The mechanism of regulatory light chain dissociation from scallop myosin

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The dissociation of the regulatory light chains from scallop myosin subfragments, on addition of EDTA, was investigated by using the fluorophore 8-anilidonaphthalene-1-sulphonate as a probe. The rate of this process (0.014 s^{-1}) was partially limited by the rate of Mg^{2+} dissociation (0.058 s^{-1}) from the non-specific high-affinity site. The dissociation of the regulatory light chain subfragment 1 was less extensive than from heavy meromyosin. Reassociation of the scallop regulatory light chain was induced on addition of Mg^{2+} , but it appeared to be limited by a first-order step. The nature of this step was revealed by the kinetics of *Mercenaria* regulatory light chain association. Scallop heavy meromyosin, denuded of its regulatory light chains, exists in a refractory state, whose reversal to the nascent state limits the rate of light chain association (0.006 s^{-1}). The formation of the refractory state is the driving force for the net dissociation of regulatory light chains from scallop heavy meromyosin. This mechanism is discussed with reference to existing structural information on light-chain-denuded myosin.

INTRODUCTION

Contraction of molluscan muscle is regulated by the direct action of Ca^{2+} on the thick filament (Kendrick-Jones & Scholey, 1981). The involvement of the regulatory light chains of myosin in this process has been demonstrated by the reversible loss of Ca^{2+} -sensitivity of ATPase activity and tension development on their removal from scallop adductor muscle (Szent-Györgyi *et al.*, 1973; Simmons & Szent-Györgyi, 1985). Scallop myosin is unusual in that the high-affinity non-specific bivalent-metal-ion-binding site on each regulatory light chain must be occupied for these subunits to remain tightly bound to the heavy chain-essential light chain complex (Bagshaw & Kendrick-Jones, 1979). Thus removal of the regulatory light chains is readily achieved by treatment with EDTA.

In the present paper we study the mechanism of the dissociation of regulatory light chains on EDTA addition, in order to gain further insight into the nature of the subunit interactions involved. A minimal scheme for regulatory light chain dissociation is given in eqn. (1):

where H represents myosin heavy chain-essential light chain complex, L the regulatory light chain and Me^{2+} the bivalent metal ion (Bennett *et al.*, 1984). In the presence of bivalent metal ions scallop myosin exists predominantly in the associated state (i.e. $\text{H} \cdot \text{L} \cdot \text{Me}^{2+}$). Addition of EDTA, to chelate the Me^{2+} , causes the equilibrium to shift towards the dissociated state (i.e. $\text{H} + \text{L}$).

Eqn. (1), however, is an incomplete description of the mechanism, and a number of additional factors need to be considered. The binding of regulatory light chain probably involves multiple interactions with the heavy chain and essential light chain (Stafford & Szent-Györgyi, 1978; Bagshaw & Kendrick-Jones, 1980; Hardwicke *et al.*, 1983), and thus its dissociation may occur in several kinetically distinct steps. Furthermore, the degree of dissociation depends critically on the molecular species involved. Regulatory light chains readily dissociate from myofibrils, myosin and HMM, whereas the extent of release from $\text{S1}^{+\text{RD}}$ is limited (Stafford *et al.*, 1979; Shpetner, 1984). At low temperature (below 10°C) one regulatory light chain appears to dissociate preferentially from the two-headed myosin species (Kendrick-Jones *et al.*, 1976; Chantler & Szent-Györgyi, 1980). A possible explanation for this form of co-operativity is that a denuded myosin head tends to associate with the other head, thereby perturbing the equilibria shown in eqn. (1) (Bagshaw, 1980; Wells & Bagshaw, 1983). Finally, light chain-heavy chain interactions are affected specifically by Ca^{2+} to achieve regulation of the ATPase activity (Szent-Györgyi *et al.*, 1973; Wells & Bagshaw, 1985). The presence of Ca^{2+} -specific sites complicates the evaluation of the pathway of regulatory light chain dissociation. Nevertheless, eqn. (1) defines the overall thermodynamic relationships and provides a framework for the characterization of the kinetic mechanism.

The first step in the release of the regulatory light chain from scallop myosin is the dissociation of the bivalent metal ion. The kinetics of this reaction are characterized in the accompanying paper (Bennett & Bagshaw, 1986). In the present study we monitor the subsequent dissociation of the light chain by using the fluorophore ANS as a probe. ANS binds rapidly, but weakly, to a site

Abbreviations used: ANS, 8-anilidonaphthalene-1-sulphonate; 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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on the denuded heavy chain-essential light chain complex, and thus it can be used to monitor the release and uptake of the regulatory light chains (Bennett *et al.*, 1984).

EXPERIMENTAL

Materials

Proteins from scallop (*Pecten maximus*) adductor muscle were prepared as described previously (Wells & Bagshaw, 1983; Bennett & Bagshaw, 1986). Regulatory light chains were prepared from the clam *Mercenaria mercenaria* (Seasalter Shellfish Co., Whitstable, Kent, U.K.) by a method based on that of Kendrick-Jones *et al.* (1976). *Mercenaria* myofibrils (2–4 mg/ml) were suspended in 40 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.0, and incubated with 5 mM-EDTA and 5 mM-5,5'-dithiobis-(2-nitrobenzoate) for 10 min at 20 °C. After centrifugation to remove the myofibrils, the light-chain fraction was recovered from the supernatant in the 40–75% saturation fraction of an $(\text{NH}_4)_2\text{SO}_4$ precipitation step. The light-chain precipitate was resuspended and dialysed against 50 mM-NaCl/25 mM-Tris/HCl/2 M-urea/0.5 mM-MgCl₂/1 mM-dithiothreitol buffer, pH 8.0. It was purified by ion-exchange chromatography on DEAE-cellulose (Whatman DE52) or on a fast-protein liquid-chromatography Mono-Q column (Pharmacia), by elution with a linear gradient of 50–250 mM-NaCl in the buffer above. The regulatory-light-chain fractions were pooled and dialysed to remove the urea. The light chain was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 75% saturation, followed by dialysis in 40 mM-NaCl/20 mM-Epps/100 μM -MgCl₂/100 μM -dithiothreitol buffer, pH 8.0. The preparation was stored frozen at –20 °C.

Fluorescence spectroscopy

Fluorescence was measured with a Baird-Atomic SFR100 spectrofluorimeter, operating in the ratio mode. Measurements were routinely carried out in a 3 ml fluorescence cell, which was maintained at 20 °C by a water-cooled block. ANS fluorescence was monitored by exciting via the protein tryptophan residues at 295 nm (5 nm slit width) and recording the emission at 460 nm (10 nm slit width) as described previously (Bennett *et al.*, 1984). In most cases manual addition of the reagents was sufficiently rapid to enable the time course of the reaction to be monitored. Where necessary (e.g. Fig. 1), a stopped-flow accessory was employed, which allowed delivery of the reagents within 50 ms. The observation cell comprised a 2 mm-internal-diameter quartz tube, mounted vertically in a 4.5 cm $\times$ 1.2 cm $\times$ 1.2 cm cell, so that it fitted the standard holder of a spectrophotometer. The reactants were fed from two 5 ml glass syringes through capillary tubing (T/Pc; Amicon-Wright) to a mixing jet fabricated in the base of the observation cell.

Data analysis

Time courses, where appropriate, were analysed by a least-squares fit to an exponential. For multi-step schemes the time courses of the formation and decay of the various intermediates were computed by numerical integration. The values assigned to the rate constants were adjusted by trial and error to give an acceptable fit to the observed profile. The computer programs used are described by Millar (1984). Calculations of concentrations,

at equilibrium, in multicomponent systems was achieved by using the iterative procedure of Storer & Cornish-Bowden (1976). Computations were carried out with an Apple II+ microcomputer.

RESULTS

Regulatory light chain dissociation

Previous studies, with ANS as a probe, indicated that regulatory light chains dissociate from scallop myosin, on addition of EDTA, over a period of several minutes (Bennett *et al.*, 1984). This is comparable with the time scale for the release of Mg^{2+} from the high-affinity non-specific site of the regulatory light chain (Bennett & Bagshaw, 1986). On the other hand, the rate of Ca^{2+} dissociation from this site is an order of magnitude faster. To ascertain the contribution of the metal ion dissociation rates to the overall release of the regulatory light chain, the ANS fluorescence profiles, on addition of EDTA to HMM preparations (preincubated with either Ca^{2+} or Mg^{2+}), were examined (Fig. 1). The observed time course with Mg^{2+} was about 3-fold slower than that with Ca^{2+} , and a lag phase was evident. The profile with Mg^{2+} (Fig. 1b) could be modelled in terms of eqn. (1) with $k_{+1} = 0.058 \text{ s}^{-1}$ (Bennett & Bagshaw, 1986) and $k_{+2} = 0.014 \text{ s}^{-1}$. The time course obtained with Ca^{2+} (where k_{+1} would not be significantly rate-limiting) suggests that k_{+2} may be slightly faster than this value. However, other processes, involving the Ca^{2+} -specific sites (see below), may contribute to the record in Fig. 1(a). For this reason most of our studies were carried out with Mg^{2+} as the pre-bound metal ion, and 100 μM -EGTA was present throughout, to chelate contaminant Ca^{2+} .

We noted previously that the amplitude of the ANS response observed with $\text{S1}^{+\text{RD}}$, on addition of EDTA, was smaller than that of myosin. Furthermore the former was very dependent on the NaCl concentration (Bennett *et al.*, 1984). For a more complete comparison, HMM was investigated because myosin itself cannot be studied in the soluble state below about 0.3 M-NaCl. Fig. 2 shows that the ANS response with $\text{S1}^{+\text{RD}}$ was smaller than that of HMM, the amplitude of the latter being almost independent of ionic strength. The smaller amplitude observed with $\text{S1}^{+\text{RD}}$ could be due, at least in part, to the limited extent of regulatory light chain dissociation characteristic of this preparation (cf. Stafford *et al.*, 1979; Shpetner, 1984). A consequence of this situation is that the observed rate constant of the ANS profile with $\text{S1}^{+\text{RD}}$ would be slightly faster, because it represents the sum of the forward and reverse rate constants. If the amplitude of the ANS response is assumed to provide a direct measure of the extent of dissociation, then, in 40 mM-NaCl, about 50% of the light chain is dissociated from $\text{S1}^{+\text{RD}}$ (total concentration 4 μM), compared with HMM. These data indicate that the overall binding constant for the regulatory light chain with $\text{S1}^{+\text{RD}}$ (K_2 , eqn. 1) is about 2 μM . This conclusion is discussed further below.

Subfragment 1 preparations lacking the regulatory domain ($\text{S1}^{-\text{RD}}$) bind very little ANS and show no fluorescence change on addition of EDTA (Bennett *et al.*, 1984).

Regulatory light chain association

Addition of excess Mg^{2+} at the end point of the EDTA-induced dissociation of the regulatory light chains

Fig. 1. Dissociation of regulatory light chains from scallop HMM pre-equilibrated with (a) Ca^{2+} or (b) Mg^{2+}

Regulatory light chain dissociation was monitored by the increase in ANS fluorescence on mixing scallop HMM with EDTA, with the use of a stopped-flow accessory. One syringe contained 7 μM -HMM heads and (a) 100 μM - CaCl_2 or (b) 100 μM - MgCl_2 and 100 μM -EGTA, and the other syringe contained 1 mM-EDTA (syringe concentrations). Both syringes contained 40 mM- NaCl , 20 mM-Epps and 20 μM -ANS at pH 8.0 at 20 $^\circ\text{C}$.

from HMM and $\text{S1}^{+\text{RD}}$ caused a reversal of the ANS response. The analysis of the mechanism of light chain re-binding is, however, complicated by the fact that at least two routes of association can occur (via reversal of steps 1 and 2 or of steps 3 and 4 in eqn. 1). We noted previously that the rate of ANS fluorescence change, on addition of Mg^{2+} , was practically independent of the regulatory light chain concentration (Bennett *et al.*, 1984). This result indicated that a first-order step limits the association of the regulatory light chain to the denuded heavy chain-essential light chain complex, and thus eqn. (1) is incomplete. With HMM, a less than 2-fold increase in the association rate was observed when the regulatory light chain concentration was increased 5-fold. The maximum rate constant observed for this process was about 0.005 s^{-1} .

In principle, the mechanism of reassociation could also be studied by increasing the regulatory light chain concentration in the absence of bivalent metal ions. With HMM, addition of 16 μM exogenous scallop regulatory light chain (the highest tested) caused no reversal of the EDTA-induced ANS response (Fig. 2a). Thus the apparent value for K_2 (eqn. 1) for this preparation is greater than 100 μM . Addition of similar concentrations of scallop regulatory light chain to $\text{S1}^{+\text{RD}}$ caused a partial reversal of the EDTA-induced response (Fig. 2b). The limited amplitude of this process, compared with the background drift, made kinetic analysis difficult. Indeed we did not detect significant reversal in our previous studies (Bennet *et al.*, 1984). If it is assumed that addition of 2 mM- MgCl_2 causes almost complete re-binding of the

regulatory light chain, then the extent of the reaction, in the absence of Mg^{2+} , indicates that the apparent value for K_2 is in the region of 10 μM for $\text{S1}^{+\text{RD}}$ (Fig. 2b). The discrepancy with the value calculated from the extent of dissociation on addition of EDTA (see above) suggests that the inherent magnitude of the ANS fluorescence change with $\text{S1}^{+\text{RD}}$ is slightly smaller than that with HMM.

Further analysis of the light chain association mechanism was aided by the fortuitous discovery that the *Mercenaria* regulatory light chain has a very high affinity

Fig. 2. Dissociation and reassociation of regulatory light chains from scallop HMM and $\text{S1}^{+\text{RD}}$

Dissociation of regulatory light chains from scallop HMM (a and c) or $\text{S1}^{+\text{RD}}$ (b and d), on addition of EDTA, was followed by ANS fluorescence. The reaction was reversed in (a) and (b) by the addition of exogenous scallop regulatory light chains, followed by excess Mg^{2+} . In (c) and (d) the reaction was reversed by the addition of *Mercenaria* regulatory light chains in the absence of Mg^{2+} . (a) 3.5 μM -HMM (heads) in 40 mM- NaCl /20 mM-Epps/100 μM - MgCl_2 /100 μM -EGTA buffer, pH 8.0, and 20 $^\circ\text{C}$ was treated with (i) 500 μM -EDTA, followed by (ii) 4 μM scallop regulatory light chain, (iii) 12 μM scallop regulatory light chain (i.e. total concentration 19.5 μM) and (iv) 2 mM- MgCl_2 . (b) As (a), but with 4 μM - $\text{S1}^{+\text{RD}}$. (c) As (a), but the initial MgCl_2 concentration was 500 μM , and 1 mM-EDTA was added at (i), followed by (v) 3.5 μM *Mercenaria* regulatory light chain. (d) As (b), but 4 μM *Mercenaria* regulatory light chain was added at (v). The time bar represents 2 min in all records. The fluorimeter settings were similar in all traces except (c), where the gain was approximately doubled. The increased sensitivity was chosen to optimize the experiment described in Fig. 3(c).

Fig. 3. Simulated and observed rate of *Mercenaria* regulatory light chain binding to scallop HMM in the absence of Mg^{2+}

(a) Computer fit of Fig. 2(c) representing the release of scallop light chains on additions of (i) EDTA, followed by (ii) binding of *Mercenaria* regulatory light chains. The first phase (i) was fitted to a two-step consecutive reaction assuming $k_{+1} = 0.058 \text{ s}^{-1}$, from which a value of k_{+2} of 0.011 s^{-1} was obtained. The second phase (ii) represents the best fit to a single exponential for Fig. 2(c) (v) and yielded a rate constant of 0.0068 s^{-1} . The incomplete re-binding in Fig. 2(c) was, at least in part, due to the sloping baseline arising from the slow continuous binding of ANS (Bennett *et al.*, 1984). (b) Computer simulation of the production of H according to eqn. (2), normalized to the same scale as the first phase of (a). This represents the predicted ANS fluorescence change according to eqn. (2) when EDTA and *Mercenaria* regulatory light chain are added simultaneously to scallop HMM. (c) Observed ANS fluorescence record (continuous line) when $3.5 \mu\text{M}$ *Mercenaria* regulatory light chain and 1 mM -EDTA were added simultaneously to $3.5 \mu\text{M}$ -HMM (heads) under conditions identical with those described for Fig. 2(c). The broken line is a computer simulation of this reaction according to eqn. (3), with the use of values for the rate constants given in the Discussion section.

for the scallop heavy chain–essential light chain complex, even in the absence of bivalent metal ions. This was demonstrated by the ANS fluorescence profile when *Mercenaria* regulatory light chains were added to EDTA-treated scallop HMM or $S1^{+RD}$ (Figs. 2c and 2d). *Mercenaria* light chain binding in the presence of EDTA was also demonstrated with scallop HMM and myosin preparations whose endogenous regulatory light chain had been removed by chymotryptic digestion (Bennett & Bagshaw, 1985). The observed rate of *Mercenaria* light chain binding to denuded HMM and myosin was about 0.006 s^{-1} and was independent of the light chain and Mg^{2+} concentration. With $S1^{+RD}$ the profile was slightly faster (0.02 s^{-1}) and was preceded by a small burst in ANS release. Such transients were invariably seen with $S1^{+RD}$ on re-binding light chains (cf. Fig. 2b and Bennett *et al.*, 1984) compared with HMM and myosin, which showed no burst, or a very small change in the opposite direction. The possible significance of this phase is discussed below.

The simplest scheme for the exchange between scallop and *Mercenaria* regulatory light chains, based on eqn. (1), comprises three consecutive reactions: (i) dissociation of

Mg^{2+} , (ii) release of scallop light chain, and (iii) binding of *Mercenaria* light chain. The ANS profile for HMM (Fig. 2c) can be fitted to this mechanism by using the following values for the rate constants (Fig. 3a):

where F represents the foreign *Mercenaria* regulatory light chain. Its binding is limited by a first-order step and therefore the process is modelled as such, rather than by a second-order rate constant. If eqn. (2) were a complete description of the kinetic mechanism then simultaneous addition of EDTA and *Mercenaria* regulatory light chain to a scallop HMM preparation pre-equilibrated with Mg^{2+} should be accompanied by a transient phase of ANS binding. This is simulated in Fig. 3(b), which shows the transient formation and decay of the denuded heavy chain–essential light chain complex (H). In practice, no such transient was detected (Fig. 3c), even though the calculated amplitude was well within the sensitivity of the method. Likewise, in similar experiments with $S1^{+RD}$ no transient phase was detected, although in this case the computed profile had a smaller amplitude.

These experiments led to the model-independent conclusion that, during the dissociation of the scallop regulatory light chain from HMM, the vacated site on the heavy chain–essential light chain complex briefly exists in a nascent state. This state is capable of the rapid uptake of a *Mercenaria* regulatory light chain (Fig. 3c). It is distinct from the subsequent refractory state of the heavy chain–essential light chain complex, which predominates on more prolonged EDTA treatment and which can only bind *Mercenaria* regulatory light chain slowly (Fig. 2c). In the simplest mechanism the observed rate of binding (0.0068 s^{-1}) under these conditions represents the first-order reversal of the refractory state to the nascent state. With $S1^{+RD}$ a detectable fraction of the heavy chain–essential light chain complex remains in the nascent state, which allows a rapid phase of regulatory light chain binding (Fig. 2d). The reversal of the refractory state also limits the rate of binding of scallop regulatory light chain on addition of Mg^{2+} . This would account for the non-dependence of this process on the regulatory light chain concentration (Bennett *et al.*, 1984).

Ca^{2+} -specific changes

Scallop myosin possesses two Ca^{2+} -specific sites that are distinct from the non-specific bivalent-metal-ion-binding sites on the regulatory light chains. The relative affinities of Ca^{2+} over Mg^{2+} for these sites are above 300 and approx. 10 respectively (Bagshaw & Kendrick-Jones, 1979). In the presence of 1 mM - Mg^{2+} and $10 \mu\text{M}$ - Ca^{2+} it may be deduced that the non-specific site is occupied predominantly by Mg^{2+} and the specific site is occupied by Ca^{2+} . Addition of $100 \mu\text{M}$ -EGTA to HMM under these conditions caused a small increase in ANS fluorescence with a time course comparable with that of the release of regulatory light chain. This process was reversed on addition of $150 \mu\text{M}$ - Ca^{2+} . Ca^{2+} binding to the specific sites occurs on the sub-second time scale (Wells *et al.*, 1985), as expected if it is to regulate the rapid contraction of the adductor muscle. The effects of EGTA

on the ANS response in the presence of 1 mM-Mg²⁺ therefore seemed paradoxical in showing the thermodynamics of the Ca²⁺-specific site but kinetics similar to those associated with the non-specific site. However, consideration of the equilibria involved indicates that Ca²⁺ binding to the specific sites can affect the degree of light chain dissociation (see the Discussion section).

There is also a possibility that scallop myosin, denuded of regulatory light chains, can undergo a slow conformational change on binding Ca²⁺ at the residual site (Chantler & Szent-Györgyi, 1980) of the heavy chain-essential chain complex. A fluorescence change was observed when Ca²⁺ was added to scallop myosin, which was denuded of its regulatory light chains and into which 4-(*N*-iodoacetoxyethyl-*N*-methylamino)-7-nitrobenz-2-oxa-1,3-diazole-labelled scallop essential light chains had been incorporated by the method of Ashiba & Szent-Györgyi (1984).

These factors potentially complicate the interpretation of the ANS fluorescence changes. We have therefore attempted a detailed analysis of the kinetic mechanism only in those experiments where the non-specific site was occupied by Mg²⁺ and contaminant Ca²⁺ was sequestered with EGTA throughout their duration.

DISCUSSION

Kinetic model

Although eqn. (1) is sufficient to describe the kinetic mechanism of regulatory light chain dissociation, on addition of EDTA to scallop HMM and S1RD, it fails to account for the kinetics of reassociation. An additional first-order step is required in the scheme. Previously we suggested that scallop regulatory light chains may dissociate by a two-step mechanism (Bennett *et al.*, 1984). Although such a mechanism is possible, our present results with the *Mercenaria* regulatory light chain are most readily accounted for by the inclusion of a refractory form of the denuded heavy chain-essential light chain complex. Eqn. (3) represents the minimal scheme for scallop regulatory light chain dissociation (L) and hybrid formation with *Mercenaria* regulatory light chain (F):

The denuded heavy chain-essential light chain complexes (H and H*) are assumed to bind ANS to give the enhanced fluorescence. H* represents the refractory state. To a first approximation the differences between S1RD and HMM can be accommodated simply by a change in the equilibrium constant for the formation of the refractory state (*K*₅). With this assumption the values of all the rate constants at 20 °C are fairly stringently defined as follows: *k*₊₁ = 0.058 s⁻¹, *k*₋₁ = 6 × 10⁴ M⁻¹·s⁻¹, *k*₊₂ = 0.014 s⁻¹, *k*₋₂ = 4 × 10⁸ M⁻¹·s⁻¹, *k*₊₅ = 0.02 s⁻¹ (S1RD) or 0.24 s⁻¹ (HMM), *k*₋₅ = 0.01 s⁻¹ (S1RD) or 0.006 s⁻¹ (HMM), *k*₊₆ < 10⁻⁴ s⁻¹ and *k*₋₆ > 10⁶ M⁻¹·s⁻¹. *k*₊₇ and *k*₋₇ need not be defined for the present discussion, but they are probably comparable with *k*₊₁ and *k*₋₁ respectively. The rationale for the assignment of these constants is as follows. *k*₊₁ is measured directly (Bennett & Bagshaw, 1986) and *k*₋₁ is calculated from the

equilibrium constant, *K*₁, of about 10⁻⁶ M (Bagshaw & Kendrick-Jones, 1979). *k*₊₂ essentially controls the rate of scallop regulatory light chain dissociation on addition of EDTA (Fig. 1*b*). *k*₋₂ is estimated, together with *k*₊₅, to give a plausible value for the apparent equilibrium constant for scallop regulatory light chain dissociation. *k*₋₅ limits the rate of re-binding of light chains to the denuded heavy chain-essential light chain complex. *k*₊₆ and *k*₋₆ must be of sufficient magnitude such that *Mercenaria* regulatory light chain binding to H can compete, both kinetically and thermodynamically, with its transition to the H* state, and thus prevent a transient in ANS binding (Fig. 3*b*; cf. Fig. 3*c*). The apparent equilibrium constant for scallop regulatory light chain binding to S1RD and HMM in the presence of EDTA is given by [*k*₊₂(1 + *k*₊₅/*k*₋₅)]/*k*₋₂ and is modelled as 1 × 10⁻⁵ M for S1RD and 1.4 × 10⁻⁴ M for HMM. The extent of regulatory light chain dissociation from HMM and S1RD is very different, as a consequence of *k*₊₅, but the rates of release and uptake, controlled primarily by *k*₊₂ and *k*₋₅ respectively under the conditions used, are rather similar. The large value (40) assigned to *K*₅ for HMM ensures that H remains a minor species under all conditions, although its existence is crucial to the rapid binding of *Mercenaria* light chains in the experiment described in Fig. 3*c*). With S1RD, *K*₅ is close to unity (both H and H* exist in significant concentration) and thus accounts of the biphasic re-uptake of regulatory light chains observed with this preparation (Fig. 2*d*). *K*₅ is decreased on increasing the ionic strength.

Eqn. (3) is incomplete in that the observed reversal of scallop regulatory light chain release, on addition of Mg²⁺ (Fig. 2*a*), occurs slightly faster than that calculated from the assigned rate constants. However, the association route via an L·Mg²⁺ intermediate has been ignored (cf. eqn. 1). Eqn. (3) does predict, however, that the overall rate of this process cannot exceed that of *k*₋₅, and this is well borne out in practice. In this regard the low value of *k*₋₂ for the scallop regulatory light chain compared with *k*₋₆ for *Mercenaria* regulatory light chain (which may approach the diffusion-controlled limit) suggests that the conformation of the former, when isolated, is critically dependent on the presence of bound metal ion. Thus in the absence of bivalent metal ions only a small fraction of the scallop light chain may exist in a conformation that is complementary to the binding site on H, thus giving a low apparent second-order association constant *k*₋₂.

Our results indicate that the affinity of the *Mercenaria* regulatory light chain for the scallop heavy chain-essential light chain complex is several thousand-fold higher than that of the scallop regulatory light chain, in the absence of bivalent metal ions (*K*₆ cf. *K*₂; eqn. 3). The homologous light chain from *Spisula solidissima* shows an intermediate affinity. On the other hand in the presence of bivalent metal ions these light chains appear to have a comparable affinity (Sellers *et al.*, 1980). In such competition experiments the order of addition and time of incubation of the various components could be of crucial importance. Under some circumstances the light chain content of the hybrid myosin produced may be under kinetic rather than thermodynamic control.

Eqn. (3) represents the simplest mechanism to account for the observed ANS profile with S1RD and HMM. The assignment of the rate constants depends to some extent on the assumption that ANS binding to H and H* is

similar and that $S1^{+RD}$ and HMM differ only in the value of K_s . In practice the lack of complete reversibility of ANS binding limits the accuracy to which the system can be defined. It is possible that regulatory light chain dissociation occurs in several steps and that *Mercenaria* light chains can bind to give a hybrid species containing the two regulatory light chains per myosin head (cf. Asakawa & Azuma, 1983) to effect rapid exchange of these subunits. However, the significant feature of the mechanism that we characterize here is the existence of a refractory state of the denuded heavy chain–essential light chain complex.

Effect of Ca^{2+}

Removal of the regulatory light chains from scallop myosin results in a change in the equilibrium constant for Ca^{2+} binding to the specific site from 10^{-7} M to 10^{-6} M (Chantler & Szent-Györgyi, 1980). This site is located on the heavy chain–essential light chain complex. Thermodynamic balance therefore requires that the regulatory light chain binds to the complex 10-fold tighter in the presence of Ca^{2+} compared with in its absence. Thus low concentrations (approx. $1 \mu\text{M}$) will be effective in stabilizing the $H \cdot L \cdot Mg^{2+}$ complex. Although the intrinsic affinity of Mg^{2+} for $H \cdot L$ is fairly high ($K_1 = 10^{-6}$ M), its apparent value is lowered by the subsequent light chain dissociation steps ($K_{app.} = K_1(1 + [K_2(1 + K_s)]/[L])$). At an HMM concentration of $4 \mu\text{M}$, the values assigned to the constants of eqn. (3) indicate that 17% of the regulatory light chain dissociate in the presence of 1 mM-Mg^{2+} . Addition of Ca^{2+} at micromolar concentrations would therefore cause light chain reassociation due to Ca^{2+} preferentially binding to the specific site of $H \cdot L \cdot Mg^{2+}$ and $H \cdot L$ states compared with the H and H^* states. The slow reversible changes in ANS fluorescence on addition of $10 \mu\text{M-Ca}^{2+}$ and $100 \mu\text{M-EGTA}$ can thus be accounted for. At higher $[Ca^{2+}]$ binding would also occur at the non-specific site ($K_1^{Mg}/K_1^{Ca} = 10$; Bagshaw & Kendrick-Jones, 1979).

The calculation that limited regulatory light chain dissociation may occur in the presence of 1 mM-Mg^{2+} has relevance to the storage conditions of scallop preparations. Traditionally EDTA was included in myosin storage media to chelate heavy-metal ions and to minimize Ca^{2+} -activated proteinase activity. This practice was modified for scallop myosin by including a $0.5\text{--}1 \text{ mM}$ excess of free Mg^{2+} to keep the regulatory light chains in the associated state (Szent-Györgyi *et al.*, 1973; Stafford *et al.*, 1979). The present conclusions suggest that it would also be beneficial to include at least $10 \mu\text{M}$ free Ca^{2+} , particularly in cases of short-term storage of dilute protein solutions that are subjected to temperatures of around 20°C . In this regard it is pertinent to note that Ca^{2+} is used for the protection against light-chain proteolysis during subfragment preparations, even in the presence of high Mg^{2+} concentrations (Stafford *et al.*, 1979; Szentkiralyi, 1984).

Single-turnover ATPase assays show that conventional HMM preparations have 20–30% unregulated molecules (Wells & Bagshaw, 1985). Although this is partly due to molecules damaged by proteolysis (which can be removed by rapid ultracentrifugation in the presence of actin; C. Wells, personal communication), there remains an upper limit for the population of regulated molecules because of the limited spontaneous regulatory light chain

dissociation that occurs in 1 mM-Mg^{2+} . This can be minimized by (i) the inclusion of Ca^{2+} until just before the assay, (ii) storage of the stock solution at high protein concentrations and low temperature, and (iii) addition of exogenous regulatory light chains to maintain a high concentration of L .

Structural implications

Eqn. (3) argues that the transition of the denuded heavy chain–essential light chain complex from the nascent to the refractory state (K_s) provides the driving force for the extensive regulatory light chain dissociation observed with HMM compared with $S1^{+RD}$. What is the structural basis of this transition? Previously one of us suggested that, in view of the tendency for light-chain-denuded preparations to aggregate intermolecularly, there might also be a tendency for the two heads of myosin to associate intramolecularly (Bagshaw, 1980). In particular, this mechanism was evoked as a modification of the ideas of Kendrick-Jones & Jakes (1976), to explain the preferential release of one regulatory light chain from myosin that was noted at 4°C (Kendrick-Jones *et al.*, 1976). Evidence in support of this hypothesis came subsequently from spin-labelling studies, which showed that, on removal of the regulatory light chains, the independent motion of the myosin heads about their flexible necks was severely restricted (Wells & Bagshaw, 1983). Furthermore, this loss of motion occurred on the same time scale as the release of regulatory light chain and was not simply a reflection of delayed aggregation. Concurrent with these studies, the electron micrographs obtained by Flicker *et al.* (1983) revealed that removal of the regulatory light chains caused a change in the form of the individual heads from pear-shaped to a more rounded shape. These workers also commented on the tendency for the neck region to ‘collapse’ on removal of the light chain. Although they did not quantify such an effect, their Fig. 6 (Flicker *et al.*, 1983) indicates that the distance between the centres of mass of the two heads decreases from an average of about 19 nm to 13 nm.

Although the interpretations suggested for these observations differ in detail [Scheme 1, (c) and (d)], they have the common theme that the denuded heavy chain–essential light chain complex undergoes a major structural change subsequent to regulatory light chain dissociation. Furthermore, they both imply that the regulatory-light-chain-binding site is unstable when vacated and that it tends to interact with another protein surface rather than remain exposed to the solvent. Although the site has been described as a ‘sticky patch’ (Bagshaw, 1980; Pastra-Landis & Lowey, 1984), it is important to stress the dynamic nature of the interactions proposed here. In this regard reversal of the refractory state to the nascent state is rapid ($t_1 = 100$ s) compared with the preparative steps involved in many structural and biochemical investigations.

Two different, but probably related, phenomena need to be accounted for with respect to regulatory light chain dissociation. The preferential release of one light chain from two-headed species, such as myosin and HMM, implies some sort of interaction between the heads. However, such a co-operative release of light chain is only clearly observed with myofibrils at low temperatures (Chantler, 1985). With myosin alone the results are more variable (cf. Wells & Bagshaw, 1983; Flicker *et al.*, 1983),

Scheme 1. Possible structures of the refractory state

On release of the regulatory light chains from HMM the neck region might undergo intramolecular association (c) or collapse (d), thereby displacing the equilibrium towards net light chain dissociation. Intramolecular association could give rise to positive co-operativity, to negative co-operativity or to random dissociation, depending on whether the vacated site on one head tends to bind to the corresponding site on the second head, or tends to bind another location or shows no preference respectively. Neck collapse might give rise to co-operative effects if one head tends to block the other sterically. Both states (c) and (d) could account for the loss of flexibility of the neck region (Wells & Bagshaw, 1983) and the decrease in the average head-head distance (cf. Flicker *et al.*, 1983). With $S1^{+RD}$ the less extensive neck region might limit the extent of its collapse (g). Both states (b) and (f) could undergo intermolecular association, which would favour light chain dissociation (cf. Pastra-Landis & Lowey, 1984). The essential light chain has been omitted from these diagrams for clarity, but it probably constitutes part of the regulatory-light-chain-binding site (Hardwicke *et al.*, 1983). The dependence of the amplitude of the observed ANS profile with $S1^{+RD}$ on the NaCl concentration suggests that the interactions involved in step 5 (eqn. 3) are, at least in part, ionic in nature.

suggesting that the actin filament may orient the heads to allow a more homogeneous interaction. At 20 °C both regulatory light chains dissociate from two-headed species and there is little suggestion of co-operativity (Chantler, 1985). There remains, however, a clear-cut difference in the behaviour of HMM and $S1^{+RD}$ at this temperature (cf. Shpetner, 1984). This may reflect the degree of interaction possible within the neck region (Scheme 1). We have not attempted to analyse the data obtained with HMM in terms of differential binding of the two regulatory light chains. The dissociation step appears to fit a single exponential after allowance is made for the lag phase due to Mg^{2+} release (Fig. 3a). In any event it would be difficult to distinguish between a biphasic response due to co-operativity from other causes, such as a heterogeneous population (Wells & Bagshaw, 1985) or slow interconversion between two refractory states. Further information on the mechanism of subunit dissociation may come from the use of 4-(*N*-iodoacetoxyethyl-*N*-methylamino)-7-nitrobenz-2-oxa-1,3-diazole-labelled *Mercenaria* regulatory light chain, which shows a large decrease in fluorescence when bound to scallop myosin.

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