Number and Location of Adenosine Triphosphatase Sites of Myosin*

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ABSTRACT: An analog of adenosine triphosphate (ATP), 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP), has been used as a chromophoric probe and as an affinity label of the ATPase sites of myosin. SH-TP is a substrate for myosin, and causes contraction of both myofibrils and glycerinated muscle fibers. When rapidly mixed with myosin, SH-TP exhibits transient decrease in the intensity of its 322-mμ absorption band; this change has been employed to estimate that the number of nucleoside triphosphate binding sites is two. At pH 8.2, after 48 hr at 0°, myosin incubated with SH-TP retains less than 5% of its original ATPase activity. Inactivation occurs by affinity labeling since (1) ATP exerts a protecting effect, (2) the monophosphate of SH-TP does not inactivate the enzyme, and (3) the number of moles of SH-TP per mole of myosin released by mercaptoethanol reduction is about 2.

Fractionation of the labeled myosin into light and heavy subunits shows that only the light subunit is labeled; the active site is therefore located at least partially on the light subunit. Reduction of the labeled light subunit with β-mercaptoethanol releases the expected 1 mole of 6-mercaptopurine riboside/mole of light subunit, suggesting that covalent bond formation occurs *via* disulfide formation with a cysteine residue.

Synthesis of 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP,[1] Murphy *et al.*, 1970) makes possible two new kinds of experiments on myosin and other ATPases: (a) The peak of the absorbance of SH-TP, at 322 mμ, is sufficiently separated from the peak of the protein absorbance to make the analog a potential "chromophoric probe" of active sites, and (b) the SH group can undergo nucleophilic displacement or mixed-disulfide formation with a protein amino acid side chain, so that SH-TP is potentially an "affinity label" (Singer, 1967) for active sites. Both of these possibilities have been realized in the present work—the former by working at a pH well below that of the mercaptopurine pK'_a for a short time, and the latter by working at a pH somewhat above, for a long time. Use of SH-TP as a chromophoric probe has provided good evidence for the duplex nature of myosin, while its use as an affinity label has located the ATPase sites among the subunits of myosin. The present use of SH-TP as an affinity label was foreshadowed by Brox and Hampton (1968), who reacted SH-MP with guanosine 5′-phosphate reductase, forming thereby a disulfide bond at the active site.

Materials and Methods

SH-TP was synthesized from the barium salt of the monophosphate, as described previously (Murphy *et al.*, 1970). For affinity labeling, a freshly prepared solution of rabbit myosin, mol wt ~ 4.8×10^5 g mole^{-1} (Tonomura *et al.*, 1966), was first dialyzed for at least 24 hr against 0.5 M NaCl–0.1 M $NaHCO_3$ (pH 8.2) at 0° (replacement of potassium by sodium ions sharply reduces triphosphatase activity of myosin). To 2 ml of 2% myosin (0.083 μmole) in this solvent was added 10.3 mg (16.6 μmoles) of SH-TP. After 48 hr at 0°, the triphosphatase activity was less than 5% that of a control sample. Part of this product was dialyzed five times against 2 l. of 0.6 M KCl–0.05 M phosphate (pH 6.5) at 4° for 24 hr each time. So-called "light subunit" was fractionated at 4° from the product by the technique of Gershman *et al.* (1966). The pH of the solution was raised to 11 by the addition of 1 M NaOH; after 10 min, the solution was neutralized and diluted by adding it to 35 ml of 0.02 M NaH_2PO_4. Centrifugation at 10,000*g* for 30 min yielded a clear supernatant and a precipitate, both of which were dialyzed against 0.6 M KCl–0.05 M phosphate (pH 6.5, 2 l.) for 24 hr; this dialysis was repeated four times. Since the resulting polypeptides were incompletely dissolved, urea (Ultra Pure, Schwarz BioResearch) was added to 8 M.

Myosin was also incubated with a 50-fold excess of SH-TP, in the presence and absence of a 200-fold excess of ATP and with a 200-fold excess of SH-MP. Protein concentrations were determined by a modified Lowry procedure (Gellert *et al.*, 1959). Triphosphatase activities were measured in 0.6 M KCl–0.05 M Tris buffer–0.01 M calcium (pH 8.0) at 25°, by measuring orthophosphate in sequential aliquots of reaction mixture (Ouellet *et al.*, 1952).

Tension developed on a thin bundle of glycerol-extracted *psoas* fibers was recorded as the electrical output from a strain gauge tensiometer. Contraction of myofibrils was observed under the low power of a microscope.

Transmittance changes at 320 mμ resulting from the rapid mixing of SH-TP and myosin were measured from photographs of memory oscilloscope traces generated by a Gibson-Durrum stopped-flow apparatus. The transmittance changes

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[1] Abbreviations used are: SH-MP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate; SH-TP, the triphosphate of SH-MP.

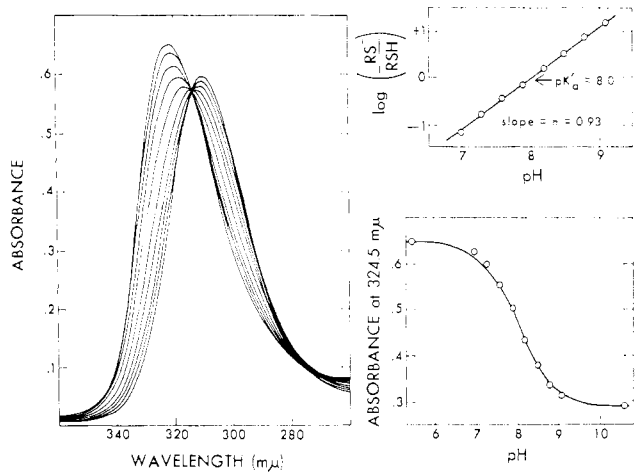

FIGURE 1: Spectral studies. Left: spectra of SH-TP at various pH values. SH-TP concentration, 28 μM; 10-mm cuvet; ionic strength, 0.1; temperature, 25°. pH: (*reading downward* at 324.5 mμ) 5.5, 7.0, 7.3, 7.6, 7.9, 8.2, 8.5, 8.8, 9.1, 10.6. Lower right: absorbance at 324.5 mμ *vs.* pH of SH-TP. Solid line is a theoretical curve, assuming a pK'_a of 8.0 and $n = 1$. Upper right: Hill plot of the above data.

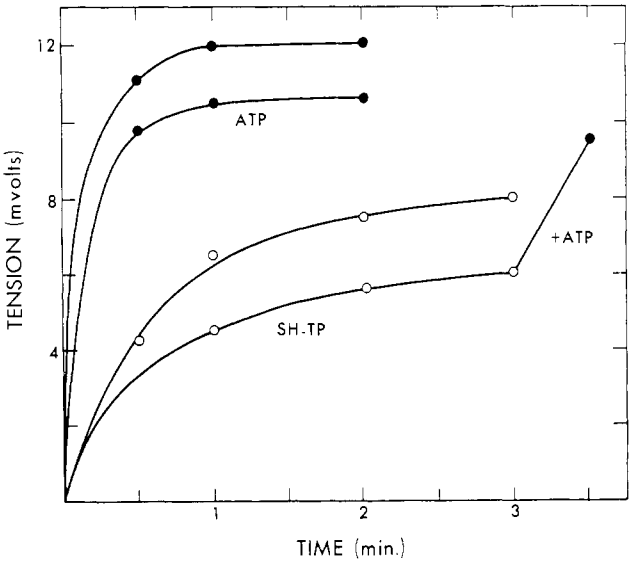

FIGURE 2: Time course of the development of tension on glycerinated muscle fibers. Buffer 0.08 M KCl–10^{-5} M Ca–1 mM Mg–0.02 M Tris, pH 7; 1 mM nucleoside triphosphate; temperature 25°.

obtained at various ligand and protein concentrations were converted into optical density units and treated as follows.

Let Δ be the optical density of the protein (concentration, (P_t)) and analog (concentration, (A_t)) after binding is at equilibrium, minus the optical density when the two are mixed but are not binding. The former is obtained from the transmittance measured immediately after mixing (before any hydrolysis) and the latter from the transmittance after all the triphosphate has been converted into nonbinding diphosphate (see Figure 3). The concentration of bound analog, (A_b), is given by $\Delta/\Delta\epsilon$, where $\Delta\epsilon$ is the difference in molar extinction coefficients between free and bound analog. Keeping (P_t) fixed

$$\lim_{(A_t)\to\infty} \frac{\Delta}{\Delta\epsilon} \equiv \frac{\Delta_{max}}{\Delta\epsilon} = n(P_t)$$

where n is the valence of protein for analog. From the second equation, $e \equiv n\Delta\epsilon = \Delta_{max}/(P_t)$. Knowing e we may henceforth write $(A_b) = n\Delta/e$, so the mass law gives for the dissociation constant

$$K_d = \frac{((A_t) - n\Delta/e)\,(n(P_t) - n\Delta/e)}{n\Delta/e}$$

This equation can be rearranged into

$$(A_t)\left(\frac{(P_t)}{\Delta/e} - 1\right) = n\left((P_t) - \frac{\Delta}{e}\right) + K_d$$

Thus in a plot of $(A_t)(((P_t)/\Delta/e) - 1)$ *vs.* $((P_t) - (\Delta/e))$ for values of (A_t) and (P_t), the slope is n and the intercept on the vertical axis is K_d.

Results

SH-TP as Substrate and Chromophoric Probe. The change with pH of the absorption spectrum of SH-TP is illustrated in Figure 1. A plot of the change *vs.* pH at 324.5 mμ yields a typical sigmoid curve, with a midpoint at 8.0. Along with a Hill plot, the curve suggests that the purine ionization is a simple, one-step transition, unaffected by the ionization occurring in the triphosphate portion of the molecule. It may be noted, however, that the ionization constant of 8.0 is significantly higher than the pK'_a of 7.6 obtained spectrophotometrically for thioinosine (Fox *et al.*, 1958); the difference is presumably due to the presence of the ionized phosphates in SH-TP.

With SH-TP (0.5 mM) as the substrate, at pH 8.0, 0.6 M KCl–0.05 M Tris–0.01 M calcium, 25°, myosin catalyzes the

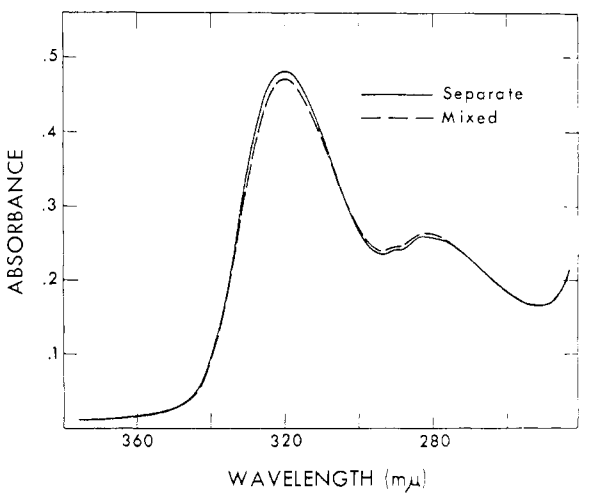

FIGURE 3: Ultraviolet absorption spectra of myosin and SH-TP. Original concentrations: myosin *ca.* 1.5×10^{-6} M; SH-TP, *ca.* 4×10^{-5} M; path lengths, 5 mm. Final concentrations: myosin, *ca.* 0.8×10^{-6} M; SH-TP, *ca.* 1×10^{-5} M; path length, 10 mm. Buffer 0.5 M NaCl–0.1 M orthophosphate, pH 6.5; temperature, 25°. (——) Spectrum before mixing and 20 min after mixing; (----) spectrum *ca.* 2 min after mixing.

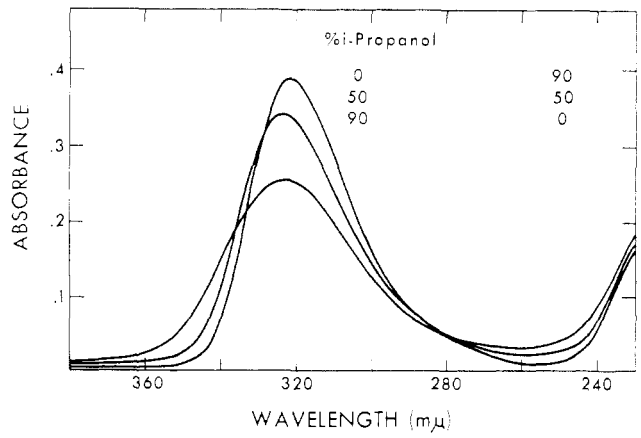

FIGURE 4: Ultraviolet absorption spectra of SH-MP in various concentrations 2-propanol. Path length, 10 mm. Buffer 0.01 M sodium acetate, pH 4.5; temperature, 25°.

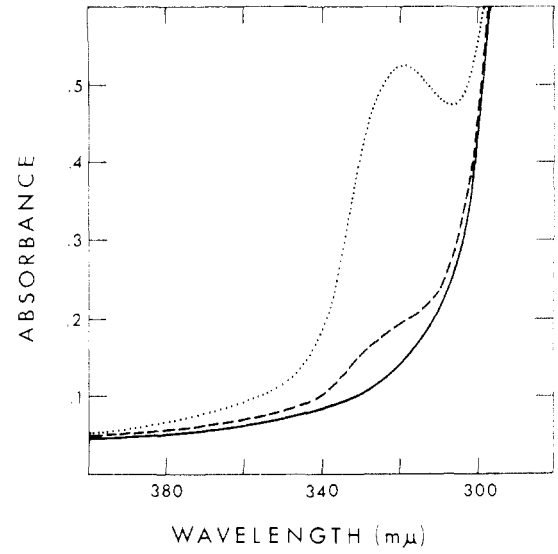

FIGURE 6: Absorption spectra of SH-TP-myosin. (——) Myosin incubated with ATP; (---) SH-TP-myosin; (· · · ·) SH-TP-myosin plus 5 μmoles of mercaptoethanol for 15 min. Protein concentration, 3.0 mg/ml; path length, 10 mm. Buffer 0.6 M KCl–0.05 M orthophosphate, pH 6.5; temperature, 25°.

release of orthophosphate at a V_{max} which is 140% that observed with ATP. It produces shortening of myofibrils, as observed by allowing a solution to diffuse into a myofibrillar suspension under a light microscope. Glycerinated *psoas* fibers, when placed in a solution containing SH-TP, develop tension (Figure 2), though neither as fast nor to the same extent as does ATP.

The spectrum of an actual mixture of myosin and SH-TP is transiently different from the optically added spectrum of myosin and SH-TP, especially near 320 and 280 mμ (Figure 3). The lesser absorbance near 320 mμ can be simulated by dissolving SH-MP in solvents of increasing 2-propanol content (Figure 4). Because the spectral differences are transient, and disappear in a time which is inversely proportional to the myosin concentration, one can suppose that only the triphosphate substrate, and not the diphosphate product, binds appreciably to myosin. This suggests the feasibility of a substrate binding analysis, provided the optical change is measured rapidly enough (in a stopped-flow apparatus), so that hydrolysis is negligible. In Figure 5 changes in transmission at 320 mμ, as a function of [SH-TP] are plotted according to the equation derived in the experimental section, which assumes that the adsorbent has n equivalent binding sites, each with dissociation constant K_d. The best-fitting straight line shows that $n = 1.8$ and $K_d = 2.5 \times 10^{-6}$ M.[2]

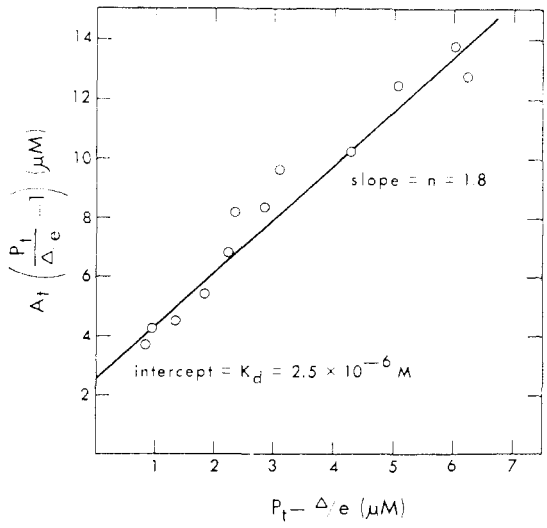

FIGURE 5: Graphical determination of the dissociation constant and number of binding sites of the myosin–SH TP system (for explanation of symbols, see Materials and Methods section). Buffer 0.5 M NaCl–0.1 M orthophosphate, pH 6.5; temperature, 25°.

SH-TP as an Affinity Label. Incubation with a 200-fold excess of SH-TP at 0° for 48 hr is sufficient to reduce the ATPase activity of myosin to less than 5% that of control. Under the same conditions, but with only a 50-fold excess of the analog, the rate of ATP hydrolysis is reduced to 40%; in the presence of a 50-fold excess of analog and a 200-fold excess of ATP, the ATPase decreases only to 90% that of the original myosin. This protecting effect of ATP, and the fact that SH-MP does not decrease ATPase activity, suggest that the inhibitory behavior of SH-TP is a reflection of a specific reaction at the active site of myosin. The stoichiometry of the labeling confirms this inference; the spectra of SH-TP myosin before and after reduction (Figure 6) can be used to calculate the amount of SH-TP bound covalently to myosin. Based on ϵ_{322} 23,100 (Hampton and Maguire, 1961), the difference at 322 mμ corresponds to 2.3 moles of SH-TP/mole of myosin.

To learn whether the labeling is specific for one or the other of the subunits of myosin, spectra were recorded before and after mercaptoethanol reduction of the separated subunits prepared from labeled myosin. The spectrum of the light subunit from myosin inactivated with SH-TP is very different

[2] Several interesting attempts to measure n using molecules similar to ATP (*e.g.*, ADP), or using ATP itself and correcting for hydrolysis, have previously been made by various authors. Such studies have led to estimates ranging from one to three. The present work, however, is the first to use a true chromophoric probe, and this feature plus the use of rapid mixing, should provide a more accurate estimate.

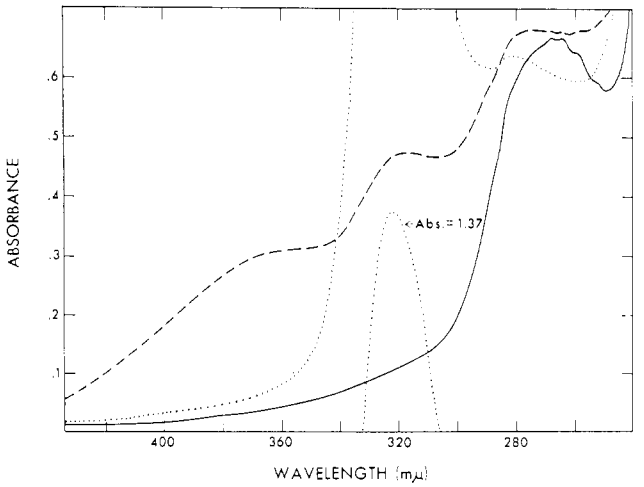

FIGURE 7: Absorption spectra of the light subunit of myosin. (——) Light subunit obtained from myosin incubated with ATP; (---) light subunit of SH-TP–myosin; (· · · ·) light subunit of SH-TP myosin plus 5 μmoles of mercaptoethanol for 15 min. Protein concentration, 0.88 mg/ml; path length, 10 mm. Buffer 0.6 M KCl–0.05 M orthophosphate (pH 6.5)–8 M urea; temperature, 25°.

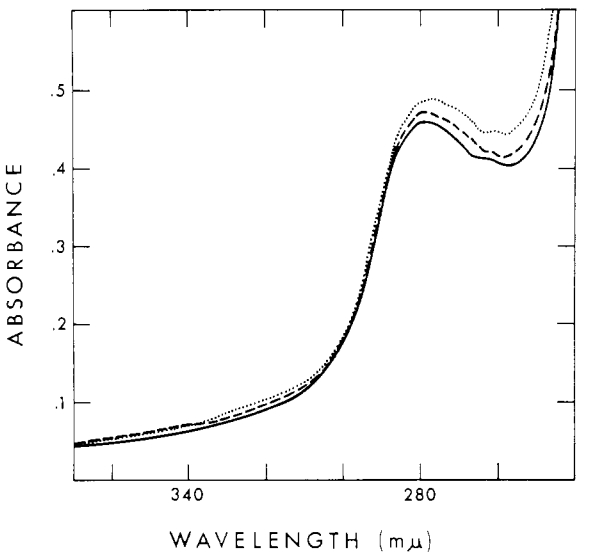

FIGURE 8: Absorption spectra of the heavy subunit of myosin. (——) Heavy subunit obtained from myosin incubated with ATP; (---) heavy subunit of SH-TP myosin; (· · · ·) heavy subunit of SH-TP myosin plus 5 μmoles of mercaptoethanol. Protein concentration, 0.84 mg/ml; path length, 10 mm. Buffer 0.5 M KCl–0.05 M orthophosphate (pH 6.5)–8 M urea; temperature, 25°.

in the 200–380-mμ range from that of (control) myosin incubated with ATP (Figure 7). A similar spectrum can be obtained when SH-TP is incubated at pH 8.2 with a 20-fold excess of cysteine ethyl ester, with cupric ion present (concentration 0.1 that of SH-TP) to catalyze the oxidation. The spectrum obtained after the addition of mercaptoethanol to the solution of light subunit (Figure 7) shows the absorbance centered near 360 mμ to be much decreased, and that at 320 mμ greatly increased. The amount of SH-TP released by the mercaptoethanol treatment is estimated from the absorbance difference at 322 mμ to be 0.9 or 1.3 moles per mole of light subunit, depending upon whether the molecular weight is accepted as being 20,000 (Gershman *et al.*, 1966), or 30,000 (Kominz *et al.*, 1959; Frederiksen and Holtzer, 1968), respectively.

When a comparison of the spectra of the heavy subunits obtained from labeled and control myosin is made (Figure 8), it can be seen that the differences are minor, and their significance is questionable, since this fraction is conceded to be contaminated by light subunit (Kominz *et al.*, 1959; Frederiksen and Holtzer, 1968). Reduction with mercaptoethanol results in but a small increase in absorbance (Figure 8), which at 322 mμ is calculated to represent less than 0.1 mole of SH-TP/mole of heavy subunit.

Discussion

The existence of a single, unperturbed, and spectrophotometrically detectable ionization in the physiological pH range suggests general utility of SH-TP as a chromophoric probe for nucleoside triphosphate sites on proteins, since it can be expected that the pK'_a will be sensitive to protein–ligand interactions. For example, preliminary experiments in this laboratory show that the pK'_a of SH-TP incorporated into F-actin is apparently the same as that of the analog in the absence of the protein, *viz.*, 8.0, implying that the purine of the nucleotide does not interact appreciably with the protein.

That SH-TP is a substrate for myosin is not surprising in view of the fact that other nucleoside triphosphates, indeed, compounds as simple as tripolyphosphate (Friess and Morales, 1955) and methyl triphosphate (Lecocq, 1968), are hydrolyzed by myosin. The contraction of myofibrils and the tension developed by muscle fibers in the presence of SH-TP is, however, more distinctive. Methyl triphosphate, for example, does not support these processes; in fact, the 2′,3′-*O*-isopropylidene derivative of SH-TP has been reported to be incapable of causing contraction of myofibrils (Tonomura *et al.*, 1967). 2′,3′-*O*-isopropylidene ATP, on the other hand, like SH-TP, does support myofibrillar shortening (Tonomura *et al.*, 1967), so it would seem that only in the absence of both the 6-amino and the 2′- and 3′-hydroxyls is the chemical-to-mechanical energy transduction seriously impaired.

The hypochromicity of the spectrum of SH-TP bound to myosin (Figure 3) and the analogous decrease observed in 2-propanol (Figure 4) suggest primarily hydrophobic binding of the purine moiety. The parallel hyperchromicity in the 280-mμ region may be attributable to displacement into the solvent of aromatic residues. Morita and Yagi (1966) report that pyrophosphate can cause an intensity increase around 280 mμ, so that it is probably the polyphosphate portion of the nucleotide which displaces aromatic residues.

X-Ray diffraction studies (Lowey and Cohen, 1962) suggest that myosin is two stranded, and electron microscopy studies (Slayter and Lowey, 1967) that it is two headed; our finding that it is two sited completes the evidence that myosin is duplex. Since the data can be fitted satisfactorily by a straight line, there is no need to assume other than that the two ATPase sites are equivalent and noninteracting. The magnitude of the dissociation constant (2.5×10^{-6} M) is quite similar to the

value of the Michaelis constant for the myosin ATPase under slowly hydrolyzing conditions (Blum, 1955). The duplex nature of myosin, which this and other studies have helped to establish, and the finding that there is one molecule of myosin per "cross bridge" (Reedy *et al.*, 1969) raise a teleological question. Assuming that the cross bridge is a form of impeller, duplex structure might be required if myosin moves along actin in "measuring worm" fashion.

The location by affinity labeling with SH-TP of the purine binding site on the small subunit of myosin agrees well with a recent report (Stracher *et al.*, 1969) showing that a lysine which was protected by ATP from chemical modification with trinitrobenzenesulfonate was located on the small subunit. It is likely that the small subunit is located near the site of energy transduction; thus, if the globular S_1 member of myosin impels, then the small subunit should be on S_1, near the S_1–S_2 junction (Huxley, 1969). It may well be that the active site is in fact constituted from the small and large subunits. This possibility is consistent with the report of complete loss of ATPase activity observed when the subunits are separated, and of partial recovery when they are recombined (Stracher, 1969). The bridging of subunits by a substrate has been shown in at least one enzyme, lactic dehydrogenase, wherein two subunits are shown by medium resolution X-ray diffraction to be bridged by NAD (Adams, 1969).

The reappearance of the spectrum of SH-TP upon addition of mercaptoethanol to both the labeled myosin (Figure 6) and the small subunit (Figure 8) shows that coupling to the protein occurs by disulfide formation with a cysteine residue. This residue is possibly the same cysteine the modification of which by *p*-mercuribenzoate eliminates the difference in pH profiles of myosin ATPase and ITPase activities (Rainford *et al.*, 1964), since this sulfhydryl would likely be near position 6 of the purine ring when the substrate is bound. On the other hand, the SH-TP-labeled cysteine may not be the S_1 or S_2 cysteines of myosin (Sekine *et al.*, 1962), since evidence placing these residues on the heavy subunit has recently been reported (Trotta *et al.*, 1968).

The existence of a large number of ATP-utilizing enzymes makes it probable that SH-TP will find widespread use in the elucidation of protein structure. That SH-TP has potential value as an affinity label for proteins other than myosin is suggested by preliminary experiments in this laboratory on creatine kinase, which is completely inactivated by incubation with a tenfold excess of SH-TP.

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