}\cdot\text{s}^{-1}$. *T*, Transmission.

regulatory light chain content, and hence their ability to bind bivalent metal ions, according to the conditions of the digestion (Margossian *et al.*, 1975; Stafford *et al.*, 1979). Subfragment 1 containing the regulatory light chain was obtained by papain digestion in the presence of $1\ \text{mM}\text{-MgCl}_2$ and $1\ \text{mM}\text{-CaCl}_2$. Such preparations are denoted $\text{S1}^{+\text{RD}}$ to distinguish them from $\text{S1}^{-\text{RD}}$, produced in the presence of EDTA, which lacks the regulatory domain. Proteolytic enzymes were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), and other reagents from BDH Chemicals (Poole, Dorset, U.K.) or Fisons (Loughborough, Leics., U.K.).

Rapid pH measurement

Bivalent metal ion dissociation, on addition of EDTA, was monitored by the accompanying release or uptake of protons by using a pH indicator. Rapid transmission changes were recorded with an Aminco-Morrow stopped-flow apparatus (1 cm optical pathlength) attached to a DW2 dual-wavelength spectrophotometer. The signal was captured by using a C. F. Palmer 8137 signal averager and displayed on a Tetrionix RM561A oscilloscope. Reactions at pH 7 were monitored with Bromothymol Blue by using the differential transmission at 618 nm and 420 nm. Reactions at pH 8 were monitored with Phenol Red by using wavelengths of 560 nm and 430 nm. The majority of the experiments were performed at pH 8.0, where the signals were less prone to drift. This pH was also optimal for the complementary studies of the dissociation of the regulatory light chain (Bennett & Bagshaw, 1986). Before use, excess buffer was removed from the protein preparations by dialysis, at 0°C , against $40\ \text{mM}\text{-NaCl}/100\ \mu\text{M}\text{-Epps}$, with a Radiometer RTS822 pH-stat being used to maintain the dialysis residue at a constant pH. In the case of scallop preparations, a low

(approx. $10\ \mu\text{M}$) concentration of Mg^{2+} or Ca^{2+} , as appropriate, was also present to maintain the regulatory light chains in an associated state (Szent-Györgyi *et al.*, 1973). The pH of each reaction mixture was checked and adjusted at 20°C just before the syringes of the stopped-flow apparatus were filled.

Attempts to rid the solutions of CO_2 were not successful, and pH transients were accompanied by an 'echo' on the seconds time scale due to re-equilibration of carbonic acid (Gutfreund, 1972):

The effect of this reaction on the transients of interest was minimized by the inclusion of $20\ \mu\text{g}$ of carbonic anhydrase (Sigma Chemical Co.)/ml.

E.p.r. spectroscopy

Mn^{2+} binding to myosin subfragments was monitored by the decrease in the characteristic e.p.r. spectrum of $\text{Mn}(\text{H}_2\text{O})^{2+}$, measured with a Varian E109 spectrometer at a microwave frequency of 9.1 GHz. The reaction was monitored by recording the intensity of the second hyperfine peak ($m_1 = +3/2$) with time, at a field strength of 3059 G. Rapid mixing was achieved by injecting the reactants from two $250\ \mu\text{l}$ glass syringes (Scientific Glass Engineering, Milton Keynes, Bucks., U.K.) through a Perspex dual-jet mixing chamber into a 1 mm-diameter quartz tube located in the cavity of the spectrometer. The first two $125\ \mu\text{l}$ portions of the mixed solutions enabled the spectrometer to be tuned at 50 mW microwave power, and the second delivery allowed the signal to be recorded within about 1 s of mixing. The quartz tube was held in a Dewar flask through which N_2 was flushed to maintain the temperature at 20°C . Before use, proteins were dialysed against the appropriate buffer, containing either

Fig. 2. Dissociation of Ca^{2+} from rabbit and scallop myosin subfragments

Ca^{2+} release was monitored by the decrease in pH on mixing the myosin subfragment with EDTA, with Phenol Red as an indicator. One syringe of the stopped-flow apparatus contained $10\ \mu\text{M}$ - CaCl_2 , 1 mg of protein/ml and the other $50\ \mu\text{M}$ -EDTA (reaction-chamber concentrations). Both syringes contained 40 mM-NaCl, 100 μM -Epps and 25 μM -Phenol Red at pH 8.0 at 20 °C. One division on the ordinate represents a change in transmission of 0.9%. (a) Rabbit papain-digest subfragment 1 ($\text{S1}^{+\text{RD}}$); (b) rabbit chymotryptic-digest subfragment 1 ($\text{S1}^{-\text{RD}}$); (c) rabbit chymotryptic-digest HMM; (d) scallop $\text{S1}^{+\text{RD}}$; (e) scallop $\text{S1}^{-\text{RD}}$; (f) scallop tryptic-digest HMM. Records (d), (e) and (f) were obtained in the presence of 20 μg of carbonic anhydrase/ml. Rabbit $\text{S1}^{-\text{RD}}$, prepared by papain digestion in the presence of EDTA, yielded a similar trace to that in (b). *T*, Transmission.

100 μM - CaCl_2 or 300 μM - MgCl_2 and 200 μM -EDTA, and then they were concentrated to 20–30 mg/ml by using a Minicon B15 ultrafiltration cell (Amicon, Stonehouse, Glos., U.K.).

Data analysis

Reactions were analysed by a least-squares fit to an exponential by using an Apple II+ microcomputer and a program based on that used by Millar (1984).

RESULTS

Ca^{2+} dissociation from EGTA

The dissociation rate of Ca^{2+} from EGTA was measured for two reasons: (1) to provide a direct measure of the process under our experimental conditions; (2) as a model system for the use of pH indicators for monitoring reactions on the seconds time scale.

At neutral pH, Ca^{2+} binding to EGTA and EDTA is accompanied by proton release:

The effective affinity of Ca^{2+} for EDTA exceeds that of EGTA under these conditions, so that mixing CaEGTA^{2-} with HEDTA^{3-} results in a net uptake of protons, limited by the rate of dissociation of Ca^{2+} from EGTA. Fig. 1 shows this reaction, as monitored with pH indicators, in a stopped-flow spectrophotometer. The effective rate constants for Ca^{2+} dissociation from EGTA were $1.0\ \text{s}^{-1}$ and $0.31\ \text{s}^{-1}$ at pH 7.0 and 8.0 respectively. These values are comparable with those calculated from the known association rate constants and equilibrium constants for CaEGTA (Smith *et al.*, 1984), bearing in mind the cumulative errors when using literature values obtained under slightly different conditions.

Table 1. Dissociation rates of bivalent metal ions from myosin subfragments

Dissociation rates were measured by ^aprotein fluorescence (data of Bagshaw & Reed, 1977), ^bpH indicator (Fig. 2) or ^cdisplacement with Mn²⁺ (Fig. 3). Abbreviation: N.D., not determined.

Protein		Dissociation rate (s ⁻¹)	
		Ca ²⁺	Mg ²⁺
Rabbit	S1 ^{+RD}	0.46 ^a	0.057 ^a
	S1 ^{+RD}	0.70 ^b	0.051 ^c
	HMM	0.77 ^b	N.D.
Scallop	S1 ^{+RD}	0.64 ^b	0.050 ^c
	HMM	0.44 ^b	0.058 ^c

Ca²⁺ dissociation from myosin subfragments

Fluorescence methods proved to be inappropriate for the study of metal ion dissociation from scallop myosin (cf. Bagshaw & Reed, 1977), and alternative probes were sought. Provided that there is some difference in the pK values of the groups involved, exchange of metal ions (Me²⁺) between a protein-bound site (Pr) and EDTA will be accompanied by a change in pH, e.g.:

This reaction was studied for myosin subfragments with a pH indicator and stopped-flow apparatus in the manner described above. As expected, metal ion release from myosin subfragments, monitored by this method, was multiphasic. All preparations investigated showed a rapid phase of proton release with a $t_{1/2}$ of less than 20 ms, assumed to reflect metal ion release from the weak sites (Bagshaw & Reed, 1976). In addition, preparations that contained the regulatory light chain showed a slow phase with a $t_{1/2}$ of 1 s when the protein was pre-equilibrated with Ca²⁺. Fig. 2 shows this phase of Ca²⁺ release for S1^{+RD} and HMM prepared from rabbit skeletal muscle and scallop adductor muscle. The corresponding S1^{-RD} preparations (Figs. 2b and 2e) showed no proton release on this time scale. Indeed, a small signal in the opposite direction was often observed, which we attribute to re-equilibration of carbonic acid (eqn. 1), following the rapid release of protons associated with EDTA chelation of the free and weakly bound Ca²⁺.

The agreement between the rate of the slow phase of H⁺ release and the change in protein fluorescence observed with rabbit S1^{+RD} (Table 1) suggests that the former signal is also monitoring Ca²⁺ release from the high-affinity, non-specific, sites. This assignment is supported by the lack of a corresponding reaction with S1^{-RD} preparations.

Attempts to monitor Mg²⁺ dissociation by using the pH indicator method failed owing to drifts over time periods of longer than about 30 s. This complication probably arose from the effects of back diffusion and dissolved CO₂. In any event, dissociation of metal ions from scallop myosin preparations, on EDTA addition, is accompanied by the dissociation of the regulatory light chains, with a rate constant of about 0.014 s⁻¹ (Bennett & Bagshaw, 1986). This step would complicate the interpretation of changes in proton concentration.

Mg²⁺ dissociation from myosin subfragments

The time scale of Mg²⁺ dissociation ($t_{1/2} = 12$ s; Bagshaw & Reed, 1977) suggested an alternative strategy for its measurement with scallop myosin subfragments. Mn²⁺ has a very high affinity for the non-specific bivalent-metal-ion-binding site (about 50-fold higher than that of Mg²⁺) and, on binding, its e.p.r. spectrum becomes extensively broadened (the amplitude of the first-derivative signal is reduced by 5-fold or more, depending on the species; Bagshaw & Kendrick-Jones, 1979). When the site is pre-occupied with Mg²⁺ the rate of Mn²⁺ binding should be limited by the dissociation of the former ion.

Fig. 3 shows the rate of dissociation of Mg²⁺ from scallop S1^{+RD} and HMM as monitored by Mn²⁺ displacement. The observed amplitude of the corresponding experiment with S1^{-RD} was much reduced.

Fig. 3. Displacement of Mg²⁺ from scallop myosin subfragments

Mg²⁺ was displaced from scallop myosin subfragments with Mn²⁺. The reaction was monitored by the decrease in the characteristic e.p.r. signal of the Mn(6H₂O)²⁺ complex, after the rapid mixing of the Mg²⁺-loaded protein with Mn²⁺ as described in the Experimental section. (a) Scallop S1^{+RD} (120 μM) in 150 μM-MgCl₂/100 μM-EDTA was mixed with 300 μM-MnCl₂. (b) Scallop S1^{-RD} (83 μM) in 150 μM-MgCl₂/100 μM-EDTA was mixed with 175 μM-MnCl₂. (c) Scallop HMM (74 μM) in 150 μM-MgCl₂/100 μM-EDTA was mixed with 150 μM-MnCl₂ (all of the above concentrations refer to the final concentration after mixing). Both syringes contained 40 mM-NaCl and 20 mM-Epps at pH 8.0 at 20 °C. The horizontal traces in (a) and (c) represent continuation of the records and define the end point of the reactions.

Low concentrations of EDTA were included in these experiments to chelate contaminant Ca^{2+} and ensure that the non-specific site was occupied with Mg^{2+} . Formation of MnEDTA did not contribute significantly to the observed transients, as indicated by the control experiment with $\text{S1}^{-\text{RD}}$. At the end point of the reactions shown in Figs. 3(a) and 3(c), the e.p.r. spectra were scanned and revealed the characteristic P-type spectrum of Mn^{2+} bound to the scallop regulatory-light-chain site (Bagshaw & Kendrick-Jones, 1979) in combination with a low concentration (5–10 μM) of free $\text{Mn}(\text{H}_2\text{O})^{2+}$. The e.p.r. spectrum of MnEDTA is too broad to detect (Bagshaw, 1977).

The rate of Mg^{2+} dissociation from the non-specific site was of the order of 0.05 s^{-1} . Comparable results were obtained with rabbit $\text{S1}^{+\text{RD}}$ (Table 1). When $\text{S1}^{+\text{RD}}$ or HMM was preloaded with Ca^{2+} , the rate of Mn^{2+} binding was much increased. The amplitude of the observed signal at 1 s from mixing, the earliest measurable, was reduced 3-fold compared with that obtained when the proteins were preloaded with Mg^{2+} . Thus Ca^{2+} displacement appeared largely complete within a few seconds, as expected from the records of Fig. 2.

DISCUSSION

The slow dissociation of Ca^{2+} from EGTA may limit the ability of this ligand to control the free Ca^{2+} concentration under certain transient conditions. Nevertheless, whereas the net release of Ca^{2+} from EGTA occurs on the time scale of seconds (Fig. 1), when CaEGTA is used as a Ca^{2+} buffer at a concentration of 1 mM, the response to a small (micromolar) change in $[\text{Ca}^{2+}]$ would occur on the millisecond time scale (cf. Smith *et al.*, 1984).

The main aim of the studies of the present paper was to use CaEGTA as a model system for an investigation of the release of bivalent metal ions from myosin subfragments. Three methods have been developed to study the latter reaction. Intrinsic protein fluorescence changes (Bagshaw & Reed, 1977) provide a convenient probe for rabbit myosin subfragments, but they are not suitable for all species. With scallop myosin, release of bivalent metal ions from the non-specific site is accompanied by little change in fluorescence as such, although subsequent dissociation of the regulatory light chains does perturb the tryptophan residues (Konno *et al.*, 1983). The pH-indicator method (Fig. 2) proved to be a satisfactory and general method for monitoring Ca^{2+} release from the non-specific site of myosins, but it was inadequate for monitoring the slower release of Mg^{2+} . For the latter ion, displacement by Mn^{2+} provided the most satisfactory approach (Fig. 3).

A consequence of the slow dissociation of Mg^{2+} from the high-affinity non-specific site of myosin (0.05 s^{-1} ; Table 1) is that this process would limit the rate of Ca^{2+} binding on activation of muscle. Thus the non-specific site, which is common to all myosins (Bagshaw & Kendrick-Jones, 1979), is not competent to trigger muscle contraction, although it might bind some Ca^{2+} during a prolonged tetanus. This conclusion was drawn previously (Bagshaw & Reed, 1977) on the basis of the change in

protein fluorescence on addition of EDTA to rabbit myosin preloaded with Ca^{2+} or Mg^{2+} . The displacement of Mg^{2+} by Mn^{2+} provides an independent check of this conclusion because the e.p.r. signal is a direct probe for the state of the metal ion rather than the protein.

Another reason for re-investigating the kinetics of bivalent metal ion dissociation was to extend the study to scallop myosin. On removal of the metal ions from the non-specific site the regulatory light chains dissociate from the heavy chains. In the accompanying paper (Bennett & Bagshaw, 1986) it is shown that the rate of release of regulatory light chain is partially limited by the rate of Mg^{2+} dissociation.

Ca^{2+} specific binding, which is responsible for activation in the myosin-linked regulation of molluscan preparations, occurs at a site separate from that investigated here. Ca^{2+} release from specific sites is not resolved by the pH-indicator method from its dissociation from other weak sites. However, protein fluorescence changes indicate that it occurs on the sub-second time scale (Wells *et al.*, 1985).

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