Fluorescence studies on the nucleotide- and Ca^{2+} -binding domains of molluscan myosin

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The effects of nucleotides and Ca^{2+} on the intrinsic tryptophan fluorescence of molluscan myosin and its proteolytic fragments were studied. By using these proteins from the scallop, *Pecten maximus*, the existence of two distinct tryptophan-containing domains was established, which respond independently to ATP and Ca^{2+} -specific binding. The latter is located in the 'neck' region of the myosin, which constitutes the regulatory domain. Subfragment 1, lacking the regulatory domain, responded only to ATP binding. On the other hand a tryptic fragment comprising the regulatory domain responded only to Ca^{2+} binding. Subfragment 1, containing the regulatory domain, responded to both ATP and Ca^{2+} , but its ATPase activity was Ca^{2+} -insensitive. By contrast, the ATPase activity of HMM was Ca^{2+} -sensitive. Increasing the ionic strength had a detrimental effect on Ca^{2+} -sensitivity, and fluorescence studies on solubilized myosin were therefore of limited value. Myosin and its fragments from other molluscan species which were investigated produced similar changes to those of *Pecten maximus*.

INTRODUCTION

The nucleotide-induced enhancement of intrinsic protein fluorescence has been of great value in the elucidation of the kinetic mechanism of the rabbit skeletal-muscle myosin ATPase pathway (Werber *et al.*, 1972; Bagshaw *et al.*, 1972; Johnson & Taylor, 1978). The binding and hydrolysis of ATP is accompanied by conformational changes in the protein that alter the environment around one or more tryptophan residues such that there is an increase in the intrinsic protein fluorescence. In addition, the existence of different levels of fluorescence enhancement for the different intermediates of the rabbit myosin ATPase pathway was a further aid to the study of the mechanism (Bagshaw & Trentham, 1974).

The potential use of this property in studying molluscan myosin has not been fully realized. Indeed, the existence of conformationally sensitive tryptophan residues, in scallop myosins at least, has remained controversial. Chantler & Szent-Györgyi (1978) reported about an 8% increase in fluorescence on binding ATP to *Aequipecten irradians* subfragment 1, but they did not observe a significant change on binding nucleotide to myosin from this species. Subsequent work by Chantler *et al.* (1981a), who used u.v. c.d., concluded that tryptophan residues were not necessarily perturbed during myosin ATPase activity. With the use of *Patinopecten yessoensis*, however, nucleotide-induced perturbations of tryptophan were observed when u.v. absorption spectroscopy (Kondo *et al.*, 1979) and fluorescence (Shibata-Sekiya, 1982; Konno *et al.*, 1983) were used.

Molluscan muscle is regulated by the direct binding of Ca^{2+} to the thick filament. The regulation of contraction can be studied *in vitro* by measuring the effect of Ca^{2+} on the actin-activated Mg^{2+} -ATPase activity (Szent-

Györgyi *et al.*, 1973). Recently we have shown by using *Pecten maximus* that scallop HMM Mg^{2+} -ATPase activity is inherently Ca^{2+} -sensitive in the absence of actin (Wells & Bagshaw, 1985). These data imply that interactions must occur between the Ca^{2+} -binding sites and ATPase sites of HMM. We have therefore examined the effect of nucleotides and Ca^{2+} on the fluorescence of scallop myosin and its proteolytic fragments by using *Pecten maximus* as a source. Subsequently, we extended these studies to other molluscan species. Some of the results in the present paper were reported in a preliminary communication (Wells *et al.*, 1985).

EXPERIMENTAL

Materials

Pecten maximus and *Chlamys opercularis* were obtained from the University Marine Biological Station, Millport, Isle of Cumbrae, Scotland, and *Mercenaria mercenaria* from the Seasalter Shellfish Company, Whitstable, Kent. *Aequipecten irradians* and *Placopecten magellanicus* came from the Marine Biological Laboratory, Woods Hole, MA, U.S.A. and were a gift from Professor A. G. Szent-Györgyi. Trypsin, chymotrypsin, papain, soya-bean trypsin inhibitor, ATP, ADP and Tes were purchased from the Sigma, Poole, Dorset, U.K., and Ap_5A from Boehringer, Lewes, East Sussex, U.K. All other reagents were obtained from BDH, Poole, Dorset, U.K. or Fisons, Loughborough, Leics., U.K.

Methods

Molluscan myosins were prepared as described by Chantler & Szent-Györgyi (1978). Scallop HMM, $\text{S1}^{+\text{RD}}$ and $\text{S1}^{-\text{RD}}$ were prepared as detailed by Wells & Bagshaw (1983). $\text{S1}^{+\text{RD}}$ was previously denoted '(Ca,Mg)-subfragment 1' and was obtained by digestion in the presence

Abbreviations used: HMM, heavy meromyosin; Ap_5A , P^1, P^5 -di(adenosine-5')-pentaphosphate; RF, regulatory fragment; $\text{S1}^{+\text{RD}}$, subfragment 1 containing the regulatory domain; $\text{S1}^{-\text{RD}}$, subfragment 1 lacking the regulatory domain; HC, heavy chain; ELC, essential light chain; RLC, regulatory light chain; mRLC, modified regulatory light chain; Mg^{2+} -ATPase, Mg^{2+} -dependent ATPase; SDS, sodium dodecyl sulphate.

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Fig. 1. SDS/polyacrylamide-gel electrophoresis of scallop myosin and its proteolytic fragments

Pecten maximus myosin and its proteolytic fragments were electrophoresed on a SDS/7.5–20%-(w/v)-polyacrylamide gradient gel as follows: (a) myosin; (b) HMM; (c) S1RD; (d) S1RD; (e) RF. S1RD and RF contain a truncated form of RLC (denoted mRLC; Stafford *et al.*, 1979). S1RD, which lacks the regulatory domain, has no intact-light-chain bands and also has a slightly smaller HC component than has S1RD (Bennett *et al.*, 1984).

of 1 mM-CaCl₂ and 1 mM-MgCl₂, whereas S1RD was denoted '(EDTA)-subfragment 1' and was obtained by digestion in the presence of 10 mM-EDTA (Stafford *et al.*, 1979). The regulatory fragment (RF) comprises a 12 kDa heavy-chain fragment, a regulatory and an essential light chain (Szentkiralyi, 1984). It was prepared by a method similar to that previously described by Szentkiralyi (1984). S1RD was digested with trypsin at a trypsin/myosin ratio of 1:250 (w/w) for 20 min at 23 °C in 40 mM-NaCl/5 mM-sodium phosphate/1 mM-MgCl₂/1.5 mM-CaCl₂, pH 7.0. The reaction was terminated with soya-bean trypsin inhibitor (5 mg/mg of trypsin) and then the RF was purified on a DEAE-cellulose (Whatman DE52) column by using a 40 mM-1 M-NaCl gradient in 20 mM-Tris/1 mM-MgCl₂/1 mM-CaCl₂, pH 8.0. The peptide compositions of proteolytic fragments of scallop myosin are shown by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) in Fig. 1. Proteins from rabbit skeletal muscle were prepared as previously described (Bagshaw, 1977).

The intrinsic tryptophan fluorescence of myosin and its proteolytic fragments was monitored by using a Baird-Atomic SFR100 spectrofluorimeter in the ratio mode at $\lambda_{\text{excitation}} = 295 \text{ nm}$ (5 nm slit width) and $\lambda_{\text{emission}} = 335 \text{ nm}$ (10 nm slit width). Measurements

were routinely carried out in a 3 ml fluorescence cell, which was maintained at 20 °C by a water-cooled block. Reactions were initiated by manual addition of the effector dissolved in a minimal volume (< 30 μl). The adenylate kinase inhibitor Ap₅A was included in critical experiments, but it did not alter the results obtained. ADP binding to scallop HMM was measured by using the Hummel-Dreyer technique (Ackers, 1973). A column (20 cm $\times$ 1 cm) of Sephadex G-50 was equilibrated with 10 mM-Tes/20 mM-NaCl/1 mM-MgCl₂/0.7 μM -Ap₅A/4.4 μM -ADP, pH 7.5, and either 100 μM -CaCl₂ or 100 μM -EGTA. HMM in the same buffer was applied to the column at room temperature and the effluent was monitored at A_{259} with a Perkin-Elmer LS5 spectrophotometer fitted with a continuous-flow cell. All other procedures used were as described by Wells & Bagshaw (1983).

RESULTS

The effects of nucleotides and Ca²⁺ on the intrinsic tryptophan fluorescence of scallop myosin and its proteolytic fragments were investigated as a probe for the study of the kinetics and regulation of the ATPase activity of this system. Fig. 2 shows the extent of

Fig. 2. Effect of ATP and Ca^{2+} on the tryptophan fluorescence of scallop myosin subfragments

The tryptophan fluorescence (F) of $\text{S1}^{+\text{RD}}$ (a, b), $\text{S1}^{-\text{RD}}$ (c, d) and regulatory fragment (e, f), all at a concentration of $2 \mu\text{M}$, was recorded with time. The buffer used was $10 \text{ mM-Tes}/20 \text{ mM-NaCl}/1 \text{ mM-MgCl}_2$, pH 7.5, at 20°C for $\text{S1}^{+\text{RD}}$ and $\text{S1}^{-\text{RD}}$ (this buffer contained 5 mM-MgCl_2 for RF). The Ca^{2+} concentration of the buffer was varied as stated below. In (a) and (c), $50 \mu\text{M-ATP}$ was added and in (e), $100 \mu\text{M-ATP}$ was added, all in the presence of $100 \mu\text{M-CaCl}_2$. In (b), (d) and (f) the buffer initially contained $10 \mu\text{M-CaCl}_2$, to which $100 \mu\text{M-EGTA}$ was added, followed by excess Ca^{2+} ($200 \mu\text{M-CaCl}_2$) at the points indicated.

fluorescence enhancement obtained on addition of ATP and Ca^{2+} to various fragments of *Pecten maximus* myosin. $\text{S1}^{+\text{RD}}$ responds to both effectors, whereas the fluorescence of $\text{S1}^{-\text{RD}}$ increases only in the presence of ATP, and the regulatory fragment (RF) is only affected by Ca^{2+} . These data therefore demonstrate that there are two distinct tryptophan-containing regions that respond to ATP and Ca^{2+} , which will subsequently be referred to as 'Trp_a' and 'Trp_b' respectively. The reversibility of these responses to both reagents is consistent with them reporting conformational changes associated with the function of the protein, rather than non-specific effects. The fluorescence enhancement, induced in subfragment 1 preparations by ATP, is maintained throughout the duration of ATP hydrolysis and therefore its profile reflects ATPase activity (Bagshaw, 1975). If more ATP is added, then the time of enhanced fluorescence is increased proportionally. The fluorescence induced by Ca^{2+} was complete at concentrations less than the

contaminant level ($\sim 10 \mu\text{M}$), in the presence of 1 mM-Mg^{2+} , and thus it represents Ca^{2+} binding to the specific regulatory sites (Szent-Györgyi *et al.*, 1973). This site is distinct from the non-specific bivalent-metal-ion site of the regulatory light chain (Bagshaw & Kendrick-Jones, 1979), which gives a fluorescence quench on binding Ca^{2+} in rabbit myosin preparations (Bagshaw & Reed, 1977). Both sites, however, are located within the regulatory fragment and are lacking in $\text{S1}^{-\text{RD}}$ (Bennett *et al.*, 1984).

Although $\text{S1}^{+\text{RD}}$ binds Ca^{2+} (Fig. 2b), the rate of ATPase activity of this fragment is independent of the presence of this cation, whereas the ATPase activity of scallop HMM is Ca^{2+} -dependent, irrespective of the presence of actin (Wells & Bagshaw, 1985). Fig. 3 shows the fluorescence profiles of these fragments in response to Ca^{2+} and ATP. Both preparations display a reversible fluorescence enhancement on binding Ca^{2+} . The profile obtained on addition of ATP to $\text{S1}^{+\text{RD}}$ is independent of Ca^{2+} (Figs. 3a and 3b), whereas the period of enhanced fluorescence of HMM is prolonged 2–3-fold in the absence of Ca^{2+} compared with that in the presence of this cation (Figs. 3c and 3d). The ATP turnover rates, calculated from the periods of enhanced fluorescence shown here (Fig. 3, legend), are in agreement with steady-state estimates of ATPase rates for these fragments, measured with a pH-stat. The fluorescence profile of HMM in the absence of Ca^{2+} also differs from the other records shown in Fig. 3 in the level to which the signal returns. With Ca^{2+} -depleted HMM, the fluorescence only falls back to two-thirds of the initial enhancement by ATP, suggesting that some residual nucleotide binding is retained.

Fig. 4 shows the effect of ADP on the fluorescence of scallop HMM. In the presence of Ca^{2+} , very little ADP binding occurs as judged by fluorescence enhancement (Fig. 4a). By contrast, in the absence of Ca^{2+} , a level of fluorescence is attained (Fig. 4b) that is comparable with that at time t_x in Fig. 3(d). Direct binding measurements, using the technique of Hummel & Dreyer (Ackers, 1973), confirmed that there is more ADP bound in the absence of Ca^{2+} (Figs. 4c and 4d). Although this finding could account for the difference in the apparent end-point fluorescence observed at t_x in Fig. 3c (cf. Fig. 3d), there is a further complication. We have shown that a typical scallop HMM preparation comprises a mixed population containing 20–30% unregulated molecules (Wells & Bagshaw, 1984a, 1985). In the absence of Ca^{2+} , the ATPase activity of this unregulated population is comparable with that of the whole preparation in the presence of Ca^{2+} (0.3 s^{-1}), whereas the regulated molecules show a very slow rate ($k_{\text{cat}} = 2 \times 10^{-3} \text{ s}^{-1}$). Fig. 3(d) can therefore be interpreted in terms of this mixed population. The decrease in fluorescence at t_x (to about two-thirds of the initial enhancement) is due to the unregulated molecules releasing ADP after ATP hydrolysis. The regulated population continues to undergo a very slow rate of phosphate release [the $\text{M} \cdot \text{ADP} \cdot \text{P}_i \rightarrow \text{M} \cdot \text{ADP}$ step ($\text{M} = \text{myosin head}$); Wells & Bagshaw, 1985]. The level of fluorescence at t_x is comparable with that attained by ADP binding in the absence of Ca^{2+} . There may be a small decrease ($< 2\%$) in fluorescence between $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ and $\text{M} \cdot \text{ADP}$, but this is not distinguishable from instrumental drift over the very slow time course of phosphate release. After phosphate release, the regulated population still binds

Fig. 3. Effect of Ca^{2+} on the tryptophan fluorescence profiles obtained on addition of ATP to scallop S1^{RD} and HMM

The tryptophan fluorescence of S1^{RD} (a, b) and HMM (c, d) were recorded with time at a protein concentration of $2 \mu\text{M}$ -heads in $10 \text{ mM-Tes}/20 \text{ mM-NaCl}/1 \text{ mM-MgCl}_2/10 \mu\text{M-CaCl}_2$, pH 7.5, at 20°C . In (a) and (c) $100 \mu\text{M-EGTA}$ was present initially, to which $200 \mu\text{M-CaCl}_2$ was added, followed by $50 \mu\text{M-ATP}$ at the times indicated. In (b) and (d), $100 \mu\text{M-EGTA}$ was added, followed by $50 \mu\text{M-ATP}$. The ATP turnover rates, calculated from the period of enhanced fluorescence subsequent to ATP addition, are as follows: (a) 0.33 s^{-1} ; (b) 0.30 s^{-1} ; (c) 0.31 s^{-1} ; (d) 0.12 s^{-1} . At the time denoted t_x in (d) the regulated fraction continues to undergo a slow single turnover (see the text).

Fig. 4. Effect of Ca^{2+} on ADP binding to scallop HMM

The tryptophan fluorescence of scallop HMM ($2 \mu\text{M}$ -heads) was recorded with time in $10 \text{ mM-Tes}/20 \text{ mM-NaCl}/1 \text{ mM-MgCl}_2$, pH 7.5, at 20°C (a) in the presence of Ca^{2+} ($100 \mu\text{M-CaCl}_2$) and (b) in the absence of Ca^{2+} ($100 \mu\text{M-EGTA}$). ADP was added in increments of (i) $2 \mu\text{M}$, (ii) $2 \mu\text{M}$ and (iii) $5 \mu\text{M}$ as indicated. ADP binding was also measured by using the Hummel–Dreyer technique (c, d). The absorbance of the eluate from a Sephadex G-50 column was monitored continuously at 259 nm after the application of $100 \mu\text{l}$ of HMM ($84 \mu\text{M}$ -heads). The bar represents an absorbance of 0.04 and corresponds to an ADP concentration of $15.8 \mu\text{M}$ in the cylindrical flow-cell used. The column and the sample were pre-equilibrated with $10 \text{ mM-Tes}/20 \text{ mM-NaCl}/1 \text{ mM-MgCl}_2/0.7 \mu\text{M-AP}_5\text{A}/4.4 \mu\text{M-ADP}$ and either (c) $100 \mu\text{M-CaCl}_2$ or (d) $100 \mu\text{M-EGTA}$. The column was run at room temperature at a constant flow rate of 0.33 ml/min . From the area of the troughs in these profiles, ADP binding to HMM was calculated to be 0.20 and 0.49 mol/mol of heads in the presence and absence of Ca^{2+} respectively.

ADP in the absence of Ca^{2+} . If Ca^{2+} is added to the preparation in Fig. 3(d) at a time after t_x , then the net fluorescence level remains approximately the same. This is because the increase in fluorescence caused by binding Ca^{2+} balances the decrease in fluorescence due to the regulated population rapidly releasing products. With

manual additions, these processes are just resolvable at lower temperatures (6°C).

Fig. 5 shows the fluorescence profile obtained by varying the Ca^{2+} concentration during the hydrolysis of a large excess of ATP by scallop HMM. Addition of this ATP produced the enhanced fluorescence, discussed

Fig. 5. Tryptophan fluorescence of scallop HMM on varying the Ca^{2+} concentration in the presence of ATP

The tryptophan fluorescence of scallop HMM ($2 \mu\text{M}$ -heads) was recorded under the same conditions as in Fig. 3. A large excess of ATP ($200 \mu\text{M}$) was added in the presence of $10 \mu\text{M}$ - CaCl_2 . During the resulting period of enhanced fluorescence, the Ca^{2+} concentration was varied by the addition of $100 \mu\text{M}$ -EGTA, followed by $200 \mu\text{M}$ - CaCl_2 .

above, which was maintained during hydrolysis. During this period the Ca^{2+} concentration was altered by adding EGTA, followed by excess Ca^{2+} . This resulted in the expected reversible response to Ca^{2+} binding, as shown in Fig. 3, superimposed on the ATP response. Therefore, although HMM displays communication between the nucleotide- and Ca^{2+} -binding sites, in that ATPase activity in this molecule is Ca^{2+} -sensitive, the responses of the Trp_a and Trp_b residues are not coupled spectroscopically. The use of vanadate (V_i) to produce a stable, long-lived nucleotide complex ($\text{M} \cdot \text{ADP} \cdot \text{V}_i$) with scallop HMM (Wells & Bagshaw, 1984b), confirmed this result. The fluorescence effects obtained by altering the Ca^{2+} concentrations were still present after a $\text{M} \cdot \text{ADP} \cdot \text{V}_i$ complex had been formed.

The selective removal of the regulatory light chains of scallop HMM has been achieved by a limited chymotryptic digestion of the preparation in the presence of EDTA (Bennett & Bagshaw, 1985). The fluorescence profile obtained on addition of ATP to this preparation was Ca^{2+} -insensitive. A Ca^{2+} -sensitive profile can be restored by reincorporating exogenous scallop regulatory light chains in the presence of Mg^{2+} .

Comparable fluorescence studies on the parent molecule, myosin, are not possible, since at 20 mM -NaCl this protein is in its filamentous form. Solubilization of the myosin was achieved by increasing the ionic strength of the buffer from 20 mM - to 500 mM -NaCl. Under these conditions, myosin from *Pecten maximus* still displayed a fluorescence enhancement with ATP, but Ca^{2+} had a decreased effect (Fig. 6). Furthermore, Ca^{2+} had less influence on ATPase activity, since the turnover time in the absence of Ca^{2+} was only prolonged by approx. 1.4-fold and the fluorescence returned to its initial value at the end of the reaction. To ascertain whether these effects were due to the increased ionic strength or intrinsic to the myosin preparation, we studied HMM at different NaCl concentrations. On increasing the NaCl concentration, the fluorescence profile observed with HMM showed a progressive loss in Ca^{2+} -sensitivity and at 0.5 M -NaCl it resembled that observed with myosin. A comparable

Fig. 6. Effect of ATP and Ca^{2+} on the tryptophan fluorescence of scallop myosin in 0.5 M -NaCl

The tryptophan fluorescence of scallop myosin ($2 \mu\text{M}$ -heads) in 10 mM -Tes/ 0.5 M -NaCl/ 1 mM - MgCl_2 , pH 7.5, at 20°C was recorded with time. In (a) $100 \mu\text{M}$ -EGTA was present initially, to which $200 \mu\text{M}$ - CaCl_2 and $50 \mu\text{M}$ -ATP was added as indicated. In (b) $10 \mu\text{M}$ - CaCl_2 was present initially, to which $100 \mu\text{M}$ -EGTA and $50 \mu\text{M}$ -ATP was added.

result for the effect of ionic strength on the ATPase activity of squid myosin was reported by Konno *et al.* (1981). The fact that 0.5 M -NaCl has a detrimental effect on the regulatory properties limits the value of experiments on solubilized molluscan myosin.

The results so far presented were obtained by using myosin and its proteolytic fragments from *Pecten maximus*. Subsequently, we extended our study to other molluscan species and also re-examined rabbit skeletal-muscle proteins, under the same conditions, for comparison. Table 1 shows the percentage fluorescence enhancements induced by ATP and Ca^{2+} for all the preparations measured. Although ATP binding induces smaller changes in the molluscan species than in rabbit, these effects are nevertheless very reproducible and of sufficient magnitude to be useful in rapid-reaction measurements. In the cases where the observed changes in fluorescence were smaller than those of *Pecten maximus*, this may reflect increased lability of the preparation rather than a genuine difference in the properties of their tryptophan residues. With *Pecten maximus* itself, variations ($\pm 10\%$) in the magnitude of the enhancement were recorded from preparation to preparation. HMM from the clam *Mercenaria mercenaria* exhibited improved Ca^{2+} -sensitivity in steady-state ATPase assays when compared with that from *Pecten maximus*. This was also reflected in a turbidity assay

Table 1. Effect of ATP and Ca^{2+} on the tryptophan fluorescence of myosin and its proteolytic fragments

The percentage enhancement of tryptophan fluorescence was measured for myosin and its proteolytic fragments from a variety of molluscan species and from rabbit skeletal muscle. All measurements were carried out in 10 mM-Tes/1 mM- MgCl_2 , pH 7.5, at 20 °C. The NaCl concentration varied as shown.

Protein	[NaCl] (mM)	Fluorescence enhancement (%)	
		ATP- induced	Ca ²⁺ - induced
<i>Pecten maximus</i>			
Myosin	500	9	1
HMM	500	8	1
	20	7	3
S1 ^{+RD}	20	9	3
S1 ^{-RD}	20	16	0
RF	500	0	2
	20	0	6
<i>Chlamys opercularis</i>			
Myosin	500	7	1
HMM	20	5	2
<i>Aequipecten irradians</i>			
Myosin	500	9	3
HMM	20	7	4
S1 ^{+RD}	20	12	3
S1 ^{-RD}	20	17	0
<i>Placopecten magellanicus</i>			
HMM	20	5	2
<i>Mercenaria mercenaria</i>			
Myosin	500	7	< 1
HMM	20	4	< 1
Rabbit skeletal-muscle			
Myosin	500	11	0
HMM	500	16	0
	20	16	0
Papain S1 ^{+RD}	20	16	0
Chymotryptic S1 ^{-RD}	20	26	0

(Wells & Bagshaw, 1984a), which indicated that the preparation contained only 10–15% unregulated (permanently activated) molecules. The observed magnitude of the fluorescence enhancement with ATP, however, was decreased, possibly due to totally inactive protein contributing to the overall fluorescence.

The difference in the percentage fluorescence enhancement of various proteolytic subfragments prepared from the same myosin preparation can be accounted for by the differences in the overall fluorescence emission of these fragments, which is determined by their tryptophan contents. This is most noticeable for the ATP-induced enhancements of HMM, S1^{+RD} and S1^{-RD} and is evident in the profiles of Figs. 2 and 3. At the same molar concentration (2 μM -heads), HMM, S1^{+RD} and S1^{-RD} exhibit fluorescence intensities in the proportions 1.00:0.80:0.46 respectively. The percentage changes induced by ATP in these preparations, 7, 9 and 16% respectively, represent the same absolute perturbation to the Trp_a residues. The reduced overall fluorescence of S1^{-RD} as compared with S1^{+RD} reflects the tryptophan-rich regulatory domain (Bennett *et al.*, 1984), which

contains the Trp_b residues. Although a larger percentage change in the fluorescence of Trp_b might be expected on Ca^{2+} binding to the isolated RF as compared to S1^{+RD}, the RF is rather labile and may exist in a slightly different conformation than when it forms part of a larger molecule.

In HMM and S1^{+RD} the enhancement induced by Ca^{2+} binding is of lower magnitude than that caused by ATP, but it is nevertheless reproducible and reversible. Increasing the ionic strength to 500 mM-NaCl decreased the enhancement by about two-thirds. This effect was more readily demonstrated with the regulatory fragment, which produces larger percentage changes. As expected, no Ca^{2+} -specific effects were seen with rabbit skeletal-muscle myosin and its subfragments, in accord with its lack of myosin-linked regulation (at least *in vitro*). A significant fluorescence change was not observed on Ca^{2+} binding to *Mercenaria mercenaria*, even though its ATPase was well-regulated. This may be due to the preparative difficulties alluded to above, as the ATP-induced fluorescence enhancement was smaller than that obtained with other species.

DISCUSSION

The conclusions of this paper are represented diagrammatically in Fig. 7 in terms of spectroscopically sensitive tryptophan-containing domains. S1^{+RD} is responsive to both ATP and Ca^{2+} , and therefore possesses both the Trp_a and Trp_b domains. S1^{-RD} responds to ATP only and RF to Ca^{2+} only and so these fragments contain Trp_a and Trp_b respectively, in

Fig. 7. Tryptophan-containing domains in scallop myosin

The relationship between the proteolytic subfragments of scallop myosin and location of the spectroscopically sensitive tryptophan-containing domains are shown diagrammatically. Trp_a responds to ATP binding and Trp_b to Ca^{2+} binding. Communication occurs between these sites in HMM to effect the Ca^{2+} -sensitivity of the ATPase activity. It is not clear to what extent communication occurs between the two heads of HMM in the absence of actin (cf. Stafford *et al.*, 1979; Chantler *et al.*, 1981b). Key to symbols: $\blacktriangle$, Trp_a; $\bullet$, Trp_b.

isolation. HMM is sensitive to both ATP and Ca^{2+} binding and, furthermore, the profile generated by this molecule shows the ATPase activity to be Ca^{2+} -sensitive. The Trp_a and Trp_b domains therefore communicate in HMM and not in $\text{S1}^{+\text{RD}}$. The double-headed structure of HMM or the intact hinge region, or perhaps both, are necessary prerequisites for a fully functioning molecule. The isolation of a homogenous single-headed HMM preparation may help to elucidate the necessary structural features for Ca^{2+} regulation. The fluorescence response to Ca^{2+} binding is consistent with the Ca^{2+} -specific binding site (Szent-Györgyi *et al.*, 1973) being involved, because the same fluorescence level exists at $10\ \mu\text{M}$ - Ca^{2+} and $100\ \mu\text{M}$ excess Ca^{2+} (Fig. 2b) in the presence of $1\ \text{mM}$ - Mg^{2+} . Increasing the $[\text{Mg}^{2+}]$ to $5\ \text{mM}$ had little effect on the fluorescence change induced by Ca^{2+} . The decreased effects of Ca^{2+} binding at higher ionic strengths (Table 1) also attain the same fluorescence level at $10\ \mu\text{M}$ - and $100\ \mu\text{M}$ - Ca^{2+} . Therefore it is likely that the decreased enhancement observed under these conditions is due to a loss of response of the Trp_b domain to Ca^{2+} binding, rather than a decrease in extent of Ca^{2+} binding *per se*.

The proximity of the Trp_a and Trp_b domains to the ATP- and Ca^{2+} -binding sites cannot be ascertained from the results presented here. It is pertinent to note, however, that the amino acid sequence of nematode myosin heavy chain (Karn *et al.*, 1982) reveals a cluster of four tryptophan residues (between positions 798–833) in the region corresponding to the difference peptide between $\text{S1}^{+\text{RD}}$ and $\text{S1}^{-\text{RD}}$ (Bennett *et al.*, 1984). If conserved, these would be candidates for the Trp_b residues. The scallop essential light chain, however, also contains a single tryptophan residue (Kendrick-Jones *et al.*, 1976), which may contribute to Trp_b . In rabbit myosin, a tryptophan residue is located in the *N*-terminal 25 kDa peptide, which is close to the ATPase site as detected by photoaffinity labelling (Okamoto & Yount, 1985), and this might correspond to the Trp_a residue.

Our data do not require a specific role for the tryptophan-containing domains in ATPase activity or its regulation. They do show, however, that conformationally-sensitive tryptophans are associated with protein function in all the species that we have studied, which conflicts with the conclusions of Chantler *et al.* (1981a). As described in the Results section, the differences in the percentage fluorescence enhancement obtained with the proteolytic fragments reflects the different tryptophan contents of these proteins. Thus $\text{S1}^{-\text{RD}}$ contains substantially less tryptophan residues than $\text{S1}^{+\text{RD}}$, and therefore the ATP-induced perturbation of the Trp_a domain results in a larger percentage change in $\text{S1}^{-\text{RD}}$ than $\text{S1}^{+\text{RD}}$, as would be expected. According to Chantler & Szent-Györgyi (1978), however, these subfragments showed very similar changes with ATP. We can provide no ready explanation for this discrepancy. We have observed significant fluorescence changes with ATP and Ca^{2+} , using the same species (*Aequipecten irradians*) under the same buffer and temperature conditions where these workers found little or no change.

The fluorescence profiles, together with other measurements, reveal some similarities and a number of differences between the ATPase mechanisms of scallop adductor myosin and rabbit skeletal-muscle myosin. The most significant difference is the Ca^{2+} -sensitivity of the former, which is characterized by a very suppressed

ATPase rate ($2 \times 10^{-3}\ \text{s}^{-1}$) of the regulated molecules in the absence of Ca^{2+} (Wells & Bagshaw, 1985). The full extent of this suppression is not evident in the fluorescence profiles of Fig. 3 or in any other steady-state assays, because of the dominant contribution of the unregulated fraction to ATP turnover. In the presence of Ca^{2+} , scallop HMM has a higher ATPase rate than that of rabbit ($0.3\ \text{s}^{-1}$ cf. $0.05\ \text{s}^{-1}$) and binds ADP more weakly. This is suggested by the return of protein fluorescence to almost its initial value when the ATP is exhausted (Fig. 3c). This weak binding, in conjunction with the fact that the fluorescence changes are smaller in scallop compared with rabbit proteins, makes the characterization of $\text{M} \cdot \text{ADP}$ fluorescence difficult, in the presence of Ca^{2+} . In the absence of Ca^{2+} , ADP binding is tighter, at least to the regulated fraction, but the analysis is complicated by the heterogeneity of the preparation. A weakening of ADP binding to regulated scallop preparations, on addition of Ca^{2+} , is consistent with an increase in its dissociation rate, as previously suggested (Wells & Bagshaw, 1985). Although the spectrofluorimeter used in the present studies could readily detect a 2% change in fluorescence over several seconds, it is not clear whether a change of this magnitude accompanies the P_i -release step in the absence of Ca^{2+} . This reaction has a half-time of about 6 min in $20\ \text{mM}$ -NaCl (Wells & Bagshaw, 1985) and therefore it is difficult to resolve this process from a decrease in fluorescence arising from photodecomposition or instrumental drift. Displacement by chromophoric nucleotide may provide larger signals to monitor the product release steps of scallop preparations (cf. Bagshaw *et al.*, 1972).

Properties common to scallop and rabbit myosin include the rapid and reversible ATP-hydrolysis step leading to the 'phosphate burst' (Marston & Lehman, 1974; Shibata-Sekiya, 1982; Wells & Bagshaw, 1985). After this step, a rate-limiting isomerization occurs, so that the apparent rates of P_i and ADP release are similar. By using a column-centrifugation technique, we have shown that, in the absence of Ca^{2+} , ^{14}C ADP release from the regulated fraction of HMM occurs with the same slow time course as ^{32}P P_i release (Wells & Bagshaw, 1985) during a single turnover of labelled ATP (C. Wells & C. R. Bagshaw, unpublished work). In such an experiment, the product ADP is released during the reaction (cf. the fluorescence assay under the same salt conditions; Fig. 3d), because the concentration of free ADP in equilibrium with the HMM is maintained close to zero.

The fluorescence changes induced by ATP and Ca^{2+} are adequate for the characterization of their rates of binding to scallop preparations using stopped-flow techniques.

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