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# Effect of Nucleotide Binding on the Proximity of the Essential Sulfhydryl Groups of Myosin. Chemical Probing of Movement of Residues during Conformational Transitions†

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ABSTRACT: The reaction of myosin with three bifunctional sulfhydryl reagents of differing cross-linking span is reported. In the absence of nucleotide only *p-N,N'*-phenylenedimaleimide with a cross-linking span of 12–14 Å can bridge between the two essential sulfhydryls of myosin. The other two reagents, 2,4-dinitro-1,5-difluorobenzene and 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone with cross-linking spans of 3–5 and 7–10 Å, respectively, react under identical conditions with the $SH_1$ sulfhydryl but do not bridge to the $SH_2$ group. In the presence of MgADP, both *p-N,N'*-phenylenedimaleimide and 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone bridge across the $SH_1$ and $SH_2$ groups indicating a closer proximity of these two sulfhydryls in the presence of bound nucleotide. These results are discussed in relation to the conformational change induced in myosin by binding of the nucleotide.

It is now well established that the binding and hydrolysis of nucleotides by myosin causes conformational perturbations of the protein which can be readily monitored by a variety of steady-state techniques such as UV[1] difference spectroscopy (Morita, 1967), fluorescence (Werber et al., 1972), ESR spectroscopy (Seidel and Gergely, 1973), and circular dichroism (Murphy, 1974). The detection of these conformational changes provided new impetus for attempts to link the

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[1] Abbreviations used: MalNEt, *N*-ethylmaleimide; pPDM, *p-N,N'*-phenylenedimaleimide; FDNB, fluorodinitrobenzene; $F_2$DNB, 2,4-dinitro-1,5-difluorobenzene; $F_2$DPS, 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone; ADP, adenosine diphosphate; UV, ultraviolet; ESR, electron spin resonance.

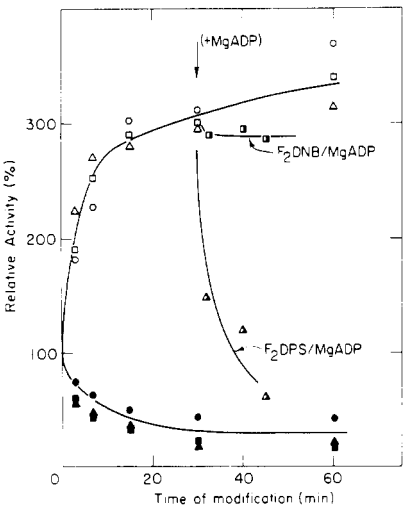


FIGURE 1: Relative ATPase activity vs. time of modification of myosin (5 mg/mL) in 0.03 M KCl, 0.025 M Tris, pH 7.9. The protein was reacted at 5 °C with a 4 molar excess of FDNB (○) and 2 molar excess of $F_2$DPS (△), and $F_2$DNB (□). The open and closed symbols represent $Ca^{2+}$- and EDTA-ATPase activities, respectively. The arrow signifies the time at which the samples were made $10^{-3}$ M in Mg-ADP and ▲, ■ represent the Ca-ATPase activity as a function of time after addition of Mg-ADP to the $F_2$DPS and $F_2$DNB systems, respectively. One hundred per cent activity for CaATPase and EDTA-ATPase correspond to 1 and 3 μmol $P_i$ $mg^{-1}$ $min^{-1}$, respectively.

energy transduction process in the muscle with conformational energy storage mechanisms (Lumry, 1974; Boyer, 1974; Volkenshtein, 1972). At the present, however, we lack basic information on the extent and localization of such changes on the globular section of the molecule, subfragment I. We are closer, perhaps, to determining their timing, i.e., when, in a single cycle of ATP binding and hydrolysis, the conformational changes occur. This is within the realm of possibility because the elementary steps of the hydrolytic reaction have been elucidated through the use of transient state methods such as chemical quenching (Lymn and Taylor, 1971) and stopped-flow fluorescence (Bagshaw et al., 1974). Though kinetic techniques allow for delineation of the intermediates of an enzymatic reaction, chemical studies (Bagshaw et al., 1975; Schaub and Watterson, 1975) are necessary to substantiate and characterize these intermediates.

The ability of myosin to hydrolyze ATP is associated with two essential sulfhydryl groups, $SH_1$ and $SH_2$, in each of the two subfragment I sections of the molecule. These groups can be specifically modified by thiol reagents. At pH 7.0, in the absence of nucleotides, the $SH_1$ group reacts with *N*-ethylmaleimide (MalNEt), while $SH_2$ is practically inaccessible to the reagent (Sekine and Kielley, 1964). However, if the $SH_1$-MalNEt myosin is treated with MalNet at pH 7.9, the $SH_2$ group becomes modified albeit at a very slow rate (Yamaguchi and Sekine, 1966). Addition of Mg-ADP to this reaction system dramatically accelerates the rate of modification of the $SH_2$ group (Yamaguchi and Sekine, 1966). Associated with these sequential modifications of the $SH_1$ and $SH_2$ groups are distinctive changes in the ATPase properties of myosin. Blocking of the $SH_1$ group causes elevation in the Ca-ATPase and a loss of EDTA-ATPase activity; subsequent blocking of the $SH_2$ group leads to a complete loss of the ability of the molecule to hydrolyze ATP.

More recent investigations (Reisler et al., 1974a; Schaub et al., 1975) have studied the effect of modifying $SH_1$ and $SH_2$ groups on the ATPase activity to determine differences in the conformations of the steady-state and product intermediates of myosin Mg-ATPase. Yet despite the information coming from these and other kinetic and conformational studies, it is difficult to estimate the magnitude of the conformational changes induced by ATP in terms of intramolecular interactions in the myosin molecule. Conceivably this is because these changes are so small as to escape detection by transport or scattering techniques.

In this communication, we show that the spatial separation of the two essential thiols of myosin is sensitive to the binding of nucleotides and that by using a series of bifunctional sulfhydryl reagents, it is possible to estimate the extent of the nucleotide induced perturbation.

## Materials and Methods

Double distilled water was used throughout. pPDM was purchased from Aldrich Chemical Co., MalNEt, $F_2$DPS, and $F_2$DNB were the products of Pierce Chemical Co., and FDNB was obtained from Eastman Chemical Co., (Rochester, N.Y.). All other reagents were of analytical grade. ADP was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Myosin was prepared by the method of Kielley and Bradley (1956), as modified by Godfrey and Harrington (1970). Myosin in 0.5 M KCl, 0.01 M EDTA, was stored in 50% glycerol at −20 °C. Before use, the myosin was exhaustively dialyzed against 0.5 M KCl, 0.05 M Tris, pH 7.9, solvent, then diluted to approximately 5-6 mg/mL with this solvent, and subsequently dialyzed against 0.03 M KCl, 0.025 M Tris, pH 7.9. Under these conditions myosin aggregates to form filaments and does not form gelatinous precipitates.

The reactions of myosin with FDNB, MalNEt, and pPDM as well as preparation of $SH_1$-MalNEt and $SH_1$-DNP myosin have been described elsewhere (Burke et al., 1973; Reisler et al., 1974a,b). All reactions were carried out at 5 °C unless specified otherwise. Stock solutions of $F_2$DNB and $F_2$DPS were prepared by dissolving the required amounts of the reagents in acetone. Stoichiometric amounts of the bifunctional reagents pPDM, $F_2$DPS, and $F_2$DNB (1 mol of bifunctional reagent/mol of myosin "head") were used in every case. Reactions were initiated by addition of the reagent with a Hamilton microsyringe. In the case of pPDM and MalNEt, the reaction was terminated by dilution of the protein in 0.5 m KCl, 0.05 M Tris, 0.002 M dithiothreitol, pH 7.9. With the fluoro reagents the reaction was terminated by precipitation followed by centrifugation. These steps were repeated twice (the dinitrophenyl derivatives undergo thiolysis in the presence of reducing reagents such as dithiothreitol and β-mercaptoethanol). Protein concentrations were obtained spectrophotometrically ($E^{1\%}_{280}$ = 5.5) for unmodified protein and by the micro Biuret method for the modified samples. ATPase assays in the presence of $Ca^{2+}$ or EDTA followed the procedure of Kielley et al. (1956) and Kielley and Bradley (1956).

## Results

(i) *Modification of the Essential Thiols of Myosin in the Absence of Nucleotide.* Prior to the use of bifunctional reagents as a probe for the spatial separation of the essential sulfhydryl groups of myosin, it is necessary to show that these reagents display the same specificity toward these sulfhydryl groups as do their monofunctional analogues. The data shown in Figure 1 provide the required verification. For the three reagents tested, FDNB, $F_2$DNB, and $F_2$DPS, the pattern of ATPase activity changes induced by their reaction with myosin is the same. The observed changes are characteristic of a modification of the $SH_1$ site; the Ca-ATPase is elevated and the EDTA-ATPase is depressed. Recent work by Bailin and Ba-

rany (1972) have demonstrated that the predominant site of modification of myosin by FDNB is the $SH_1$ thiol with very little reaction occurring at free amino groups. Moreover, we have found that this specificity of FDNB for the $SH_1$ site is not altered even in the presence of Mg-ADP, Mg-ATP, Ca-ADP, and Ca-ATP (Reisler et al., 1977). In the present study we have employed stoichiometric amounts of the bifunctional reagents (1 mol of bifunctional reagent per mol of myosin "head") and yet the observed changes in ATPase activities were identical with those obtained on modification of the protein with FDNB. We, therefore, infer that these reagents are at least as specific for the $SH_1$ site as their monofunctional analogue FDNB.

The reaction of myosin with pPDM and MalNEt under the same conditions is shown in Figure 2A. The changes in ATPase activities induced by MalNEt attest to modification of the $SH_1$ site. With the bifunctional reagent, pPDM, we find that the Ca-ATPase is initially elevated but on subsequent standing it is progressively lost. We interpret such a change in Ca-ATPase activity as indicating that the $SH_1$ site is initially monofunctionally blocked at a rapid rate and then the bifunctional reagent bridges to the $SH_2$ site at a much lower rate (Reisler et al., 1974b). In the absence of nucleotide only pPDM, of the three bifunctional reagents employed, can covalently bridge $SH_1$ to $SH_2$ (Figures 1 and 2). Except for the bridging, the reactions of MalNEt and pPDM with myosin are similar, although the bifunctional reagent displays somewhat lower reactivity toward the $SH_1$ groups than MalNEt.

The effect of magnesium pyrophosphate on the reaction of myosin with both MalNEt (see Watterson et al., 1975) and pPDM is to increase the rate at which the $SH_1$ groups are modified (Figure 2B). The increased reactivity of these groups reflects perturbation of the active site which may arise from simple change of environment due to the binding of magnesium pyrophosphate or from a local transition in the position of certain residues.

All of the modifications reported here were carried out at low ionic strength and low temperature conditions where the reactivity of nonessential thiols is strongly repressed (Schaub et al., 1975). In all experiments the amount of bifunctional reagent employed is sufficient to react with only two sulfhydryl groups in each myosin head (i.e., 1 mol of bifunctional reagent per mol of myosin head), while that of the monofunctional reagents is twice as much to maintain a constant stoichiometry of functional groups per myosin head. As shown in Figure 1, the loss in EDTA-ATPase activity with the mono or difluoro reagents indicates that over 70% of the myosin population has been reacted monofunctionally at the $SH_1$ site, leaving less than 0.3 mol of the bifunctional reagent per myosin head either free or reacted with nonessential groups. The fact that the Ca-ATPase remains elevated following the modifications with the bifunctional fluoro derivatives indicates that they are unable to covalently bridge between the blocked $SH_1$ group and the $SH_2$ site.

Their failure to bridge could arise from either of two reasons which immediately came to our mind. First, the free fluoro group of the reagent, monofunctionally attached to the $SH_1$ site, might have been lost by hydrolysis or, alternatively, the distance between the two essential thiol groups may be too large for a given reagent to bridge over it. Fortunately, we can easily check these possibilities by adding Mg-ADP ($10^{-3}$ M) to the $F_2$DPS and $F_2$DNB systems after their reaction with the protein is well advanced (at the time indicated by the arrow in Figure 1). Since Mg-ADP is known to expose and perturb the $SH_2$ site (Yamaguchi and Sekine, 1966), it should consequently promote bridging of $SH_1$ and $SH_2$, and we would know

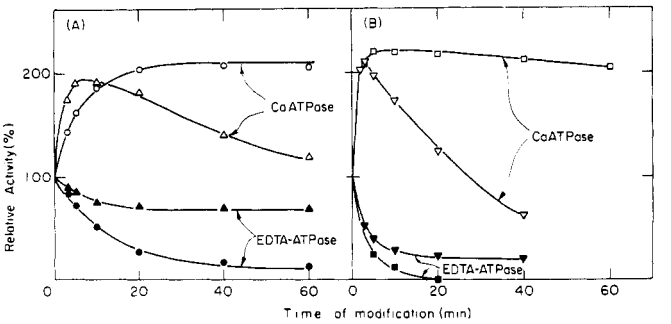


FIGURE 2: Relative ATPase activity vs. time of modification of myosin (5 mg/mL) in 0.03 M KCl, 0.025 M Tris, pH 7.9, reacted at 5 °C with (A) 4 molar excess of MalNEt (○, ●) and 2 molar excess of pPDM (△, ▲); (B) in the presence of $10^{-3}$ M magnesium pyrophosphate: 4 molar excess of MalNEt (□, ■) and 2 molar excess of pPDM (▽, ▼). The open and closed symbols represent $Ca^{2+}$- and EDTA-ATPase activities, respectively.

that the free fluoro group on the attached bifunctional reagent had not undergone hydrolysis. The result of this experiment is shown in Figure 1. Addition of Mg-ADP to the $F_2$DPS-myosin system after 30 min of reaction causes a rapid drop in the Ca-ATPase activity. This indicates that the residual fluoro group of the $F_2$DPS attached to $SH_1$ is still present and available for reaction with the protein. The observed bridging to the $SH_2$ group could be due either to its increased reactivity, induced by binding of Mg-ADP, or could arise from a change in the spatial distance between $SH_1$ and $SH_2$ or perhaps from both of these factors. To examine these possibilities we have used $SH_1$MalNEt and $SH_1$DNP myosin to check the reactivity of the $SH_2$ thiol toward $F_2$DPS and FDNB in the presence and absence of Mg-ADP. In neither case could we detect any modification at the $SH_2$ site. Apparently, binding of Mg-ADP to myosin does not stimulate monofunctional modification of the $SH_2$ group by either FDNP or $F_2$DPS. The ability of Mg-ADP to induce intramolecular $SH_1$-$SH_2$ bridging via $F_2$DPS must be then modulated by steric factors. It is likely to be caused by a shift in the $SH_1$ and $SH_2$ spacing to within the cross-linking range of the bifunctional reagent.

In the myosin-$F_2$DNB system the addition of Mg-ADP causes no significant change in the Ca-ATPase activity. We conclude from this result that the Mg-ADP induced conformational change does not allow the $SH_1$ and $SH_2$ groups to come into the cross-linking range of $F_2$DNB. Hydrolysis of the free fluoro group of monofunctionally attached $F_2$DNB is considered unlikely in view of the results obtained with $F_2$DPS. Also unlikely, though not impossible, is the reaction of the free fluoro group with an adjacent nonessential thiol group which in turn would preclude the bridging to $SH_2$.

(ii) *Modification Reaction in the Presence of MgADP.* Figure 3 shows the changes in $Ca^{2+}$- and EDTA-ATPase activities of myosin accompanying modification of the protein by $F_2$DPS, $F_2$DNB, and FDNB, under the same conditions as described in Figure 1 except for inclusion of $10^{-3}$ M Mg-ADP in the reaction system. In the presence of nucleotide only, $F_2$DNB mimics the reaction pattern of the monofunctional analogue FDNB; in both cases the Ca-ATPase activity is elevated and the EDTA-ATPase is decreased which signifies modification of the $SH_1$ site. In both systems, no time dependent decrease of Ca-ATPase can be detected suggesting, that $F_2$DNB is unable to bridge $SH_1$ and $SH_2$ even in the presence of nucleotide.

The modification of myosin by $F_2$DPS in the presence of Mg-ADP proceeds in a markedly different fashion (Figure 3). There is no evidence for an initial elevation of the Ca-ATPase

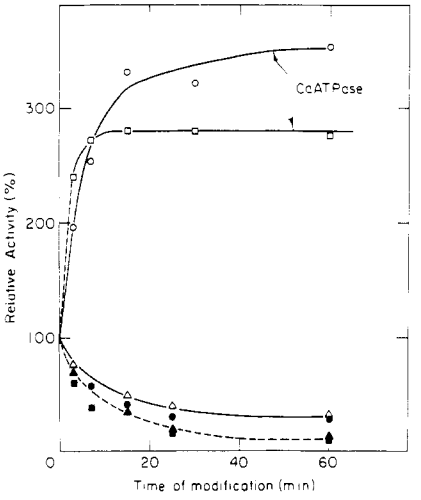


FIGURE 3: Relative ATPase activity vs. time of modification of myosin (5 mg/mL) in the presence of $10^{-3}$ M Mg-ADP. Solvent conditions are the same as in Figure 1. The protein was reacted with a 4 molar excess of FDNB (○, ●) and 2 molar excess of $F_2$DPS (△, ▲) and $F_2$DNB (□, ■). The open and closed symbols represent $Ca^{2+}$- and EDTA-ATPase activities, respectively.

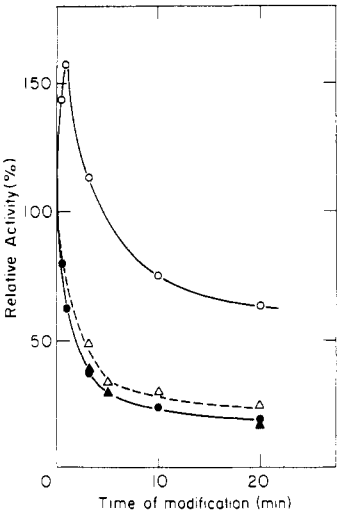


FIGURE 4: Relative ATPase activity vs. time of modification of myosin (5 mg/mL) reacted in the presence of $10^{-3}$ M Mg-ADP with a 4 molar excess of MalNEt (○, ●) and 2 molar excess of pPDM (△, ▲). The open and closed symbols represent $Ca^{2+}$- and EDTA-ATPase activities, respectively. Solvent conditions are the same as in Figure 1.

activity and in fact the Ca- and EDTA-ATPases decrease at almost identical rates. This result does not preclude initial attachment of the bifunctional reagent to the $SH_1$ site provided the next step of bridging to the $SH_2$ site is much faster.

The data in Figure 4 represent the reaction of myosin with pPDM and its monofunctional analogue MalNet in the presence of millimolar Mg-ADP. The $SH_1$ and $SH_2$ sites react with MalNEt sequentially since the Ca-ATPase activity is initially elevated and declines rapidly. With the bifunctional reagent, the Ca-ATPase decreases at a rate similar to the rate of EDTA-ATPase loss. Although it is possible that an initial elevation of Ca-ATPase may have been overlooked, due to insufficient time resolution, the extremely rapid drop in this activity together with the employed stoichiometry of pPDM to myosin can only indicate covalent bridging between $SH_1$ and $SH_2$ groups.

Thus in contrast to the modification reaction in the absence of nucleotide, the sulfhydryls $SH_1$ and $SH_2$ are very rapidly bridged by both $F_2$DPS and pPDM suggesting closer proximity of these thiols in the presence of Mg-ADP.

## Discussion

The results presented up to now enable us to correlate the conformational perturbation induced by binding of Mg-ADP to myosin with changes in the spatial proximity of its essential sulfhydryls. The bifunctional reagents employed apparently show a very high specificity for these sulfhydryls since stoichiometric amounts of the reagents produce near stoichiometric extents of modification of the $SH_1$ site of myosin. (For a summary of ATPase activities and their relation to the $SH_1$ and $SH_2$ groups, see Reisler et al., 1974a; Schaub et al., 1975.) In consequence we can rule out the possibility of significant modification at nonessential sulfhydryl sites.

The three bifunctional reagents differ in their cross-linking span from 3-5 Å for $F_2$DNB and 7-10 Å for $F_2$DPS to 12-14 Å for pPDM. The range of their cross-linking spans enables a quantitative estimate of the proximity of the essential sulfhydryls in the presence and absence of nucleotide. In the absence of nucleotide, only the largest of these reagents is capable, albeit poorly, of bridging between $SH_1$ and $SH_2$. This implies that the two sulfhydryl groups can reside at least temporarily 12-14 Å apart, presumably closer to the upper limit. The inability of $F_2$DPS to bridge between these groups confirms that they cannot approach each other to within 7-10 Å or less, and they must reside further apart.

The binding of Mg-ADP to myosin is known to cause a conformational change in the subfragment 1 region of the molecule. This change can be detected by UV absorption (Morita, 1967), fluorescence measurements (Werber et al., 1972), CD (Murphy, 1974), and by alterations in the modification pattern of essential thiols (Reisler et al., 1974a; Schaub et al., 1975). In terms of the spatial proximity of these essential thiols, our results (Figures 3 and 4) indicate that both $F_2$DPS and pPDM bridge between them in the presence of MgADP. The ability of $F_2$DPS to bridge the two sulfhydryls in the presence of Mg-ADP suggests that these groups may now approach one another to a distance of 7-10 Å or even less. In the absence of nucleotide their separation, as concluded above, is about 14 Å. We may estimate, thus, that the conformational shift induced by binding of Mg-ADP to myosin, as reflected in the change of the separation between the essential thiols, amounts to about 4-7 Å. We cannot exclude the possibility that this shift is even somewhat larger, although the modification reaction of myosin with $F_2$DNB does not support such a conclusion. The latter reagent fails to bridge in the presence of Mg-ADP between $SH_1$ and $SH_2$ groups, suggesting that the distance between them is larger than 5 Å. Yet, it is conceivable that steric reasons other than simple distance considerations prevent the bridging by $F_2$DNB.

One may possibly take a different view of our results by attributing the Mg-ADP induced briding of the essential thiols not to the movement of $SH_2$, but solely to its increased reactivity. Such an alternative explanation has to assume a variable separation of the two thiols ("breathing" of their interspacing) to accommodate for their cross-linking by reagents of different length. This requirement of considerable "breathing" of the $SH_1$-$SH_2$ interspacing seems to us to be more demanding and less realistic than the explanation evoking Mg-ADP induced shift in the separation of $SH_1$-$SH_2$ groups. Moreover, the fact that we could not modify, using FDNB, $F_2$DPS, and $F_2$DNB, the $SH_2$ group of $SH_1$ blocked protein, even in the presence of Mg-ADP, can also be taken against the simple reactivity explanation.

The conformational changes which occur during trans-

duction of chemical energy into mechanical work in muscle are undoubtedly more complex than the transitions induced by binding of Mg-ADP to myosin. Unfortunately, the chemical approach taken in this work to probe the conformational changes of myosin through its reactive essential thiols cannot be applied in a simple and straightforward fashion to the Mg-ATP/actomyosin system where the thiol groups are partially protected from modification. The results of this work, however, succeeded to provide a quantitative estimate of the perturbation induced by nucleotide binding to myosin. The magnitude of the perturbation, a small shift of 4-7 Å in the distance between the $SH_1$ and $SH_2$ groups, explains also, assuming that the change is localized to the vicinity of the thiol sites, why methods monitoring gross conformational properties of a molecule do not detect this conformational change (Burke et al., 1976).

Acknowledgments

The authors wish to thank Dr. William F. Harrington for helpful discussions and Mrs. Helen Russell for technical assistance.

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