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Photochemical Mapping of the Active Site of Myosin [and Discussion]

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Photochemical mapping of the active site of myosin

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SUMMARY

The active sites of myosin from skeletal, smooth and scallop muscle have been partly characterized by use of a series of photoreactive analogues of ATP. Specific labelling was attained by trapping these analogues in their diphosphate forms at the active sites by either cross-linking two reactive thiols (skeletal myosin) or by formation of stable vanadate-metal ion transition state-like complexes (smooth muscle and scallop myosin). By use of this approach combined with appropriate chemistry, several key residues in all three myosins have been identified which bind at or near the adenine ring, the ribose ring and to the γ-phosphate of ATP. This information should aid in the solution of the crystal structure of the heads of myosin and in defining a detailed structure of the ATP binding site.

1. INTRODUCTION

Muscle myosin is the best characterized of the many molecular motors now known to exist in various cells (Vale & Goldstein 1990). This is true both for historical reasons (it was the first motor discovered) and for practical reasons (gram quantities can be isolated in a pure form in less than two days). This ease of preparation, coupled with myosin's obvious importance and ease of assay, has made it the subject of hundreds of studies in the past five decades. Despite this intense interest, we still lack a molecular explanation for force generation. Attention has focused on the head of myosin called subfragment-1 (S1) because it contains both the ATPase and actin binding sites. More recently studies *in vitro* have shown that S1 alone in the presence of ATP can move actin filaments (Toyoshima *et al.* 1987), a result which suggests that a molecular explanation of movement will require mainly the structure of S1 and F-actin. Fortunately the molecular structure of co-crystals of G-actin and DNase I has recently been solved (Kabsch *et al.* 1990) and this has been extended to a reasonable structure of F-actin (Holmes *et al.* 1990). S1 was crystallized in 1984 (Rayment & Winkelmann 1984), and a low resolution structure (≈25 Å†) has been determined from electron microscopy of sectioned crystals (Winkelmann *et al.* 1991).

As an alternative method of structural characterization, we have employed the technique of photoaffinity labelling (Bayley & Knowles 1977; Bayley 1983) to characterize the composition and nature of the ATP binding site. Myosin is an ideal enzyme to study by this approach, as large quantities are often needed to ensure that enough labelled peptide can be isolated for characterization. Also, myosin is a highly non-specific triphosphatase. It will hydrolyse the terminal phosphate from essentially any substituted triphosphate, including simply tripolyphosphate. This suggested that photoaffinity analogues of ATP would bind efficiently to the active site and this proved to be the case. To improve the specificity of binding and photolabelling we have taken advantage of myosin's ability to bind ADP analogues tightly at the active site (called 'trapping') under certain conditions. Two methods of trapping have proved most useful. First, nucleotides can be trapped by cross-linking the two reactive thiols of skeletal myosin (in rabbit skeletal myosin, Cys_{697} and Cys_{707}) with bifunctional reagents (Wells & Yount 1979) such as Co(III) phenanthroline or *p*-phenylenedimaleimide (reviewed in Wells & Yount 1982). A second more general trapping method, which works for all myosins that we have studied, is to form a transition state-like complex of vanadate (V_i) with the β-phosphate of ADP and a divalent metal ion (Goodno 1982). It is essential that the divalent metal ion is a transition metal, e.g. Co^{2+} or Mn^{2+} (Grammer *et al.* 1988; Cremo *et al.* 1988) to prevent a vanadate-promoted photo-oxidation of active-site side chains (see below). These two trapping procedures decrease the off-rate of ADP (and most ADP analogues) by a factor of greater than 10^4, which allows the nucleoside diphosphate–myosin complexes to be purified by centrifugal gel filtration before activation of the photoprobes by irradiation. Without this prior trapping and purification procedure, we have observed significant non-specific photolabelling by essentially every probe utilized. Finally, advances in peptide isolation by newer high performance liquid chromatography (HPLC) techniques and in peptide microsequencing by the pulsed-liquid methodology

† 1 Å = 10^{-10} m.

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have been essential features of our photolabelling work.

2. STRUCTURE OF PHOTOAFFINITY LABELS

The structures of the ATP photoprobes used to date are shown in figure 1. Five of the probes react with amino acid residues at the adenine binding site (purine probes). These include UTP and ATP, which both photolabel smooth muscle myosin directly with short-wavelength irradiation (Maruta & Korn 1981; Garabedian & Yount 1990, 1991). The other purine probes contain aryl azides which photolabel myosin via an intermediate nitrene. Of these the non-nucleoside ATP analogue, NANTP, has been used the most because it normally traps well and gives the highest photo-incorporation. Both 2-N_3ATP and 8-N_3ATP trap well but typically give lower covalent labelling. An additional complication is that the resulting labelled peptides are often unstable. Note that although the structures shown are the triphosphate forms it is the diphosphates which are stably trapped in each case.

Two ATP analogues are designed to probe at or near the ribose ring. With myosin, most is known about Bz_2ATP (Mahmood *et al.* 1989; Cole & Yount 1990) which labels via a free radical mechanism involving the benzophenone carbonyl and nearby C-H bonds. The other analogue, NANPAP-ATP (see below), was first synthesized and used to photolabel myosin by Guillory & Jeng (1977) and later by Szilagyi *et al.* (1979). A disadvantage to these two photoprobes is that their reactive groups are somewhat removed from the 2′(3′) oxygens of the ribose ring (6–7 Å for Bz_2ATP and up to 10 Å for NANPAP-ATP).

The single probe for the phosphate binding site is the Mg^{2+} complex of ADP and vanadate (V_i). A possible structure of the complex is shown with the vanadate depicted as a trigonal–bipyrimidal adduct with ADP. This transition state-like structure is believed to exist at the active site of myosin, based on its unusual stability (Goodno 1982) and an increased absorbance of the S1·MgADP·V_i complex at 310 nm which we attribute to the vanadate–phosphate adduct (Grammer *et al.* 1988). The photoreaction occurs between the trapped V_i and adjacent amino acids, presumably those which normally bind to the γ-phosphate of ATP.

3. EXPERIMENTAL STRATEGY IN PHOTOLABELLING

Purified myosins and their S1 subfragments are incubated with the appropriate radioactive ATP photoaffinity analogues in the dark and trapped either by: (i) cross-linking of SH_1, SH_2, or (ii) by addition of vanadate (V_i) and a divalent metal as described above. In the latter method, time is allowed for the triphosphate derivative to be hydrolysed before V_i is added. The divalent metal used is either Co^{2+} or Mn^{2+}, both of which quench the known photoreaction of vanadate with myosin (see below). After the ADP (or analogue) is trapped, EDTA and excess ATP are added to complex excess divalent metal and to block non-specific binding of the ATP analogues. The trapped myosin or S1 complexes are then purified by one (sometimes two) centrifugal gel filtration step(s) which leaves the myosin (S1) complexes undiluted and essentially free of extraneous photosensitive reagents.

Samples are typically irradiated on ice with UV light in the 300–400 nm range. Light below 300 nm is removed by two pyrex filters because skeletal muscle myosin is particularly sensitive to inactivation by low-wavelength UV. Depending upon the chemical nature of the analogue used, irradiation times may vary from 3–40 min. For direct photoaffinity labelling, e.g. the use of unmodified UDP or ADP with gizzard myosin, unfiltered UV light is used and the uracil or adenine rings react directly with the active site residue(s).

The amount of photo-incorporation is quantified by acid precipitation of a portion of the modified myosin (S1) and by measuring the radioactivity of the precipitate. The amount of covalent labelling by trapped photoprobes may vary from a few percent to as high as 70%. The remaining photolabelled complex is generally partly denatured in 1 M urea and digested with multiple additions of trypsin to break all arginyl or lysyl bonds. The resulting tryptic peptides are then purified by a series of reversed phase HPLC columns under differing pH conditions. Overall yields of pure labelled peptides are typically about 10%. Peptides are sequenced by pulsed liquid methods. It has been possible on occasion to identify directly the radioactive photolabelled amino acid(s) during the appropriate sequencing cycle if the phosphates can be removed by prior alkaline phosphatase treatment (Garabedian & Yount 1990). Otherwise the location of the photolabel is detected by the absence of the expected amino acid phenylthiohydantoin derivative during a given cycle in the pulsed-liquid sequencing procedure. In some cases, e.g. [^{3}H]-Ser-180 of skeletal myosin (see below), the modified residue was detected directly as the appropriate radioactive serine phenylthiohydantoin derivative.

4. LOCATION OF PHOTOLABELLED RESIDUES

The specific amino acids that are modified in several myosins by various photoprobes are given in table 1. We have most information about skeletal myosin. The two azide-containing analogues, NANTP and 2-N_3ATP both photolabel Trp-130 in the heavy chain. Molecular modelling shows that the photoreactive azide groups of these two probes are within 0·1 nm of each other if they bind in their most extended conformation, i.e. 2-N_3ATP in the *anti* conformation. A third azide analogue, 8-N_3ATP, photolabels specifically the NH_2-terminal 23 kDa tryptic peptide (J. Grammer & R. Yount, unpublished results), but due to the low percentage photo-incorporation (3–4%), specific labelled residues have not yet been identified.

Interestingly, chemical modification of Trp-130 with dimethyl (2-hydroxy-5-nitrobenzyl) sulphonium

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Table 1. *Summary of myosin photolabelling results*

myosin	analogue	trapping method	residues photolabelled heavy chain	references
skeletal	NANDP	SH_1-SH_2	Trp-130	Okamoto & Yount (1985)
	2-N_3ADP	SH_1-SH_2	Trp-130	Yount *et al.* (1987)
	Bz_2ADP	SH_1-SH_2	Ser-324	Mahmood *et al.* (1989)
	Mg·ADP·V_i	vanadate	Ser-180	Cremo *et al.* (1989)
photomodified S1 (skeletal)	Mg·ADP·V_i	vanadate	Ser-243	Grammer & Yount (1991)
cardiac	NANDP	SH_1-SH_2 or vanadate	Trp-129	Okamoto & Yount (unpublished results)
smooth (gizzard)	NANDP	Co^{2+}·vanadate	23 kDa	Okamoto *et al.* (1986)
	2-N_3ADP	Co^{2+}·vanadate	23 kDa	Grammer & Yount (unpublished results)
	Bz_2ADP	Co^{2+}·vanadate (6S)	Pro-324	Cole & Yount (1990)
	MgBz_2ADP·P_i	10S conformation	Pro-324	Cole & Yount (1990)
	UDP	Co^{2+}·vanadate	Glu-185	Garabedian & Yount (1990)
	ADP	Co^{2+}·vanadate	Glu-185	Garabedian & Yount (1991)
	Mg·ADP·V_i	vanadate	Ser-179	Cole & Yount (1991)
scallop (*Aquepectin*)	NANDP	Mn^{2+}·vanadate (no Ca^{2+})	Arg-128	Kerwin & Yount (unpublished results)
	NANDP	Mn^{2+}·vanadate (10^{-5} M Ca^{2+}; 2×10^{-3} M Mg^{2+})	Cys-198 Arg-128	Kerwin & Yount (unpublished results)

bromide (Peyser *et al.* 1990) has little effect on the actin-activated MgATPase activity of S1. This has caused these authors to question if Trp-130 is at the active site. However, several lines of evidence suggest that it is. First, both 2-N_3ATP (E. Pate & R. Yount, unpublished results) and NANTP (Pate *et al.*, 1991) support generation of tension by skinned muscle fibres in a manner almost identical to ATP. The diphosphate forms are stably trapped ($t_{1/2} > 5$ days at 0°C), and both give high photo-incorporation yields from trapped complexes (20% for 2-N_3ADP and 50–60% for NANDP). NANDP also specifically photolabels the analogous Trp in cardiac myosin (Trp-129) in high yield (*ca.* 70%). Most recently, NANDP trapped on scallop myosin (table 1) photolabels Arg-128 with high efficiency (*ca.* 37%). Arg-128 is in the identical sequence location of the heavy chain of scallop myosin as Trp-130 is in skeletal muscle myosin. It seems reasonable that Arg-128 and Trp-130 are in similar locations in the active site of these two myosins and perform similar functions. It is unlikely that two amino acids with such chemically diverse side chains would have been photolabelled so specifically in high yield if this were not true. It may be that part of the indole ring of Trp-130 in skeletal myosin is near the surface and can be chemically labelled without affecting the actin-activated MgATPase activity of S1. At the present time, these apparently conflicting results remain unresolved.

Table 1 also shows that two ribose-modified photoprobes, Bz_2ATP and NANPAP-ATP, photolabel Ser-324 and Lys-189 in skeletal myosin, respectively. These two residues are in the 50 kDa and 23 kDa major tryptic heavy chain fragments, respectively, and this illustrates that both these two major fragments (some would call them domains) are likely to contribute to the ATPase site. NANPAP-ATP was the first photoaffinity label to be used on myosin (Guillory & Jeng 1977) and was first shown to label the 23 kDa NH_2-terminal tryptic heavy chain fragment (Szilagyi *et al.* 1979). However, our photolabelling studies with vanadate-trapped NANPAP-ADP show significant photolabelling not only of the 23 kDa fragment mentioned above but also the 50 kDa tryptic fragment (D. Kennedy & R. Yount, unpublished results). Although the specific residue photolabelled in the 50 kDa fragment is not yet determined, these results show that portions of the 23 kDa and 50 kDa fragments must be adjacent to each other at the active site. Finally, the observation that Cys-697 (SH2) from the 20 kDa tryptic peptide can be chemically cross-linked to Lys-184 (or Lys-189) (Sutoh and Hiratuska 1988) suggests that this peptide is also near the active site. Thus all three major tryptic S1 heavy chain fragments, the 23 kDa, 50 kDa and 20 kDa (in that order from the NH_2-terminus), are likely to be at or near the ATP-binding site of skeletal myosin.

(*a*) *Smooth muscle myosin*

The ATPase site of gizzard myosin has been less well characterized. Interestingly, direct photolabelling of the purine binding site can occur with both trapped UDP or ADP. Both nucleotides photolabel Glu-185 in the heavy chain. The photo-activatable groups are probably the 5,6 double bond of UTP and the 7,8

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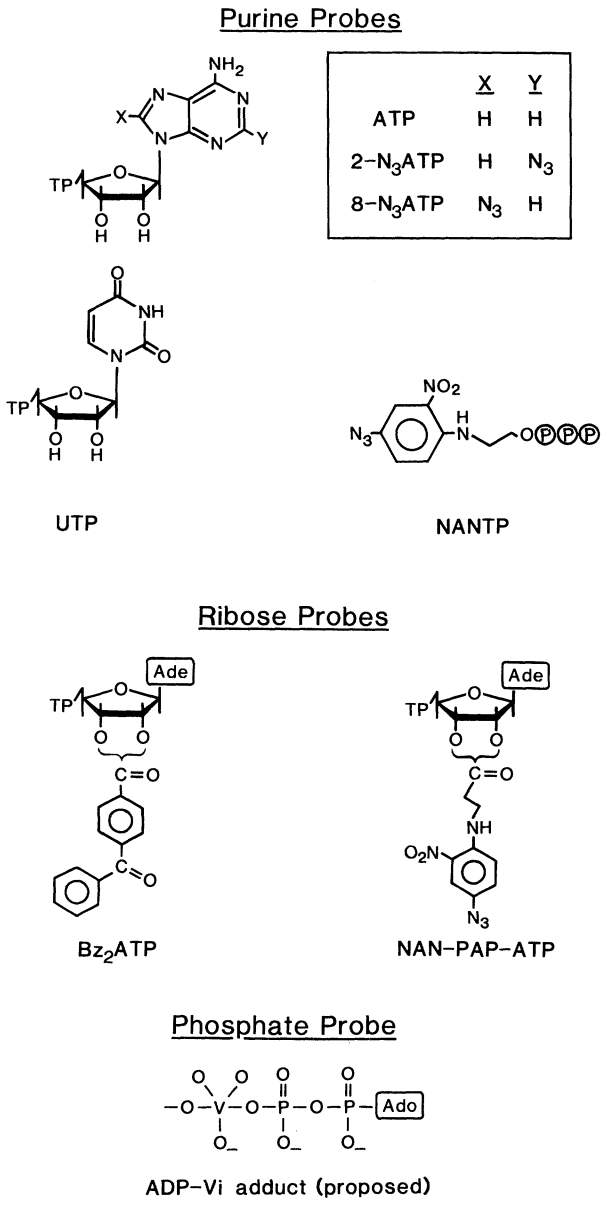

Figure 1. Structures of photoreactive ATP analogues. Abbreviations are: 2-N_3ATP, 2-azido-adenosine triphosphate; 8-N_3ATP, 8-azidoadenosine triphosphate; NANTP, 2-[(4-azido-2-nitrophenyl) amino] ethyl triphosphate; Bz_2ATP, 3′(2′)-0-(4-benzoylbenzoyl) triphosphate; NAN-PAP-ATP, 2′(3′)-0-{3-[(N-4-azido-2-nitrophenyl) amino] propionyl} ATP.

nitrogen–carbon double bond of ATP (Garabedian & Yount 1991). UDP and ATP do not photolabel skeletal S1 (< 1%), possibly because a less reactive valine replaces Glu-185 in the heavy chain sequence.

The conversion of the extended 6S form of gizzard myosin to the folded 10S form is a third method of trapping nucleotide, which is stabilized at the active site as ADP·P_i by this conformational change (Cross *et al.* 1987). This same conformational change also traps Bz_2ATP as Bz_2ADP·P_i (Cole & Yount 1990). Bz_2ADP, trapped either in the folded 10S form or with Co^{2+}·V_i in the extended 6S conformation, photolabels the same residue, Pro-324. The labelling of this residue (closely analogous to the Ser-324 of skeletal myosin) suggests that both the skeletal and smooth muscle heavy chains fold in a similar manner. In addition, NANPAP-ADP also photolabels both the 26 kDa (NH_2-terminal tryptic peptide) and 50 kDa (central tryptic peptide) of the heads of gizzard myosin (D. Cole & R. Yount, unpublished results), suggesting further similarity with skeletal myosin.

Other photolabeling studies (Okamoto *et al.* 1986) had initially suggested that both the essential light chain (LC_{17}) as well as the heavy chain were photolabelled by trapped NANDP. A re-investigation of this labelling (J. Grammer & D. Cole, unpublished results) suggests that the light chain labelling is non-specific and probably occurred because untrapped NANDP was not completely removed. Residues near the NH_2-terminus of LC_{17} are preferentially photolabelled by traces of [^{32}P] NANDP even when the active site is blocked by CoADP·V_i. This unusual observation shows that care must be taken in interpretations when low levels of photo-incorporation are observed; here *ca.* 2–3% of LC_{17} was photolabelled with the *ca.* 11% of the heavy chain labelled. The photolabelled residue(s) in the heavy chain has not been determined because the photo-adduct is unstable to normal purification conditions. The same low photo-incorporations and instability (e.g. to heat and β-mercaptoethanol treatment) has been observed for comparable 2-N_3ADP photolabelled gizzard myosin preparations. This low incorporation may reflect the fact that the gizzard myosin heavy chain has a glutamine where skeletal and scallop myosins have Trp and Arg, respectively.

(*b*) *Scallop myosin*

This has been photolabelled with trapped NANDP as reported above (table 1). In the absence of Ca^{2+} and Mg^{2+} in low salt (myosin is filamentous), NANDP labels almost exclusively Arg-128. However, if Ca^{2+} is titrated in, the major site of labelling shifts to Cys-198. Here photo-incorporation is high (20–30%) and non-specific labelling by trace amounts of untrapped NANDP, as observed with gizzard myosin, can be ruled out. These results support the concept that Ca^{2+} binding to the essential light chain in the regulatory domain (Kwon *et al.* 1990; Nyitray *et al.* 1991) induces a conformation change which directly affects the active site. This result shows that in favourable cases the photolabelling approach can be used to detect conformation changes and to identify specific residues involved in these changes.

5. VANADATE PHOTOMODIFICATION OF MYOSIN

Vanadate (V_i) was shown by Gibbons and co-workers (Gibbons *et al.* 1987) to promote the photocleavage of dynein in the presence of ATP (ADP) and Mg^{2+}. At the same time we observed independently (R. Mahmood & R. Yount, unpublished results) that $MgBz_2$ADP·V_i·myosin complexes were unstable to

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light. Further investigation (Grammer *et al.* 1988) showed that irradiation of the MgADP·V_i·S1 complex did not cleave the heavy chain but rapidly released ADP and V_i ($t_{1/2} \approx 2$ min) with a concomitant increase in the absorbance of 270 nm and a four- to five-fold activation of the CaATPase activity. These effects could be reversed by reduction with $NaBH_4$, and the residue modified was localized to the 23 kDa tryptic NH_2-terminal fragment (Cremo *et al.* 1988). Reduction with [^{3}H]$NaBH_4$ allowed identification of Ser-180 in the 23 kDa fragment as the residue photomodified (Cremo *et al.* 1989). Because this Ser is in the GESGAGKT consensus sequence defining a phosphate-binding loop, and is found in all myosin heavy chains, it is likely Ser-180 binds directly to the γ-phosphate of ATP.

The heavy chain of photomodified S1 (myosin) can be cleaved by a second irradiation after retrapping of ADP and V_i at the active site. The best evidence shows the side chain serine aldehyde produced by the first irradiation (Cremo *et al.* 1988) is oxidized to a carboxylic acid (Grammer & Yount 1990) with subsequent chain cleavage at Ser-180. The amino group of Ser-180 remains with the α-carboxyl of Glu-179 to yield a carboxyl terminal amide. The NH_2-terminal Gly-181 at the cleavage site is also blocked by one to two carbon fragments originating from Ser-180 (J. Grammer, unpublished results). Surprisingly, during the photocleavage reaction a second serine (Ser-243) is also photo-oxidized (Grammer & Yount 1991). This serine is the first serine in a highly conserved region of the 50 kDa tryptic fragment, NSSRFGKFI, which occurs in all myosin heavy chains sequenced to date. These results support the previous photolabelling studies with Bz_2ADP and NANPAP-ADP that the 23 kDa and 50 kDa fragments are close to each other in the active site, and suggest a role for the high degree of conservation of the sequence around Ser-243.

The heavy chain of S1 is cleaved in two places when irradiated with V_i and no added nucleotides at neutral to alkaline pH (Mocz 1989; Cremo *et al.* 1990). At pH 6, a third cleavage site yields an apparent 31 kDa NH_2-terminal tryptic peptide (Muhlrad *et al.* 1991). It is possible this fragment may represent photocleavage at Ser-243. Cleavage also occurs at the active site and near the actin-binding site close to the 50–20 kDa junction. By blocking the active site of S1 with ATP at higher pH values, it is possible to observe specific cleavage near the 50–20 kDa junction (C. Cremo, unpublished results). Thus vanadate-promoted cleavage can be used much as a chemical restriction enzyme to give specific polypeptide fragments.

Recent studies on vanadate photocleavage of adenylate kinase suggest that the chain is cleaved at Pro-17 (Cremo *et al.* 1992). This Pro is part of the glycine-rich P-loop now believed to bind the phosphate portion of ATP (see Tsai & Yan 1991, and references therein) and occurs in a sequence position analogous to Ser-180 in myosin's heavy chain. These results show that photocleavage studies with vanadate alone, without nucleotides, can give information about phosphate-binding residues. They also show that photocleavage can occur at residues other than serine.

6. CONCLUSION

The use of photochemical mapping has provided new information about the amino acid composition of the ATP-binding site in several myosins. Residues at or near the purine ring, the ribose ring and γ-phosphate of ATP have been identified. 'Active-site trapping' has increased the specificity of photolabelling and the percentage covalent modification by stabilizing the various photoprobes at the active site. Residues from both the 23 kDa and the 50 kDa major tryptic fragments from the heavy chain of subfragment-1 have been implicated as being at or near the ATP-binding site. Identification of these residues should provide critical information to aid in the definition of the ATP-binding site of S1 by X-ray crystallography now in progress (I. Rayment). Finally, preliminary studies with scallop myosin have shown it is possible to detect Ca^{2+}-dependent conformational changes at the active site by photochemical techniques. This latter observation suggests this approach may also be useful in studying myosins which are regulated by phosphorylation of regulatory light chains.

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Discussion

R. S. Goody (*Max-Planck-Institut für medizinische Forschung, Heidelberg, F.R.G.*). Myosin is known to bind pyrophosphate very strongly, so in the mixture of vanadates does the divanadate bind to the myosin active site?

C. Cremo. From binding studies using vanadium-51 nuclear magnetic resonance (NMR) we think that the tetravanadate binds. Vanadate is added to a very concentrated solution of the protein, and binding is indicated by which species in the spectrum broadens. Apparently we are not in agreement with Muhlrad's group, who thinks that perhaps some of the dimer also binds, but my evidence is that the tetramer is the predominant form binding at the active site. It is probable that all forms can bind, but it is the tetramer that is the predominant one.

R. S. Goody. I have a comment on the adenylate kinase results. The original structure, which got the ATP and the AMP sites wrong, was wrong to some extent for understandable reasons, because AMP can bind in the ATP sites. There is NMR evidence which is much more consistent with what is now known to be the correct mode of binding, consistent with the author's work as well.

C. Cremo. It is consistent with the ras structure too.

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T. J. Mitchison (*Department of Pharmacology, University of California, San Francisco, U.S.A.*). Ian Gibbons showed the famous vanadate cleavage site in dynein. I was wondering if the chemistry of that has been elucidated as well?

C. Cremo. There appear to be two classes of cleavage sites in dynein. One appears to be very similar to the cleavage site that we have shown, and that appears to occur through a nucleotide–vanadate complex, although the complex is not as tight as it is in myosin. The other site is analogous to our V2 site, and Gibbons called it V2 in his system too. My feeling on that site in dynein is that it is probably one of those other nucleotide binding sites that have now been shown from the recent sequence of dynein. In our case, the V2 site is very close to a long string of lysines near the tryptic cleavage site. My guess is that vanadate is binding specifically there in the tetrameric form because there is good charge–charge interaction.

D. R. Trentham (*National Institute for Medical Research, Mill Hill, London, U.K.*). The authors have probably seen from ^{18}O exchange experiments that there is evidence for the bound phosphate group being able to rotate freely in the active site, although it is not free to dissociate. Do the authors have any clues about that mechanism from their vanadate work?

C. Cremo. No, I think it is difficult to extend the vanadate work to that kind of discussion, because the bond length in the vanadate is longer than in phosphate, and that may alone explain the difference in its ability to form this type of complex. I know Dr Trentham wants the structure of the vanadate complex to be determined. It is a difficult experiment that would require doing a vanadium 51 NMR experiment in the solid state, but I plan to do that experiment.

P. L. Dutton (*Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, U.S.A.*). What happens if the authors alter the active ring of NANDP, for example moving the azide around relative to the nitro group. Does this alter the kinetics but not the labelling, or is something else labelled?

C. Cremo. We have never done that experiment for one very good reason. Unless you have the meta position of the nitro relative to the azide as in NANDP itself to activate the azide, you will deactivate the azide and are unlikely to get any photolabelling, so it is not a very interesting experiment. I do not have any data on the kinetics.

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