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Spatial Proximity of the Two Essential Sulfhydryl Groups of Myosin†

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ABSTRACT: A bifunctional analog of NEM, *p*-phenylenedimaleimide, shows specificity similar to the monofunctional reagent toward the two essential sulfhydryl groups of myosin, SH_1 and SH_2. The dimaleimide reagent, which has a cross-linking span of 12–14 Å, can bridge between these thiol groups thus establishing their steric proximity. This finding provides experimental support for a proposed mechanism of ATP hydrolysis which requires that these two SH groups be in appropriate spatial arrangement to form a cyclic ternary complex with the metal substrates.

We have recently proposed that the two essential sulfhydryl groups of myosin, generally referred to as SH_1 and SH_2 (Sekine and Kielley, 1964; Yamaguchi and Sekine, 1966), participate in the formation of a cyclic ternary complex with MgATP (Burke *et al.*, 1973; Reisler *et al.*, 1974) and that the stability of this complex is responsible for the well-known inhibition of ATP hydrolhsis in the presence of 1 mM Mg^{2+}. Mandelkow and Mandelkow (1973) have proposed that the myosin-MgATP complex is in the form of enzyme-metal-substrate, rather than enzyme-substrate-metal based on recent fluorometric studies. Their studies also reveal independence of the two "heads" in the interaction with MgATP. Actin apparently activates the MgATPase of myosin by binding at, or in the vicinity of, the SH_1 site, thus disrupting the cyclic complex at this site (Burke *et al.*, 1973; Reisler *et al.*, 1974; see also Seidel, 1973). Evidence supporting this mechanism for activation of ATP hydrolysis was obtained from a study of the influence of various ligands on the reaction between NEM[1] and myosin (Reisler *et al.*, 1974).

In this communication we wish to demonstrate that the two essential sulfhydryl groups of myosin, SH_1 and SH_2, participating in the proposed cyclic ternary complex with MgATP are in close proximity to each other during their interaction with the Mg-nucleotide complex. Steric proximity of these groups is a prerequisite for formation of the proposed cyclic complex with MgATP and the results to be presented below show that a bifunctional reagent, *N,N'-p*-phenylenedimaleimide, with a cross-linking span of 12–14 Å and a specificity for SH_1 and SH_2 groups of myosin similar to that of NEM, can bridge between these groups.

Materials and Methods

ATP of highest available purity was purchased from Sigma Chemical Co., St. Louis, Mo. Inorganic salts and reagents were analytical grade. NEM was the product of Eastman Kodak Co. (Rochester, N. Y.) and *p*-phenylenedimaleimide was obtained from Aldrich Chemical Co. The preparation of myosin has been described elsewhere (Godfrey and Harrington, 1970).

SH_1-NEM and SH_2-NEM modified myosins were prepared as described earlier (Reisler *et al.*, 1974). Reaction of myosin with *N,N'-p*-phenylenedimaleimide (dissolved in acetone) was carred out in exactly the same way as the modification with NEM. The effect of these modifications on ATPase acitivty of myosin was tested as reported by Sekine and Kielley (1964).

Purification of SH_1-phenylenedimaleimide myosin to remove the unreacted bifunctional reagent was achieved with a Sephadex G-25 column equilibrated with pH 7.0 solvent (0.5 M KCl–0.05 M Tris). Myosin samples reacted for 30 min at pH 7.0 with an eightfold molar excess of the bifunctional reagent over protein were loaded on the column and eluted within 15 min. The loss in CaATPase activity was followed as a function of time at pH 7.0, 7.9, and in the presence of 1 mM MgADP (pH 7.9). All modifications were carried out at 5°.

The Ca and EDTA activities of myosin were measured at 37° employing the procedures of Kielley and Bradley (1956) and Kielley *et al.* (1956).

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[1] Abbreviation used is: NEM, *N*-ethylmaleimide.

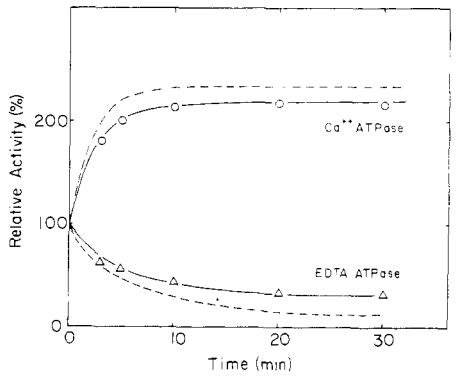

FIGURE 1: Relative ATPase activities of myosin reacted at pH 7.0 with an eightfold excess of NEM (- - - -) or *p*-phenylenedimaleimide (—) plotted as a function of time of modification.

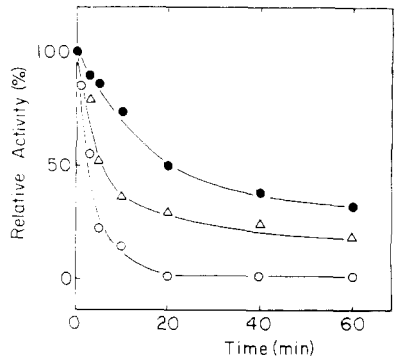

FIGURE 3: Relative Ca^{2+} ATPase activity of SH_2-NEM modified myosin reacted with an eightfold molar excess of *p*-phenylenedimaleimide at pH 7.0 (●), pH 7.9 (△), and pH 7.9 in the presence of 1 mM MgATP (○). 100% ATPase activity of SH_2-NEM myosin corresponds to 0.95 μmol of P_i mg^{-1} min^{-1}.

Results and Discussion

The reaction of myosin with NEM has been characterized in detail over the past years both with respect to its catalytic consequences and the nature of the modified site on the protein (Sekine *et al.*, 1962; Sekine and Kielley, 1964; Yamashita *et al.*, 1964; Yamashita *et al.*, 1965; Yamaguchi and Sekine, 1966; Reisler *et al.*, 1974). A clear correlation has been established between the titration of the two essential sulfhydryl groups SH_1 and SH_2 with NEM and the observed changes in the activity properties of myosin. Blocking of the SH_1 group of myosin with NEM results in a marked increase in CaATPase and a decrease in EDTA (K^+) ATPase activities (Sekine *et al.*, 1962). Subsequent modification of the second essential sulfhydryl group, SH_2, leads to a complete loss of both activities (Sekine and Yamaguchi, 1963; Yamaguchi and Sekine, 1966). We have employed these changes in ATPase activities of myosin in the present study to establish that a bifunctional analog of NEM, *N,N'-p*-phenylenedimaleimide, reacts with myosin in a similar fashion to the monofunctional NEM.

Figure 1 shows that under conditions employed for modification of SH_1 by NEM (pH 7.0, 8:1 molar ratio of NEM to myosin) the reaction with *p*-phenylenedimaleimide results in a concomitant increase in CaATPase and decrease in EDTA (K^+) ATPase activities. The rate and extent of these changes are somewhat smaller with *p*-phenylenedimaleimide than with NEM, possibly due to a lower reactivity of the former toward SH_1 groups of myosin. The overall effect, however, is the same.

Evidence that the bifunctional maleimide can react with both SH_1 and SH_2 groups was obtained from modification with this reagent of prelabeled SH_1-NEM and SH_2-NEM myosin samples (prepared as described by Reisler *et al.*, 1974). Figure 2 demonstrates that at pH 7.0 and at four- and eightfold molar excess of the reagent over protein, *p*-phenylenedimaleimide, does not affect the active site of SH_1-NEM myosin. When the pH is raised to 7.9 some loss in CaATPase is observed; this loss is complete and rapid when the modification is performed in the presence of 1 mM MgADP. Thus the reaction pattern follows very closely the reaction of NEM with SH_1-NEM myosin, as originally observed by Yamaguchi and Sekine (1966). The effect of *p*-phenylenedimaleimide on SH_2-NEM myosin, shown in Figure 3, resembles again the reaction of NEM with the SH_2-labeled protein (Seidel, 1969; Reisler *et al.*, 1974). The modification, as followed by a loss in CaATPase activity, proceeds quite effectively at pH 7.0, is accelerated at pH 7.9 and even more so in the presence of MgADP. This pattern of modification, however, differs significantly from the blocking of SH_1-NEM myosin (Figure 2).

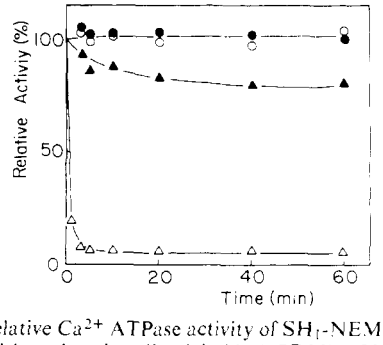

FIGURE 2: Relative Ca^{2+} ATPase activity of SH_1-NEM modified myosin reacted with *p*-phenylenedimaleimide (pPDM). 100% ATPase activity of SH_1-NEM myosin corresponds to 2 μmol of P_i mg^{-1} min^{-1}. pH 7.0, 4-(●) and 8-(○) fold molar excess pPDM/myosin; pH 7.9, (▲) fourfold molar excess pPDM/myosin; pH 7.9 (△) 1 mM MgADP, fourfold molar excess pPDM.

The results presented in Figures 1-3 establish the similarity between the reaction of NEM and *p*-phenylenedimaleimide with myosin and indicate that both reagents exhibit comparable specificity toward SH_1 and SH_2 groups of the protein.

We have observed previously (Reisler *et al.*, 1974) that modification of SH_1 groups of myosin by NEM can be achieved more readily at pH 7.9 than at pH 7.0. When modification is conducted (at pH 7.9) with *p*-phenylenedimaleimide the rapid initial elevation of CaATPase is followed by a slow decrease in this activity (Figure 4). This behavior is unlike that observed with NEM (upper dashed line in Figure 4), where the elevated CaATPase activity remains invariant with time (~60 min). A decrease in CaATPase activity is normally observed upon modification of the SH_2 group subsequent to a prior blocking of the SH_1 group (Yamaguchi and Sekine, 1966). The initial decrease in EDTA (K^+) ATPase activity, shown in Figure 4, is a measure of myosin molecules which have reacted with the bifunctional reagent. Within a few minutes EDTA ATPase reaches a constant value depending on the molar excess of the reagent over protein. Apparently at this stage the reagent has been exhausted in competitive reactions. The decrease in CaATPase activity mentioned above is confined to those molecules which have been initially modified at SH_1 since the EDTA ATPase remains unaltered over the examined time interval. Had the loss in CaATPase been due to a slower reaction of SH_2 groups on previously unmodified myosin molecules an additional decrease in EDTA ATPase would have been observed (Seidel, 1969; Reisler *et al.*, 1974).

The behavior of Ca and EDTA ATPases in Figure 4 cannot, however, exclude a hypothetical two-step process: blocking of

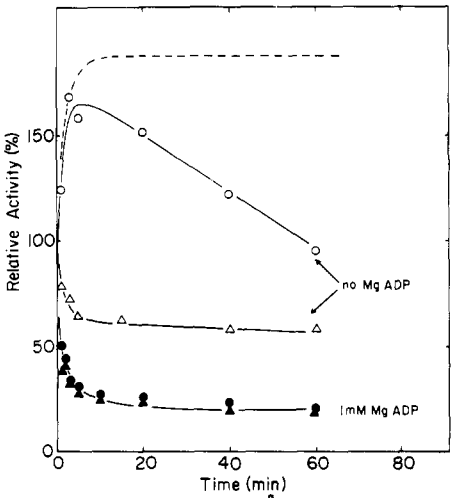

FIGURE 4: Relative Ca^{2+} ATPase (○, ●), and EDTA ATPase activity (△, ▲) of myosin reacted with a fourfold molar excess of *p*-phenylenedimaleimide. The dashed line represents the Ca^{2+} ATPase activity of myosin reacted with a fourfold molar excess of NEM.

SH_1 group followed by modification of SH_2 group on the SH_1-labeled myosin. To exclude such a possibility myosin samples modified with the dimaleimide reagent were diluted after achieving maximum elevation of their CaATPase. The rate of the activity loss (in CaATPase) was found to be independent of the protein concentration in the range of 1–10 mg/ml. Consequently the activity decrease could not be attributed to a separate reaction of the bifunctional reagent with SH_2, but rather to an intramolecular process.

To eliminate the possibility that disulfide bond formation is responsible for the loss in CaATPase activity of SH_1-phenylenedimaleimide myosin, the modified protein was treated (on reaching its elevated CaATPase level) with a 500-fold molar excess of β-mercaptoethanol which was subsequently removed by dialysis. This treatment should block any residual free and monofunctionally substituted dimaleimide precluding any further reaction of this reagent. It should not, however, prevent disulfide bond formation. The above treatment resulted in an almost complete stabilization of the elevated CaATPase activity indicating that disulfide formation (if it occurs) is not responsible for the loss in CaATPase. It appears, therefore, that the intramolecular reaction which takes place is the bridging by the dimaleimide between the SH_1 and SH_2 groups of myosin.

To substantiate the last conclusion that SH_2 groups react with the bifunctional maleimide attached to SH_1 groups we attempted to show that the loss in CaATPase activity of SH_1-dimaleimide myosin follows the pattern established for blocking of SH_2 groups on SH_1-NEM myosin. To achieve this we have isolated SH_1-phenylenedimaleimide myosin (prepared at pH 7.0 by reacting the protein for 30 min with an eightfold molar excess of the regent) from the unreacted bifunctional reagent on a G-25 Sephadex column equilibrated at pH 7.0. Following this separation the loss in CaATPase of the purified SH_1-dimaleimide myosin was examined at pH 7.0, pH 7.9, and in the presence of MgADP (pH 7.9). The results presented in Figure 5 demonstrate that the decrease in CaATPase activity parallels the behavior observed upon blocking of SH_2 groups in SH_1-NEM myosin (compare also Figure 2). In the present case the modification of SH_2 groups cannot be ascribed to excess bifunctional reagent, since the excess unreacted reagent was removed by gel filtration. Thus the loss in CaATPase activity of SH_1-dimaleimide myosin arises from the intramolecular bridging of SH_1 and SH_2 groups *via* the dimaleimide reagent.

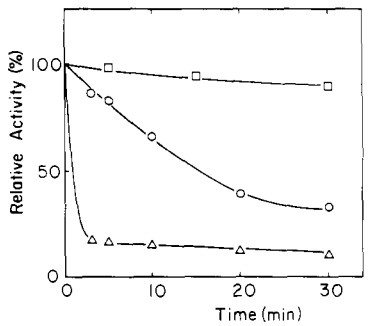

FIGURE 5: Loss in Ca^{2+} ATPase activity of SH_1-*p*-phenylenedimaleimide myosin purified on Sephadex G-25 column plotted as a function of time. 100% ATPase activity of SH_1-*p*-phenylenedimaleimide corresponds to 1.8 μmol of P_i mg^{-1} min^{-1}. Experiments carried out at pH 7.0 (□); pH 7.9 (○); and pH 7.9, 1 mM MgADP (△).

In our previous work (Reisler *et al.*, 1974) we have demonstrated that MgADP exposes the SH_2 group of native myosin to reaction with NEM while protecting the SH_1 group from the modification. When the same reaction in the presence of MgADP is performed with the dimaleimide reagent the results obtained are quite revealing. As shown in Figure 4 both Ca and EDTA ATPase activities decrease at the same rate and to the same extent. Such behavior is consistent with formation of a dimaleimide bridge between SH_1 and SH_2 groups of myosin. The reaction apparently proceeds through blocking of SH_2 groups followed by immediate bridging to SH_1. Any conceivable sequential modification of the two groups would lead to separate Ca and EDTA activity curves. Thus it is not surprising that the simultaneous decrease in Ca and EDTA ATPases has not been achieved by modification of myosin with NEM. The latter reagent exhibits preferential specificity toward either SH_1 or SH_2 group according to the reaction conditions.

Our preliminary results demonstrate that the reaction of *p*-phenylenedimaleimide with a proteolytic fragment of myosin, subfragment I, proceeds in identical fashion with that with myosin indicating that the bridges formed between the SH_1 and SH_2 groups reside within each "head" of the myosin molecule and not between two adjacent "heads." Since the dimaleimide bridge allows for a cross-linking span of 12–14 Å, the two essential sulfhydryl groups of myosin are located 12–14 Å apart or, more precisely, may reside temporally within that distance during their interaction with the Mg-nucleotide complex. Moreover, it seems possible that flexibility of some segments of the peptide chain may allow the SH_1 and SH_2 groups to come into an even closer spatial relationship. Experiments employing bifunctional reagents of different cross-linking span should allow the spatial freedom of these groups to be established as well as the range of their closest approach. Such studies including peptide analysis of products of the reaction of myosin with bifunctional maleimide are currently under investigation in our laboratory. The already established proximity of SH_1 and SH_2 groups is consistent with and supports the previously postulated specific interaction of MgATP with the two sulfhydryl sites to form a ternary cyclic complex.

The finding that the two sulfhydryl groups SH_1 and SH_2 are located with 12–14 Å is of considerable interest in view of the recent low resolution structure studies of adenylate kinase (Schulz *et al.*, 1973). It appears from this work that the two thiol groups of the molecule are located at about this distance on either side of a 10 Å deep cleft thought to be the active site region. The two SH groups react at substantially different rates with the fluorogenic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Price, 1972). Reaction of the "fast" group

with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole leads to complete inactivation of the enzyme, while adenine nucleotide substrates produce a differential effect on the rate of reaction of the two groups reminiscent of the reactivity of the two SH groups of myosin in the presence of ADP and ATP.

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Oxidation and Sulfonation of the Highly Reactive Sulfhydryl Groups of Muscle Phosphorylase *b*†

Alfred Schwab‡ and Aaron Lukton*

ABSTRACT: The reaction of muscle phosphorylase *b* with a threefold molar excess of *o*-iodosobenzoate leads to aggregation of the enzyme and a 94% loss of enzyme activity. Substrates and AMP have little or no effect on the inactivation of phosphorylase *b* by *o*-iodosobenzoate. Dithiothreitol restores the activity of *o*-iodosobenzoate-inactivated phosphorylase *b* to 92% of the control activity and causes reformation of a dimeric structure which is similar to that of the native enzyme. Treatment of oxidized phosphorylase *b* with $^{35}SO_3^{2-}$ results in the sulfonation of 1.3–1.6 sulfhydryl groups/molecule of phosphorylase *b* dimer. The S-sulfonated enzyme has 1.3–2.0 fewer sulfhydryl groups available for rapid reaction with iodoacetamide than native phosphorylase *b*. Phosphorylase *b* that has 3.0–3.2 rapidly reacting sulfhydryl groups alkylated, by reaction with iodoacetamide, retains 73–85% of its activity after reaction with *o*-iodosobenzoate. It is concluded that those sulfhydryl groups of phosphorylase *b* that react rapidly with iodoacetamide can be oxidized by *o*-iodosobenzoate and subsequently sulfonated by reaction with sulfite ion. The inactivation of phosphorylase *b* by *o*-iodosobenzoate is attributed to the change in the state of aggregation of the enzyme that occurs when the highly reactive sulfhydryl groups are oxidized. S-Sulfonated phosphorylase *b* has 59–68% of the control activity and, in the absence of sulfite ion, sediments in the ultracentrifuge as a mixture of dimer (major component) and tetramer. S-Sulfonation of the highly reactive sulfhydryl groups of phosphorylase *b* leads to a loss in the homotropic cooperativity of AMP sites and a threefold increase in the K_m for AMP; these effects can be reversed by dithiothreitol.

Battell *et al.* (1968a) found that the reaction of two sulfhydryl groups of rabbit skeletal muscle phosphorylase *b* with iodoacetamide, or with various other sulfhydryl reagents, does not cause any loss of enzyme activity. Recently, Zarkadas *et al.* (1970) have reported that 3.1 sulfhydryl groups of freshly prepared phosphorylase *b* can react rapidly with iodoacetamide without loss of enzyme activity.

Gold and Blackman (1970) have shown that up to 3.6 sulfhydryl groups of phosphorylase *b* are exceptionally reactive with 2,4-dinitrochlorobenzene and have concluded that these sulfhydryl groups are identical with those that react rapidly with iodoacetamide. However, dinitrophenylation of the rapidly reacting sulfhydryl groups of phosphorylase *b* leads to a decrease in the affinity of the enzyme for AMP and glucose 1-phosphate, and a small decrease in the V_{max} (Gold, 1968). Apparently, modification of the same sulfhydryl groups of phosphorylase *b* with different reagents can lead to different effects on enzyme activity.

Aggregates of phosphorylase *b* that disappear upon addition of mercaptoethanol are sometimes seen in the ultracentrifuge (Madsen and Cori, 1956; Seery *et al.* (1967)). Battell *et al.* (1968b) have suggested that such aggregates are due to the formation of intermolecular disulfides by the highly reactive sulfhydryl groups. These investigators also suggested that the reactive sulfhydryl groups may play a role in regulating the ac-

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