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# Effect of Nucleotides and Pyrophosphate on Spin Labels Bound to $S_1$ Thiol Groups of Myosin*

J. C. Seidel,† M. Chopek,‡ and J. Gergely

ABSTRACT: Titration of myosin with the spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, indicates the presence of two rapidly reacting thiol groups per molecule which are presumably the so-called $S_1$ thiol groups. Labeling of these groups results in the well-known increase in $Ca^{2+}$-activated adenosine triphosphatase activity and loss of $K^+$-activated adenosine triphosphatase characteristic of the reaction of myosin with sulfhydryl modifiers. Strong immobilization of the spin label accompanies this reaction as indicated by electron spin resonance spectra. The addition of adenosine triphosphate, inosine triphosphate, adenosine diphosphate, or pyrophosphate increases the mobility of spin labels bound to $S_1$ groups, the maximum changes in electron spin resonance spectra being obtained in the presence of 2 moles of pyrophosphate/mole of enzyme. The effect of adenosine triphosphate is also observed in the absence of activating divalent or monovalent cations and after all enzymatic activity has been inhibited with *N*-ethylmaleimide. Tryptic digestion of spin-labeled myosin to heavy meromyosin (HMM) or digestion of HMM to subfragment 1 does not alter the electron spin resonance spectrum or the spectral change produced by adenosine triphosphate. The present results suggest that each of two heavy chains of myosin contains one $S_1$ thiol group and one enzymatic site. The change in electron spin resonance spectra produced by adenosine triphosphate is interpreted to represent a change in the structure of myosin near the nitroxide radicals bound to $S_1$ thiol groups. Since adenosine triphosphate produces no gross change in the structure of myosin as measured by optical rotatory dispersion (Gratzer, W. B., and Lowey, S. (1969), *J. Biol. Chem. 244*, 22), it is concluded that the conformational change is confined to a relatively small region of the molecule and that the $S_1$ groups are located close to the adenosine triphosphate binding site.

It is generally believed that there are two classes of thiol groups in myosin whose blocking affects its ATPase activity. Reaction of thiol groups in one class ($S_1$)[1] with reagents such as NEM[2] or *p*-mercuribenzoate results in the increase of the $Ca^{2+}$-stimulated ATPase activity and inhibition of the $K^+$-EDTA activated activity (Kielley and Bradley, 1956). Additional reaction of thiol groups in the second class ($S_2$) leads to inhibition of both types of ATPase activity. Recent work in our laboratory has shown that, notwithstanding these differences between $S_1$ and $S_2$, blocking of either group individually has the same effect on ATPase activity (Seidel, 1969). The technique of spin labeling (Stone *et al.*, 1965; Hamilton and McConnell, 1968) provides a method for probing the environment of the thiol groups involved in these effects and investigating their relationship to the binding and hydrolysis of ATP. The method involves binding of a

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† This work was done during the tenure of an Established Investigatorship of the American Heart Association, Inc.

‡ Part of this work was presented in partial fulfillment of the requirements of the degree A.B. with honors in biochemical sciences, Harvard University.

[1] The $S_1$ thiol groups are those rapidly reacting thiol groups whose modification results in an increase in $Ca^{2+}$-activated ATPase activity and loss of $K^+$-ATPase activity. The $S_2$ groups are those which on modification lead to a loss of $Ca^{2+}$-activated ATPase of $S_1$-modified myosin.

[2] Abbreviations used are: NEM, *N*-ethylmaleimide; HMM, heavy meromyosin; TES, *N*-tris(hydroxymethyl)methyl-2-ethanesulfonic acid.

nitroxide free radical (spin label) to the protein; the electron spin resonance spectrum of the spin-labeled protein reflects changes in the mobility of the bound spin label. The aim of the present study was to spin label the $S_1$ thiol groups of myosin in order to probe for possible substrate induced changes in the structure of myosin.

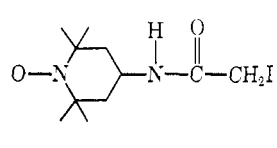
While this work was in progress two reports on spin-labeled myosin appeared. Quinlivan *et al.* (1969) have shown that the electron spin resonance spectrum of myosin labeled with a nitroxide derivative of iodoacetamide indicates the presence of both strongly and weakly immobilized labels, and extensive reaction with *p*-mercuribenzoate increases the weakly immobilized component of the spectrum. Cooke and Morales (1969) found that ATP had no effect on the electron spin resonance spectrum of myosin labeled with a nitroxide analog of NEM.

In this study we have defined conditions for the specific labeling of $S_1$ groups and find that ATP and ATP analogs increase the mobility of spin labels attached to these groups of myosin or its enzymatically active fragments, HMM and subfragment 1.

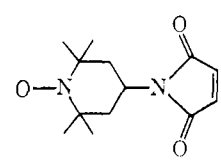
## Methods

Myosin was prepared by the method of Tsao (1953) with EDTA present during all steps of the preparation (Sreter *et al.*, 1966; Nauss *et al.*, 1969). Myosin was stored in 50% saturated ammonium sulfate at −15 to −10° and was dialyzed before use to remove ammonium sulfate. Heavy meromyosin was prepared as described previously (Nauss *et al.*, 1969) by digestion of either native or spin-labeled myosin with trypsin (200:1 myosin to trypsin weight ratio) for 10 min at 25° and purification by ammonium sulfate fractionation. Subfragment 1 was prepared by digestion of spin-labeled HMM with trypsin in a ratio of 15:1 (w/w) for 25 min at 25° and purified by gel filtration on a Sephadex G-200 column (Young *et al.*, 1965; Nauss *et al.*, 1969).

*Synthesis of Spin Labels.* *N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, label A, was synthesized as described by McConnell and Hamilton (1968). *N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, label B, was synthesized according to the procedure for the synthesis of the pyrrolidine analog (Griffith and McConnell, 1966), with slight modifications. Closure of the maleimide ring was accomplished by refluxing in acetic anhydride at 100° for 3 hr. The product (mp 93°) was purified by column chromatography on a silicic acid column with ethyl acetate as eluent. *Anal.* Calcd for $C_{13}H_{19}O_3N_2$: C, 62.13; H, 7.62; N, 11.15. Found: C, 61.74; H, 7.62; N, 10.68.



A



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*Determination of the Kinetics of the Reaction of Spin Labels with Myosin.* The reaction of label A with myosin was carried out in a solution containing 10 mg of myosin/ml, 0.5 M KCl, and 0.02 or 0.04 M Tris at pH 8.0 and the reaction with label B in 0.5 M KCl and 0.02 M TES at pH 7.0. Both reactions were carried out at 0°. The spin labels were dissolved in ethanol and diluted with buffer so that the ethanol concentration in the reaction mixture was 0.1%. For ATPase determinations the reaction was stopped with $10^{-3}$ M 1,4-dithiothreitol. For determination of bound spin label the reaction was stopped with 50% ethanol or 50% saturated ammonium sulfate at 0°, and the mixture was immediately centrifuged. The electron spin resonance spectrum of the supernatant was recorded within 15 min. Under these conditions the spectrum consists of three sharp peaks characteristic of the nitroxide label in aqueous solution (Stone *et al.*, 1965) with no broadening due to immobilization of the label. The amplitude of the peak at the lowest field strength is linearly related to concentration and was chosen as a measure concentration of unbound label. The concentration of bound label was determined by the difference. Electron spin resonance spectra were recorded with a Varian X-band spectrometer at room temperature.

Precipitation of the protein had to be carried out under neutral or alkaline conditions because of the acid lability of the nitroxide radical. The most satisfactory method for the precipitation of myosin was with 50% saturated ammonium sulfate at pH 7.0 and 0°. No further precipitation was obtained by adding trichloroacetic acid (5% v/v, final concentration) to the supernatant solution after ammonium sulfate precipitation, and ammonium sulfate produced no distortion or loss of signal. After precipitation with 50% (v/v) ethanol, a small amount of protein could be precipitated from the supernatant with trichloroacetic acid, particularly if the solution was warmed slightly above 0°. The use of β-mercaptoethanol or 1,4-dithiothreitol to stop the reaction of label with myosin resulted in a substantial loss of the electron spin resonance signal in the time required to measure the spectrum. This loss of signal seems to be related to finding that the nitroxide radical can oxidize thiol groups (Morrisett and Drott, 1969). It appears that the oxidation of thiol groups is accompanied by reduction of the nitroxide to a diamagnetic species. Glutathione (0.1 mM) could be used to stop the reaction if the spectra were recorded within 30 min.

*Spin Labeling of $S_1$ Groups of Myosin and HMM.* After the stoichiometry of the reaction of label A with myosin and HMM was established, the following conditions were routinely used to spin label the $S_1$ groups of myosin or HMM. Myosin (20 mg/ml; $4 \times 10^{-5}$ M) was reacted with $8 \times 10^{-3}$ M label A in 0.45 M KCl and 0.04 M Tris at pH 8.0 and 0° for 40–60 min, followed by dialysis overnight at 4° against 500–2000 volumes of a solution containing 0.5 M KCl, 1 mM TES, and 0.1 mM EDTA (pH 7.0). HMM (20–35 mg/ml) was labeled with a twofold molar excess of label A in 0.04 M Tris (pH 8.0) at 0° for 40–60 min and dialyzed as above against 0.01 M Tris (pH 7.5) containing 0.1 mM EDTA. The molecular weight of HMM was taken as 350,000 (Nauss *et al.*, 1969).

*ATPase activity* was determined by measurement of the released inorganic phosphate according to Fiske and Subbarow (1925). The assay medium contained either 10 mM $CaCl_2$, 5 mM ATP, 0.05 M Tris, and 0.025 or 0.6 M KCl for Ca-activated ATPase or 0.6 M KCl, 5 mM ATP, 5 mM EDTA, and 0.05 M Tris for $K^+$-activated ATPase. Myosin concentration was 0.1 mg/ml, incubations were carried out

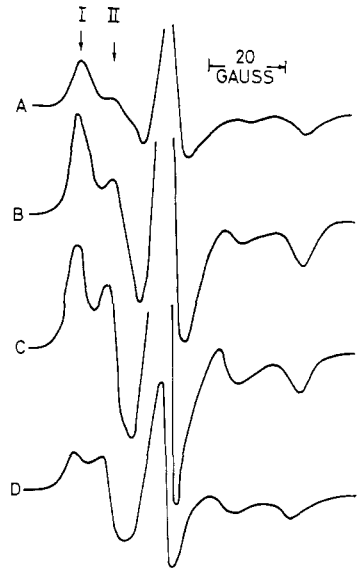


FIGURE 1: Spectra of myosin spin labeled with label A or B at varying ratios of label to myosin. Reaction of myosin (10 mg/ml) with label A orB was carried out in a solution containing 0.5 M KCl and 0.02 M Tris (pH 8.0 for label A or pH 7.0 for label B) for 40 min at 0° and dialyzed overnight against 1000 volumes of a solution containing 0.5 M KCl and 2 mM TES (pH 7.0) at 4°. The spectral peak labeled I arises from labels which are strongly immoblized and the peak labeled II arises from labels which are weakly immobilized. (A) $2 \times 10^{-5}$ M label A (1 mole added per mole of myosin) (B) $5 \times 10^{-5}$ M label A (2.5 moles/mole of myosin), (C) $10^{-4}$ M label A (5 moles/mole of myosin), and (D) $2 \times 10^{-5}$ M label B (1 mole/mole of myosin). Molar ratios refer to the amount of label added.

at pH 7.5 and 25°, and the reaction was stopped by adding an equal volume of 10% trichloroacetic acid.

*Chemicals.* Nucleotides were obtained from P-L Biochemicals and NEM from Calbiochem. 4-Iodoacetamidosalicylic acid was obtained from Nutritional Biochemical Corp. and recrystallized from $H_2O$ before use.

## Results

Myosin was allowed to react with varying concentrations of either label A or B and the electron spin resonance spectra were recorded after removal of unreacted label by dialysis. The electron spin resonance spectra of myosin usually show the presence of both strongly immobilized and weakly immobilized labels. Peaks I and II (Figure 1) correspond to strongly immobilized and weakly immobilized labels, respectively. With addition of 1 mole of label A/mole of myosin the electron spin resonance spectrum indicates that all labels are strongly immobilized (Figure 1). As the ratio of label A to myosin is increased the spectra show both strongly immobilized and weakly immobilized components, and the relative height of the peak corresponding to weakly immobilized labels increases upon increasing the ratio of the label to myosin. The electron spin resonance spectrum of myosin labeled with equimolar concentrations of label B shows both strongly and weakly immobilized labels (Figure 1D). Changes in the ATPase activities of myosin preparations whose spectra are shown in Figure 1 are qualitatively the same as those observed with other thiol reagents (Table I).

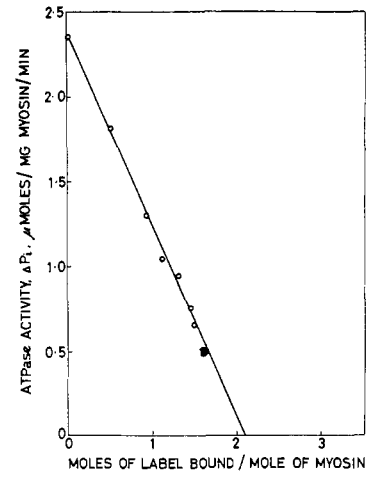


FIGURE 2: Loss of $K^+$-activated ATPase activity and binding of spin label to myosin. Reaction of myosin (10 mg/ml) with label A was carried out in a solution containing 0.5 M KCl, 0.02 M Tris, and $5 \times 10^{-5}$ M label A at pH 7.0 and 0°. Samples were taken for determination of ATPase activity and electron spin resonance spectrum as described in Methods. The last sample was taken 90 min after initiation of the reaction.

However, at the same label to myosin ratios label A produces a greater loss of $K^+$-ATPase and a greater increase in $Ca^{2+}$-ATPase than label B.

The number of $S_1$ thiol groups per mole of myosin can be estimated from the linear loss of $K^+$-activated ATPase activity with increasing amounts of spin label bound to myosin (Figure 2). Extrapolation of this curve indicates that complete loss of $K^+$-ATPase activity requires binding of 2 moles of label A/500,000 g of myosin.

Since the labels which react most rapidly with myosin and produce a change in enzymatic activity are strongly immobilized it appears that the number of $S_1$ thiol groups could also be estimated using the height of the strongly immobilized peak of the electron spin resonance spectrum as an indicator. These experiments were done with HMM

TABLE I: Effect of Spin Labels on ATPase Activities of Myosin.[a]

| Moles of Label Added/ $5 \times 10^5$ g of Myosin | ATPase Act. ($\Delta P_i$, μmoles/mg of myosin min) | | | |
|---|---|---|---|---|
| | Label A | | Label B | |
| | $K^+$ | $Ca^{2+}$ | $K^+$ | $Ca^{2+}$ |
| 0 | 1.7 | 0.29 | 1.7 | 0.29 |
| 1 | 1.3 | 0.91 | 1.7 | 0.45 |
| 2.5 | 0.8 | 1.48 | 1.0 | 0.43 |
| 5 | 0.3 | 1.46 | 0.7 | 0.63 |

[a] Myosin was incubated with spin label as described under Methods for 40 min at 0° and dialyzed overnight against 1000 volumes of 0.5 M KCl–2 mM TES (pH 7.0). ATPase activity was measured as described under Methods except that the $Ca^{2+}$-ATPase media contained 0.6 M KCl.

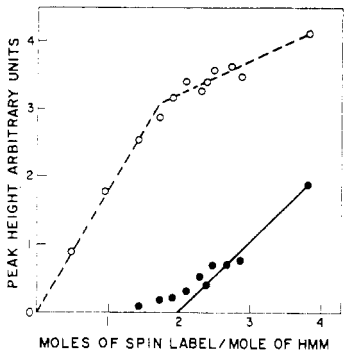


FIGURE 3: Dependence of peak heights in electron spin resonance spectra of labeled HMM on concentration of added label. Reaction of HMM (37 mg/ml) was carried out in 0.1 M Tris (pH 8.0) with varying concentrations of label A for 1 hr at 0° and electron spin resonance spectra were immediately recorded. (○) Height of strongly immobilized peak and (●) height of weakly immobilized peak.

in order to use higher protein concentrations. Varying concentrations of label A were added to HMM and the electron spin resonance spectra were recorded after the reaction was essentially complete. The plot of the height of the strongly immobilized peak against concentration of added spin label consists of two linear regions with a break at 1.7 moles of label/350,000 g of heavy meromyosin (Figure 3). The height of the weakly immobilized peak is also plotted against concentration of added label. This curve rises slowly up to about 2 moles of label/mole of HMM and more rapidly at higher concentrations of label. When the steeper portion of the curve is extrapolated to the abscissa it intersects at a value of 2.0 moles of label/mole of HMM (Figure 3). These results are consistent with the view that 2 moles of label A react rapidly with $S_1$ thiol groups giving rise to strongly immobilized labels while weakly immobilized labels are bound to less reactive groups.

The electron spin resonance spectrum of $S_1$-labeled myosin (Figure 4) is not changed on tryptic digestion to HMM (Figure 5) except for a slight decrease in the height of the weakly immobilized peak which may represent a small loss of label bound to the light meromyosin portion of myosin. Direct labeling of HMM prepared from unlabeled myosin also results in a similar electron spin resonance spectrum. The electron spin resonance spectrum of subfragment 1 (Figure 6) prepared from spin-labeled HMM is essentially the same as that of HMM.

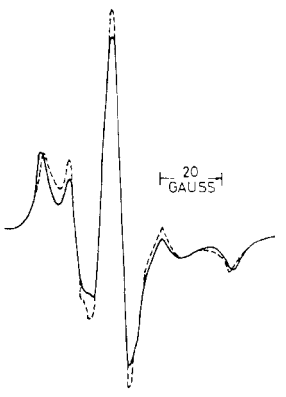


FIGURE 4: Effect of ATP on electron spin resonance spectrum of $S_1$-spin-labeled myosin. Myosin was spin labeled as described in Methods with 2 moles of label A/mole of myosin. Spectra were recorded in a solution containing 0.4 M KCl–0.02 M Tris–0.02 M imidazole (pH 7.0) at room temperature. (——) No addition and (– – –) 5 mM ATP.

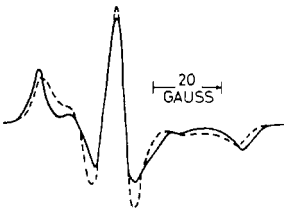


FIGURE 5: Effect of ATP on electron spin resonance spectrum of $S_1$-spin-labeled HMM. HMM was spin labeled as described under Methods with 1.5 moles of label A/mole of HMM. Spectra were recorded in a solution containing 0.04 M Tris (pH 8.0) and 9.6 mg of HMM/ml. (——) No addition and (– – –) 5 mM ATP.

The effect of ATP on the electron spin resonance spectrum of myosin having $S_1$ groups labeled with label A is shown in Figure 4. On addition of ATP the ratio of the height of the strongly immobilized peak to the height of the weakly immobilized peak decreases and splitting of the spectrum decreases from ~65 to ~61 gauss, indicating an increased mobility of labels attached to $S_1$ groups. This effect is independent of the degree of labeling and is the same whether myosin is labeled with 0.8 or 1.6 moles of label A per mole of myosin (Table II). If $S_1$ groups are blocked with iodoacetamide or 4-iodoacetamidosalicyclic acid and the myosin then reacts with label A, spectra indicating almost exclusively the presence of weakly immobilized labels are observed and no effect of ATP is found (Figure 7). The effect of ATP on the electron spin resonance spectrum of $S_1$-spin-labeled myosin is reversed on removal of ATP by dialysis against 0.5 M KCl and can be observed again on subsequent addition of ATP.

The effect of ATP on $S_1$-labeled HMM (Figure 5) or subfragment 1 (Figure 6) is essentially the same as on labeled myosin resulting in an increased mobility of labels. ITP, ADP, or $MgPP_i$ produces the same change in the electron spin resonance spectrum as does ATP, while AMP or MgAMP

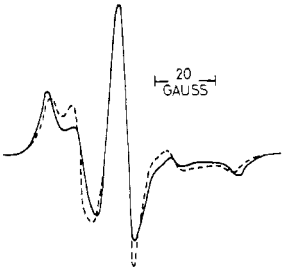


FIGURE 6: Effect of ATP on electron spin resonance spectrum of $S_1$-spin-labeled subfragment 1. Subfragment 1 was prepared from $S_1$-spin-labeled HMM labeled with label A as described under Methods. Spectra were recorded in a solution containing 0.2 M Tris (pH 7.5) containing 31 mg of subfragment 1/ml. (——) No addition and (– – –) 5 mM ATP and 1 mM $MgCl_2$.

TABLE II: Effect of ATP on Electron Spin Resonance Spectra of Myosin Labeled to Different Extents with Label A.[a]

| Molar Ratio of Added Spin Label to Myosin | Addition | Ratio of Peak Heights, I:II | Approx Separation of Peaks I and IV (gauss) |
|---|---|---|---|
| 1.6 | None | 1.72 | 65 |
| 1.6 | 5 mM ATP | 1.39 | 62 |
| 0.8 | None | 2.32 | 65 |
| 0.8 | 5 mM ATP | 1.66 | 62 |

[a] Myosin was labeled as described under Methods and dialyzed to remove unreacted label. Spectra were recorded in solutions containing 16 mg of myosin/ml, 0.4 M KCl, and 0.02 M TES at pH 7.0. Peak IV is the peak at the extreme right of the spectra.

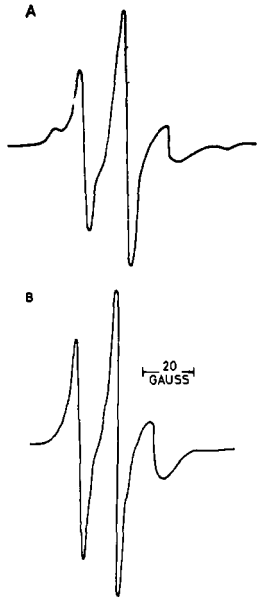


FIGURE 7: Electron spin resonance spectrum of myosin spin labeled following reaction with iodoacetamidosalicylic acid. Myosin (22 mg/ml) was incubated with iodoacetamidosalicyclic acid in 0.5 M KCl and 0.04 M Tris (pH 8.0) for 60 min at 0°. One-tenth volume of label A was added and the solution was kept at 0° for another 60 min. This solution was dialyzed overnight against 500 volumes of a solution containing 0.5 M KCl, $10^{-3}$ M TES, and $10^{-4}$ M EDTA (pH 7.0) at 4°. (A) Reaction of 3 moles of iodoacetamidosalicyclic acid and 2 moles of label A per mole of myosin. (B) Reaction of 5 moles of iodoacetamidosalicylic acid and 4 moles of label A per mole of myosin.

has no effect. Table III presents a set of experiments with spin-labeled HMM; essentially the same results were obtained with spin-labeled myosin. Addition of either 5 mM $Mg^{2+}$ or 5 mM EDTA does not alter the effect of 5 mM ATP or ADP, but $Mg^{2+}$ slightly increases the effect of $PP_i$. Addition of either $Mg^{2+}$ or EDTA alone does not alter the electron spin resonance spectrum of spin-labeled myosin or HMM. It appears also that activating monovalent cations are not required for the spectral change produced by ATP since the effect is observed with HMM in the absence of monovalent cations other than 10 mM Tris (Figure 5).

The effect of varying $PP_i$ concentrations on the electron spin resonance spectrum of $S_1$-labeled myosin is shown in Figure 8. The isosbestic points in the spectra suggest that binding of $PP_i$ produces a transition from one distinct state or conformation of spin-labeled myosin to another without there being any intermediate states. The number of binding sites for $PP_i$ involved in the spectral change can be estimated from these data. Addition of 1 mole of $PP_i$/mole of HMM produces about one-half the maximal spectral change, while addition of either 2 or 3 moles of $PP_i$ per mole of HMM produces the same maximal change in the spectrum.

It seemed of interest to see if reaction of the $S_2$ thiol groups leading to loss of ATPase activity led to changes in the protein

TABLE III: Effect of Nucleotides and Pyrophosphate on Electron Spin Resonance Spectra of $S_1$-Labeled HMM.[a]

| Addition | Ratio of Peak Heights, I:II | Approx Separation of Peaks I and IV (gauss) |
|---|---|---|
| None | 2.7 | 64 |
| ATP | 1.8 | 61 |
| ADP | 2.0 | 61 |
| AMP | 2.5 | 65 |
| ITP | 1.8 | 62 |
| $Mg^{2+}$ + $PP_i$ | 2.0 | 61 |

[a] HMM was spin labeled with 2 moles of label A/mole of HMM. Spectra were measured in a solution containing 0.02 M TES and 20–22 mg of HMM/ml at pH 7.0. Nucleotides, potassium pyrophosphate, and $MgCl_2$ were added at 5 mM final concentration.

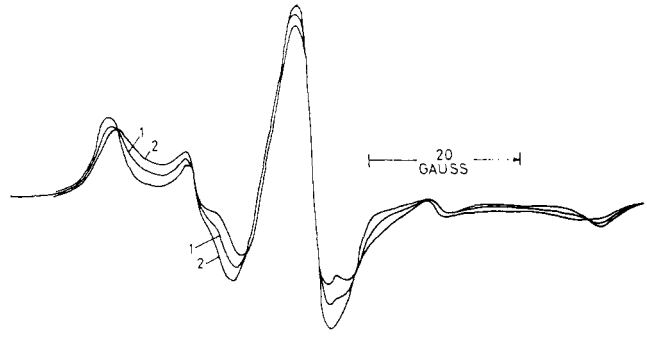


FIGURE 8: Effect of pyrophosphate on electron spin resonance spectrum of $S_1$-spin-labeled HMM. HMM was labeled as described under Methods with 2 moles of label A/mole of HMM. Spectra were recorded in a solution containing 0.08 M Tris (pH 7.5), 1 mM $MgCl_2$, and 39 mg of HMM per ml ($1.1 \times 10^{-4}$ M). Curves were traced twice and were found to be exactly reproducible. Unlabeled curve, no pyrophosphate; curve 1, $10^{-4}$ M potassium pyrophosphate; curve 2, $2 \times 10^{-4}$ M and $3 \times 10^{-4}$ M potassium pyrophosphate.

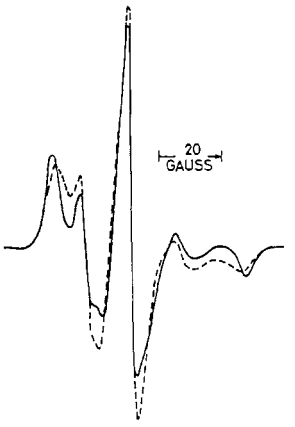


FIGURE 9: Effect of ATP on the electron spin resonance spectrum of $S_1$-spin-labeled myosin after inhibition of $Ca^{2+}$-ATPase activity with NEM. Myosin was spin labeled with 2 moles of label A/mole of myosin and dialized as described under Methods. This $S_1$-labeled myosin (18 mg/ml, $3.6 \times 10^{-4}$ M) in a solution containing 0.45 M KCl, 4.5 mM ADP, 4.5 mM $CaCl_2$, 0.04 M Tris, and $3.6 \times 10^{-5}$ M NEM at pH 7.5, was incubated at 0° for 40 min and dialyzed overnight at 4° against 667 volumes of a solution containing 0.5 M KCl, 1 mM TES, and 0.1 mM EDTA (pH 7.0). Spectra were recorded in a solution containing 0.42 M KCl, 0.04 M Tris (pH 7.5), and 14 mg of myosin per ml. (——) No addition and (– – –) 5 mM ATP.

TABLE IV: Effect of NEM on ATPase Activity of Spin-Labeled Myosin.[a]

| Treatment of Myosin | ATPase Act. ($\Delta P_i$, μmoles/mg of myosin min) | | |
|---|---|---|---|
| | $K^+$ | $Ca^{2+}$ | $Ca^{2+}$ + KCl |
| None | 2.18 | 1.08 | 0.37 |
| Label A | 0.46 | 0.93 | 2.13 |
| Label A, NEM | 0.00 | 0.00 | 0.02 |

[a] The myosin used for recording the spectra shown in Figure 9 was used in this experiment. The conditions of modification are described in the legend to Figure 9.

that would be reflected in changes of the mobility of labels attached to $S_1$ groups, and whether the interaction of myosin with ATP might be affected. This effect was investigated by treating $S_1$-spin-labeled myosin with 10 moles of NEM/mole of myosin in the presence of ADP (Sekine and Yamaguchi, 1963). This produces 95% inhibition of the $Ca^{2+}$-activated ATPase (Table IV) but does not alter the shape of the electron spin resonance spectrum (Figure 9) or change the effect of ATP on the spectrum.

## Discussion

The technique of spin labeling and its applications have been extensively discussed in several recent review articles (Hamilton and McConnell, 1968; Griffith and Waggoner, 1969). Since the usefulness of this technique is greater when the labeling can be limited to a single amino acid residue per mole or per subunit of protein, our initial experiments with myosin were designed to determine whether such limited labeling could be achieved. The thiol groups of myosin which are involved in the change in enzymatic activity are among the most reactive groups in the molecule (Sekine *et al.*, 1962) and appear to be the logical choice for specific labeling. To quantitate the binding of spin label to myosin the protein is precipitated, the amplitude of the electron spin resonance (which is directly proportional to the concentration of the free labels in the supernatant) is measured and the concentration of bound label obtained by difference. The complete loss of $K^+$-activated ATPase activity on binding of 1.9–2.3 moles of label/mole of myosin and the rapid reaction of 1.4–2 moles of spin label/mole of HMM giving rise to a spectrum characteristic of strongly immobilized labels suggests the presence of two $S_1$ groups per mole. Both of these groups belong to the same class of thiol groups on the basis of their rate of reaction with label A and the mobility of spin labels bound to them. The identical behavior of both of these groups on spin labeling suggests that both groups are involved in the loss of $K^+$-activated ATPase activity on reaction with thiol reagents.

Nucleotide, $PP_i$, and metal binding studies indicate the binding of 2 moles of ligand/mole of myosin or HMM with each site having essentially the same affinity (Kiely and Martonosi, 1968, 1969; Nauss *et al.*, 1969; Lowey and Luck, 1969). In contrast, Morita (1969) reports that a maximal ultraviolet difference spectrum of HMM is produced by 2 moles of ATP or 1 mole of ADP per mole of protein and concludes that the two nucleotide binding sites have different affinities for ADP, only one site being involved in the generation of the difference spectrum. This does not agree with the report of Lowey and Luck (1969) who find the binding of 2 moles of ADP/mole of myosin, with both binding sites having the same affinity. Our results show that when increasing amounts of ATP or $PP_i$ are added to spin-labeled myosin or HMM, the change in the electron spin resonance spectrum becomes maximal with 2 moles of ligand/mole of protein. These facts as well as the stoichiometry of the ATPase inhibition would be consistent with a myosin structure having two equivalent heavy chains (Lowey and Cohen, 1962; Lowey and Luck, 1969; Weeds and Hartley, 1968), each containing one active site and one $S_1$ group.

ATP produces a change in the electron spin resonance spectrum of spin-labeled myosin which indicates an increase in the mobility of strongly immobilized spin labels. Reaction with label A after blocking of $S_1$ groups with iodoacetamide or 4-iodoacetamidosalicyclic acid produces an electron spin resonance spectrum, indicating almost complete absence of strongly immobilized labels. Thus under the conditions of labeling used here all strongly immobilized labels are attached to $S_1$ groups and it follows that ATP affects the mobility of labels bound to $S_1$ groups.

ATP has been reported to change the ultraviolet absorption of HMM (Morita and Yagi, 1966), the optical rotation (Tonomura *et al.*, 1963; Kay *et al.*, 1964), dye binding (Gratzer and Lowey, 1969), the fluorescence of 8-anilino-1-naphthalenesulfonate bound to myosin (Cheung, 1969), and the reactivity of myosin with NEM (Sekine and Yamaguchi, 1963) or 1-fluoro-2,4-dinitrobenzene (Barany *et al.*, 1969). Recently Gratzer and Lowey (1969) reported that they were unable

to find changes in the optical rotatory dispersion of myosin on addition of ATP, indicating the absence of any extensvie change in the myosin molecule induced by ATP. The observed increase in mobility of spin labels attached to $S_1$ groups of myosin on addition of ATP suggests the presence of a conformational change near the $S_1$ groups of myosin. Since no detectable change in optical rotation accompanies the addition of ATP, such a conformational change must be confined to a relatively small portion of the myosin molecule. Thus it seems probable that the $S_1$ groups are located close to the ATP binding site of myosin.

In contrast to the effect of ATP on myosin spin labeled with label A, Cooke and Morales (1969) found no effect on the electron spin resonance spectrum of myosin which had reacted with the maleimide spin label (label B). In preliminary experiments we also found no effect of ATP on myosin spin labeled with label B. This suggests that the binding of label B to myosin is not confined solely to $S_1$ groups.

The fact that ATP alters the electron spin resonance spectrum of spin-labeled myosin which has reacted with NEM to the extent that all ATPase activity is inhibited indicates that ATP binds to myosin even when both $S_1$ and $S_2$ groups are blocked and that these thiol groups are not directly involved in ATP binding. This confirms the earlier finding of Martonosi and Meyer (1964) and that of Kielley *et al.* (1967) that myosin having both $S_1$ and $S_2$ groups blocked with NEM with concomitant loss of all ATPase activity is still able to bind ATP. It also follows that the effect of ATP on the electron spin resonance spectrum of spin-labeled myosin depends only on the binding of ATP and does not involve its hydrolysis.

Reaction of $S_2$ groups with NEM under conditions leading to complete loss of ATPase activity does not change the electron spin resonance spectrum of label A attached to $S_1$ thiol groups. In contrast, inhibiton by *p*-mercuribenzoate of $Ca^{2+}$-activated ATPase of myosin spin labeled with *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide is accompanied by a marked change in the electron spin resonance spectrum (Quinlivan *et al.*, 1969). This difference may be due to the fact that a large molar excess of *p*-mercuribenzoate was added sufficient to react with groups other than those affecting activity, and/or that Quinlivan *et al.*, used the label containing the five-membered nitroxide ring while we used the six-membered nitroxide label. McConnell and coworkers (Ogawa *et al.*, 1968) have demonstrated differences in the behavior of these two labels with hemoglobin, and Taylor *et al.* (1969) have reported differences in the reactivity of these two labels with creatine kinase.

The binding of $PP_i$ to myosin requires the presence of a divalent cation (Kiely and Martonosi, 1968), the binding of ADP requires a divalent cation at high ionic strength (Kiely and Martonosi, 1969; Lowey and Luck, 1969), and the binding of ATP to myosin is reduced in the absence of divalent cation (Schlisefeld and Barany, 1968). Our results with $S_1$-spin-labeled myosin show binding of ATP or ADP in the absence of divalent cations which might imply that chemical modification of $S_1$ thiol groups may abolish the requirement for divalent cation in ATP or ADP binding. The effect of chemical modification of myosin on the divalent cation requirement for ATP binding is being investigated in greater detail.

Tryptic digestion of $S_1$-spin-labeled myosin to HMM slightly reduces the proportion of weakly immobilized labels but does not otherwise alter the spectrum. Digestion of spin-labeled HMM to subfragment 1 apparently does not alter the enzyme conformation in the region of nitroxide radicals attached to $S_1$ groups. Trotta *et al.* (1968) report that an enzymatically active subfragment 1 which does not contain the regulatory sulfhydryl groups can be obtained by tryptic digestion of HMM. The presence of spin label in our subfragment 1 preparation does not support this conclusion. A more detailed study of the tryptic digestion of spin-labeled myosin will be the subject of a future communication.

## Acknowledgments

We wish to acknowledge the excellent technical assistance of the Misses Johanna Fine and Josaphine Donahue and Mr. Stephen Brown. We wish to thank Professor H. M. McConnell for making the methods of synthesis of spin labels available prior to publication and Dr. W. C. Landgarf of Varian Associates for supplying samples of spin labels.

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# Synthesis of 1-Halo Analogs of DL-Glycerol 3-Phosphate and Their Effects on Glycerol Phosphate Dehydrogenase*

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ABSTRACT: 1-Fluoro, 1-chloro, and 1-bromo analogs of DL-glycerol-3-P have been synthesized from the corresponding epihalohydrins with either phosphoric acid or dibenzylphosphoric acid. The 1-halodeoxyglycerol phosphates so obtained were demonstrated to be analogs of glycerol-3-P rather than glycerol-2-P by nuclear magnetic resonance spectra and by synthesis from 1-halodeoxyglycerol and dibenzylphosphorochloridate. 1-Fluorodeoxy-DL-glycerol-3-P is a substrate for rabbit muscle nicotinamide–adenine dinucleotide (NAD)-linked glycerol-3-P-dehydrogenase with an $K_m$ (app) of 8 mM at pH 7.5 and 6.5 mM at pH 9.0. 1-Chlorodeoxy- and 1-bromodeoxy-DL-glycerol-3-P do not appear to be substrates for glycerol 3-phosphate dehydrogenase unless 0.1 M hydrazine is present. All three halo analogs are substrates for calf intestine alkaline phosphatase.

None of the three halo analogs of DL-glycerol-3-P appears to be an irreversible inhibitor of NAD-linked glycerol-3-P-dehydrogenase, at either pH 7.5 or 9.0, although nonspecific organic phosphate incorporation is observed with the chloro and bromo analogs, particularly at pH 9.0. We suggest that the 1-fluoro analogs of L-glycerol-3-P or of dihydroxyacetone-3-P or derivatives of them may be useful as agents selectively toxic to cells lacking NAD-linked glycerol-3-P-dehydrogenase, which is a characteristic of many rapidly growing cancer cells.

Cytoplasmic NAD-linked glycerol-3-P-dehydrogenase is present at a very low level or is entirely absent from a wide variety of human cancer types (Delbrück *et al.*, 1959; Boxer and Shonk, 1960; Boxer and Devlin, 1961; Angeletti *et al.*, 1960; Sacktor and Dick, 1960; Foster and Taylor, 1966; Shonk and Boxer, 1964; Shonk *et al.*, 1964, 1965a,b, 1966). Instances where the enzyme level is not depressed in cancer cells are also known (Bär *et al.*, 1963; McKee *et al.*, 1968). In the cases where there is a significantly lower level of glycerol-3-P-dehydrogenase in cancer cells when compared with corresponding normal cells, it may be possible to exploit the glycerol-3-P-dehydrogenase deficiency in the selective destruction of the cancer cell. This selective destruction might be achieved by employing potentially toxic substrate analogs that could be detoxified by the action of the enzyme. 1-Fluoro analogs of either L-glycerol-3-P or of dihydroxyacetone-3-P might be useful in this selective chemotherapeutic approach. These analogs are of particular interest because the 1-fluoro analog of glycerol is already known to have a toxicity approaching that of fluoroacetate in rats (O'Brien and Peters, 1958). Dephosphorylation of the 1-fluoro analog of L-glycerol-3-P by phosphatases might be expected to produce the toxic 1-fluoro analog of glycerol in situations where the 1-fluoro-1-deoxy-L-glycerol-3-P cannot be metabolized by alternative means. One such possible alternative fate of 1-fluoro-1-deoxy-L-glycerol-3-P might be oxidation to the 1-fluoro analog of dihydroxyacetone-3-P if the fluoro analog of glycerol-3-P were a substrate for glycerol-3-P-dehydrogenase. On the other hand, the 1-fluoro analog of dihydroxyacetone-3-P might itself be toxic, and reduction to 1-fluoro-1-deoxyglycerol-3-P, if catalyzed by glycerol-3-P-dehydrogenase, might reduce the toxicity of 1-fluorodeoxy-

* From the Biochemistry Committee and the Department of Zoology, Syracuse University, Syracuse, New York 13210. *Received March 11, 1970.* This work was supported by Grant CA-10250 from the National Cancer Institute of the National Institutes of Health, and by Grant GB-7673 from the National Science Foundation.