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PHOTOAFFINITY LABELLING WITH AN ATP ANALOG OF THE N-TERMINAL PEPTIDE OF MYOSIN*

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SUMMARY: Photoaffinity labelling of tryptic and chymotryptic heavy meromyosin with 3'O-3-[N-(4-azido-2-nitrophenyl) amino]propionyl-adenosine 5'-triphosphate (arylazido-β-alanine ATP) resulted in incorporation of radioactivity and inhibition of the ATPase activity. ATP prevented the reaction with the photoaffinity label, as shown by the lack of incorporation of ^{3}H and intact ATPase activity. On the tryptic digestion of either type of photoaffinity labeled HMM the label was found in a 25K peptide identifiable with the N-terminus of the myosin heavy chain (Lu et al., Fed. Proc. 37, 1695, 1978). The results are discussed in the light of previous localization of the reactive thiol groups, SH-1 and SH-2 (Balint et al., Arch. Biochem. Biophys. 190, 793, 1978).

The use of proteolytic enzymes has made it possible to begin a mapping of the topography of that portion of the myosin molecule that contains the active sites. The original proposal (2) for the connectivity of the fragments obtained by tryptic digestion based on the temporal sequence of their appearance and the effect of Ca^{2+} or Mg^{2+} on the proteolytic process has recently been refined. A 20-21K fragment containing the two so-called essential sulfhydryl groups of the ATPase has been identified, and it has been suggested that it is separated from the amino terminus of the heavy chain by a peptide stretch of about 70K daltons (3). In view of the suggested proximity of the essential, SH-1 and SH-2, thiol groups to the ATP binding site (see e.g. 4) it appeared of interest to attempt to identify those fragment(s) that are sufficiently close to ATP to permit the reaction with an aryl-azido[^{3}H]ATP derivative (5) as a photoaffinity label. The

*A preliminary report of these results was presented (1).

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present results show that $N_3NpApr[^3H]ATP^{**}$ binds to HMM and upon illumination forms a covalent bond with it; the binding to myosin and the accompanying inhibition of ATPase activity are prevented by ATP. On proteolytic digestion of the labelled myosin the label is found in the N-terminal 25K fragment of the molecule, which is separated by a sequence of at least 51K (6) from the fragment that contains SH-1 and SH-2.

EXPERIMENTAL PROCEDURES

Preparations: Myosin and T-HMM were prepared as described by Balint et al. (2) and C-HMM according to Weeds and Taylor (7). The photoaffinity label analog of ATP was prepared according to Jeng and Guillory (5) on the basis of previously published procedures for the preparation of N-(4-azido-2-nitrophenyl)-β-alanine (8) and utilizing carbodiimidazole (9) in coupling it to ATP. In the synthesis of the ^3H-compound, 20 µg of [2,3-^3H]ATP (ICN), 41.6 Ci/mmol, was mixed with 10 mg of unlabelled Na-ATP before coupling, and the ethanol in which the radioactive material had been dissolved was removed by lyophilization.

Photoaffinity labelling: To 3 ml of a T-HMM or C-HMM solution (6 mg/ml) containing 40 mM KCl, 5 mM imidazole HCl, pH 7.0, 0.1 g (filtered, wet wt.) of Dowex 2X8 was added while N_2 was passed over the solution to prevent oxidation of -SH groups. We found that the use of Dowex was more convenient than charcoal (cf. ref. 5). After removal of Dowex by filtration 0.3 ml of the filtrate was placed in a semimicro cuvette, light path 1 cm, and $N_3NpApr[^3H]ATP$ was added. Its molar ratio to myosin heads was usually about 5:1 (M_r for HMM was taken as 3.6×10^5). The best results were obtained if the label was added in several smaller portions (1.5-5.0 µl), each addition being followed by irradiation with ultraviolet light (200 W, distance 15 cm, 0-4°). Excess label was removed by adding 0.1 g (wet wt. after filtration) of Dowex 2X8 to the cuvette. After 20-30 min. stirring with a magnetic stirrer while N_2 was being passed over the solution, the solution was separated from Dowex with the use of a Pasteur pipet containing a small absorbent cotton plug. All operations were carried out at 2-5°.

ATPase activity was measured in a solution, total volume 4.0 ml, containing 10 mM $CaCl_2$, 50 mM imidazole, pH 7, 50 mM KCl, 1 mM ATP and 40-100 µg of protein, 20°. The incubation time was three minutes and the liberated phosphate determined by the method of Fiske and SubbaRow (10). Protein was determined according to Itzhaki and Gill (11).

Digestion of labelled samples with trypsin: The digestion was carried out in a solution (total volume 0.2-0.25 ml) containing 40 mM KCl, 15 mM imidazole HCl, pH 7.0 and 2 mM EDTA. Sigma type III trypsin was added at a trypsin/ protein ratio of 1:100 (w/w). Samples were taken at various times and treated with an equal volume of a solution containing 2% Na-dodecyl SO_4, 2% mercaptoethanol and 30% glycerol and kept for 2 min at 70°. This procedure is adequate for the inactivation of trypsin but it is mild enough not to hydrolyze the label. Polyacrylamide gel electrophoresis in the presence of Na dodecyl SO_4 was carried

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** Abbreviations used: $N_3NpApr[^3H]ATP$, 3'O-3-[N-(4-azido-2-nitrophenyl) amino] propionyl-adenosine 5'-triphosphate (arylazido-β-alanine ATP); HMM, heavy meromyosin; T-HMM, tryptic heavy meromyosin; C-HMM, chymotryptic heavy meromyosin; SDS, sodium dodecyl sulfate.

out according to Weber and Osborn (12) except that the concentration of phosphate buffer was 30 mM instead of the originally suggested 100 mM. Gels were stained with Coomassie blue.

Distribution of radioaffinity on gels: For determination of the distribution of radioactivity one of two procedures was used: (i) Two identical samples were placed on two gels and electrophoresed simultaneously. One gel was first stained and then destained; the other gel was left unstained. The unstained gel was cut into 2 mm slices and the radioactivity of the slices was counted as described below; or ii) Only one sample was used and the stained gel itself cut into segments that somewhat varied in size; however, the cuts were made so that stained bands stayed within one segment. In this procedure the pieces were weighed, the sum of the weights obtained and the length of each piece taken to be proportional to the ratio of its weight to the total.

The gel pieces were placed in counting vials to which 0.5 ml of Soluene (Packard) was added. The vials were kept at 45° for 2-3 hours. This procedure rendered the gel pieces first opaque and then transparent but they did not go into solution. However, it was found that the labelled ATP did pass into solution. After cooling to 25° the solution was neutralized by the dropwise addition of glacial acetic acid and 10 ml of scintillation solution containing 6 g of diphenyloxazole and 0.1 g of p-bis[2-(5-phenyloxazolyl)]-benzene in 1 liter of toluene were added. Radioactivity was determined in a Beckman LC-100 scintillation spectrometer.

RESULTS

Table I illustrates the effect of photoaffinity labelling on the activity of HMM. The ATP analog was added in three portions, each addition followed by incubation in the dark and then irradiation. This technique was found to be preferable to adding the total amount at once. If the analog was added without irradiation no inhibition of ATPase activity was observed. Similarly, irradiation in the absence of the analog did not produce inhibition. The analog itself, if not irradiated, could serve as a substrate, although its rate of hydrolysis was about 1/10 that of ATP. To determine whether reaction with the photoaffinity label was specific we examined the effect of ATP upon it. As shown in Table I, the presence of 5 mol of ATP per mole of myosin head almost completely abolishes incorporation of the photoaffinity label and prevents the inhibition of ATPase produced by photoaffinity labelling. The specific character of the reaction with the photoaffinity label is also shown by the fact that the ratio of the fraction of sites reacted and the inhibition of ATPase activity is close to 1.

Photoaffinity labelled tryptic and chymotryptic HMM was subjected to further tryptic proteolysis to see if the fragment(s) containing the SH-1 and SH-2 groups could be identified. Tryptic HMM contained 140-150K, 80K, 74K and 51K

TABLE I

Effect of Photoaffinity Labelling on ATPase Activity

Mol of label/ mol of HMM	Inhibition of ATPase Activity		Incorporation	
	-ATP	+ATP	-ATP	+ATP
	%	%	%	%
3	30	3	24	3
6	45	0	46	4
9	57	2	49	4

To 0.3 ml of a solution of chymotryptic HMM (6 mg/ml) containing 40 mM KCl, 5 mM imidazole HCl, pH 7.0, the ATP analog $N_3NpApr[^3H]ATP$ was added in the total amounts indicated. The actual addition took place in three equal steps, 5 µl vol. each. After each addition incubation for 1 min in the dark was followed by irradiation for 1.5 min (200 W UV lamp at a distance of 15 cm). The temperature of the samples was kept between 0 and 4°C. Unreacted label was removed as described in Experimental Procedures. In the experiments with ATP 5 mol were added per mol of HMM. The % of incorporation was calculated from the specific radioactivity assuming that there was one binding site per myosin head. Counts were corrected for radioactivity in controls that were not illuminated. ATPase activity of untreated HMM, determined at 20°, was 0.98 µmol min^{-1} mg^{-1}. For measurements of radioactivity 20 µl samples were mixed with 0.5 ml of Soluene; after adjusting the pH to neutrality with the use of an indicator paper by the dropwise addition of glacial acetic acid 10 ml of the counting mixture used for counting of gels was added. The procedure was standardized by counting 20 µl of a 100-fold diluted stock solution of the ATP analog in the Soluene system. Overall counting efficiency was 25%.

peptides and a smaller peptide, 18K, probably a light chain (Fig. 1). Radioactivity was chiefly present in the 74K peptide which had been previously identified as the N-terminal one (2). Radioactivity was also present in the 140K fragment, presumably the heavy chain moiety of HMM. Some radioactivity appeared in the 18K peptide too. In the course of digestion the previously described (2) peptides with M_r of 60K, 37K, 28K and 21K appeared. As shown in Fig. 2, the radioactivity passed through the 74K peptide to the 25K peptide. The 18K peptide disappeared in the course of digestion. It should be noted that no significant amount of radioactivity appeared in the other peptides including the 21K peptide, which has been identified as containing the SH-1 and SH-2 thiol groups (3). Chymotryptic HMM showed essentially the same pattern except that initially more radioactivity was present in the 140-150K band (Fig. 3A). This is consistent with the known fact (7) that chymotryptic digestion leaves a larger fraction of

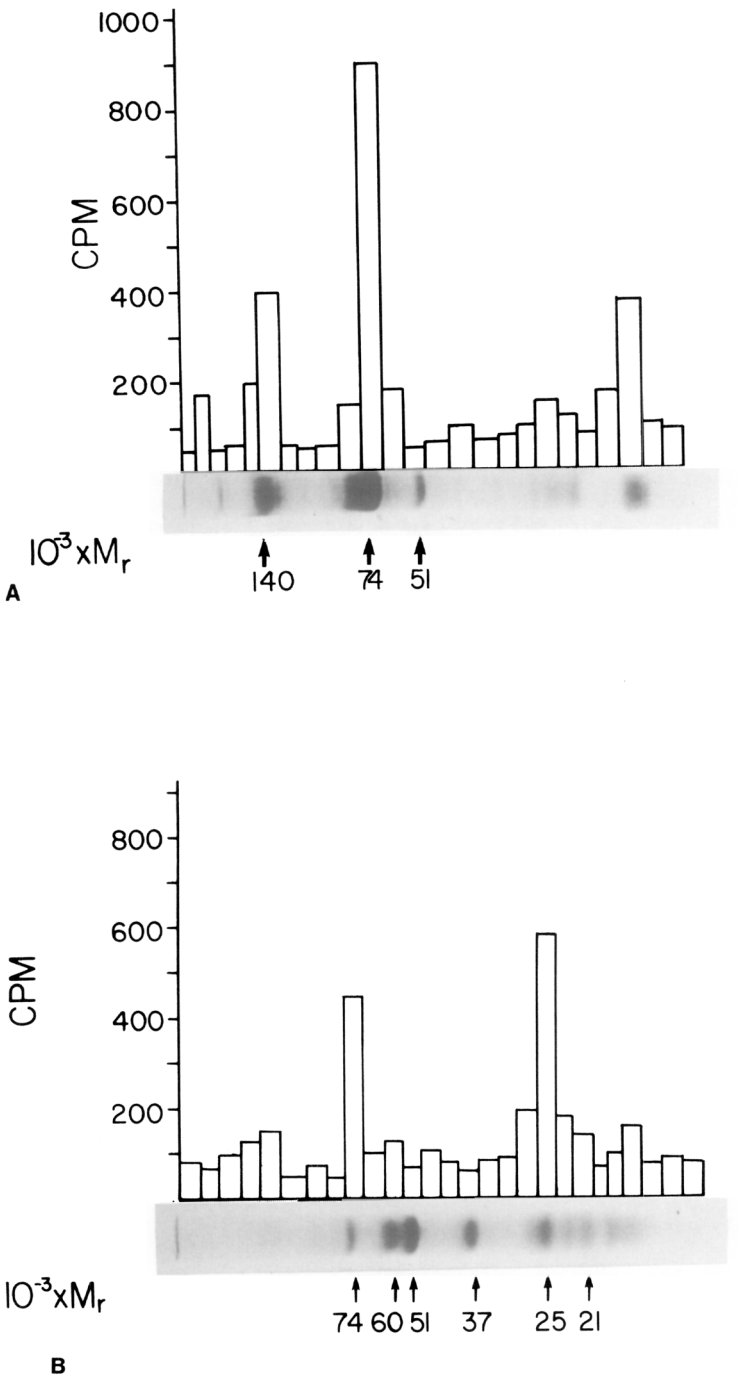
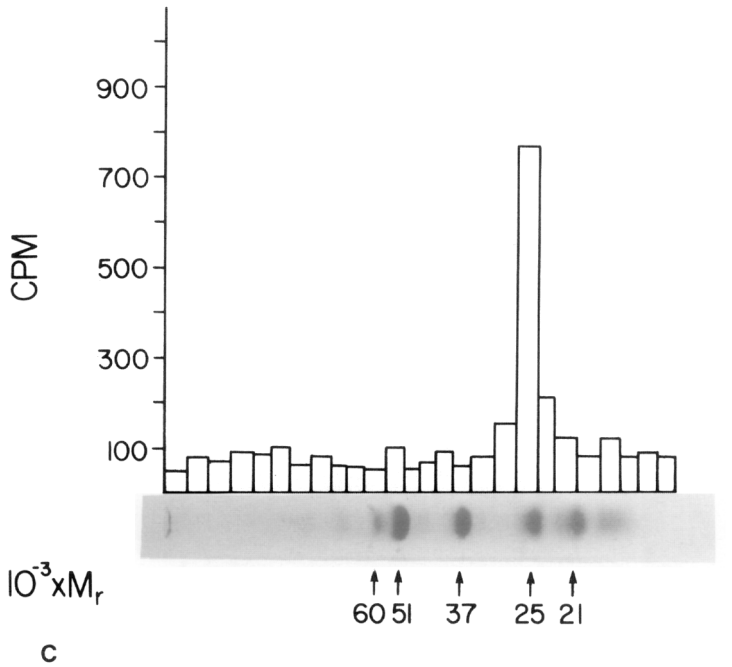


Fig. 1. SDS-gel electrophoresis of tryptic digests of photoaffinity-labelled tryptic HMM. The amount of protein applied to each gel corresponded to 60 µg of the original HMM. Photoaffinity labelling was carried out by adding the photoaffinity label $N_3NpApr[^3H]ATP$ in a 6:1 molar ratio as



described in the legend to Table I in three equal portions. A) T-HMM; B) 10 min tryptic digest of T-HMM; C) 30 min tryptic digestion of T-HMM. The distribution of the radioactivity in the gels was determined with the use of procedure (ii) (see Experimental Procedures).

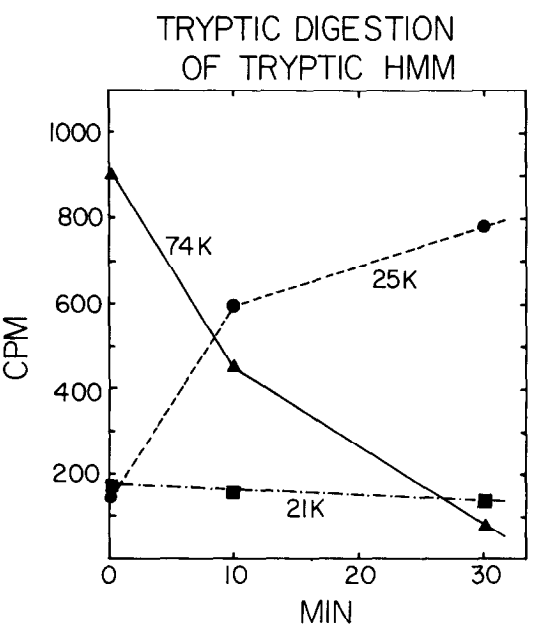


Fig. 2. Time course of incorporation of radioactivity into tryptic fragments of photoaffinity labelled tryptic HMM. The data shown in Fig. 1 were used.

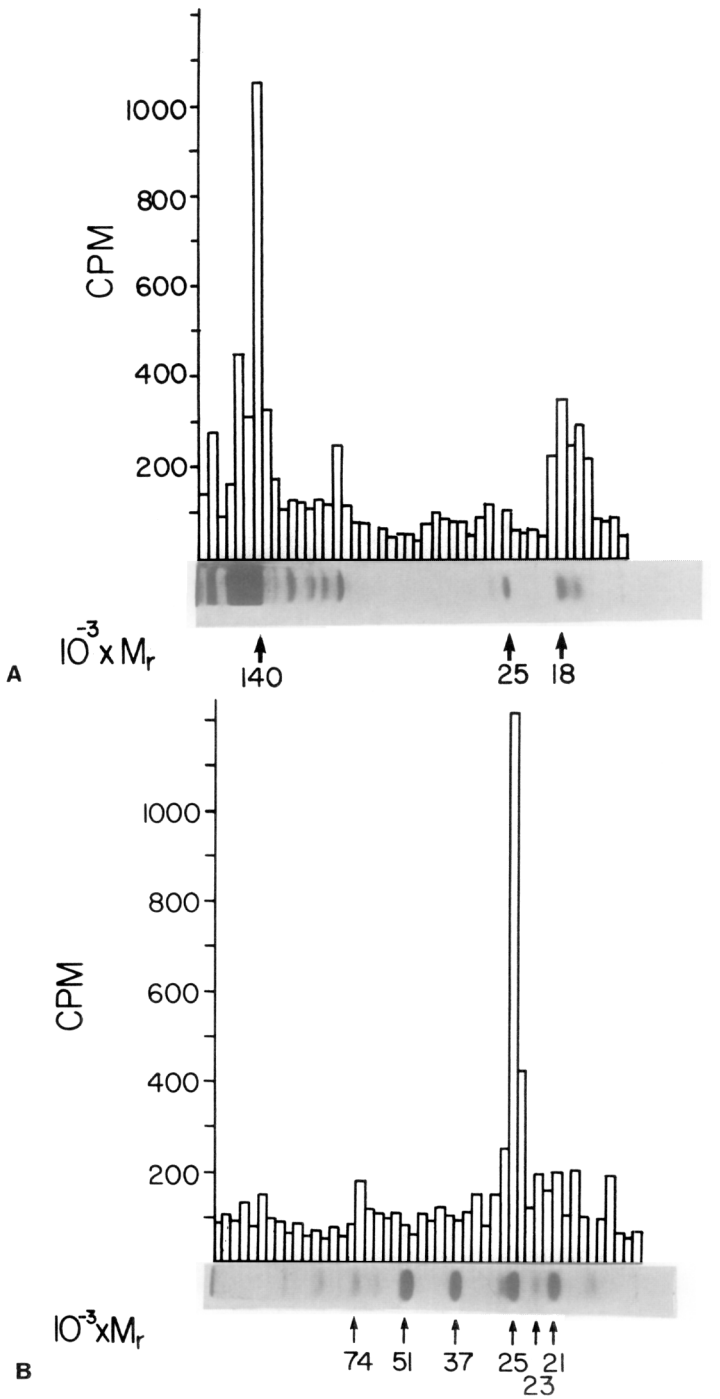
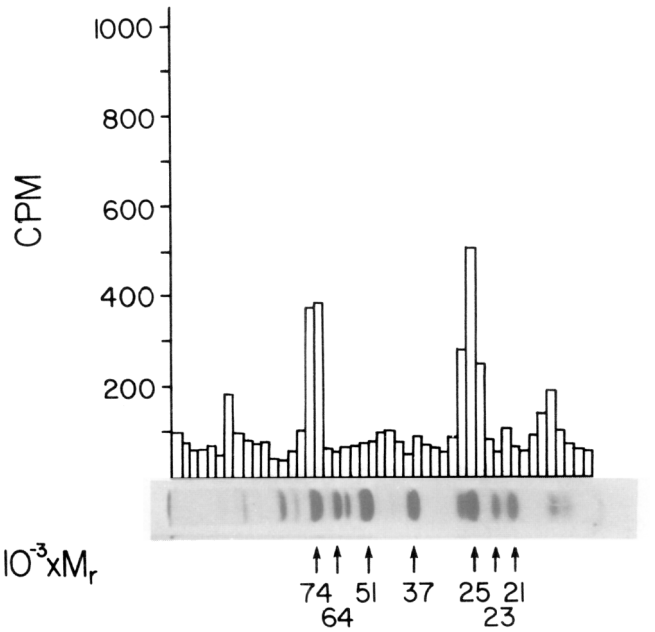


Fig. 3. SDS gel electrophoresis of tryptic digests of photoaffinity labelled chymotryptic HMM. The amount of material applied to each gel corresponded to 40 µg in the original HMM. Photoaffinity labelling was carried out with 7.2 mol of N_3NpApr[^{3}H]ATP per mol of HMM divided into



three equal portions (see legend to Table I). A) C-HMM; B) 10 min tryptic digest of C-HMM; C) 30 min tryptic digest of C-HMM. The distribution of the radioactivity in the gels was determined with the use of procedure (i) (see Experimental Procedures).

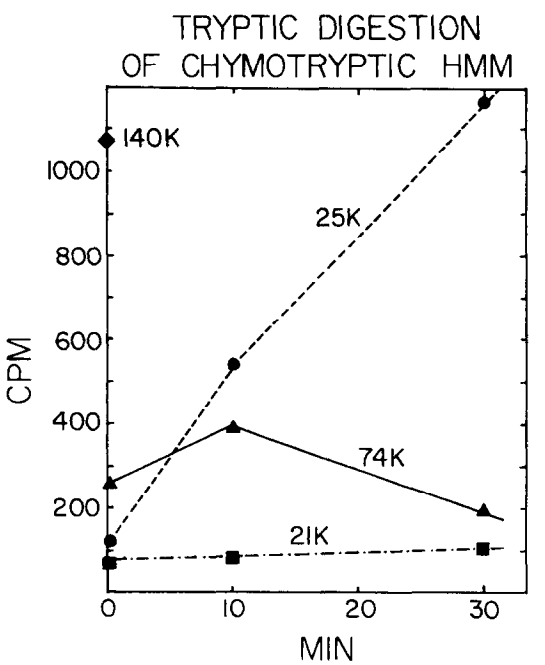


Fig. 4. Time course of incorporation of radioactivity into tryptic fragments of photoaffinity labelled chymotryptic HMM. The data shown in Fig. 3 were used.

the heavy chain intact in HMM. Upon tryptic digestion of chymotryptic HMM the radioactivity again ended up in the 25K fragment, with essentially none in the 21K peptide.

DISCUSSION

The results presented in this paper show that the azido derivative of ATP first used by Jeng and Guillory (5), $N_3N_pApr[^3H]ATP$, reacts with myosin upon irradiation in a specific manner. This is evidenced by the essentially 1:1 relation between incorporation of the radioactive analogue and ATPase inhibition and the prevention of inhibition by adding ATP. Upon tryptic digestion of chymotryptic or tryptic HMM the radioactivity was eventually found in the 25K peptide. This peptide has previously been assigned to the N-terminal half of the myosin heavy chain (2) and has recently been identified as containing the blocked N-terminus of the chain (6). No radioactivity appeared in the 21K fragment which contains the two reactive sulfhydryl groups (3) as well as the 3-methylhistidine residue (6). Recent work from our laboratories (6) indicates that the fragment containing the sulfhydryl groups whose participation in the ATP binding site has been suggested by various lines of evidence (3,4) is separated from the N-terminal 25K fragment, which now has been shown to interact with the photoaffinity analogue of ATP, by a stretch of about 50,000 daltons. While the precise mapping of the regions surrounding the ATP bound to myosin will require considerably more work, the present studies point to the possibility of two spatially separated regions of the myosin head being involved in the active site.

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References

1. Szilagyi, L., Balint, M., Sreter, F.A. and Gergely, J., Fed. Proc. (1978) 37, 1695.

2. Balint, M., Sreter, F.A., Wolf, I., Nagy, B. and Gergely, J. (1975) J. Biol. Chem. 250, 6168-6177.
3. Balint, M., Wolf, I., Tarcsafalvi, A., Gergely, J. and Sreter, F. (1978) Arch. Biochem. Biophys. 190, 793-799.
4. Elzinga, M. and Collins, J.H., Proc. Natl. Acad. Sci. (U.S.) 74, 4281-4284.
5. Jeng, S.J. and Guillory, R.J. (1975) J. Supramolec. Struct. 3, 448-468.
6. Lu, R., Sosinski, J., Balint, M. and Sreter, F. (1978) Fed. Proc. 37, 1695.
7. Weeds, A.G. and Taylor, R.S. (1975) Nature 257, 54-56.
8. Fleet, G.W.J., Knowles, J.R. and Porter, R.K. (1972) Biochem. J. 128, 499-508.
9. Gottikh, B.T., Krayevsky, A.A., Tarussova, N.B., Purygin, P.P. and Tsilevich, J.L. (1970) Tetrahedron 26, 4419-4433.
10. Fiske, H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375-400.
11. Itzhaki, R.F. and Gill, D.M. (1964) Anal. Biochem. 9, 401-410.
12. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.