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Effect of Bridging the Two Essential Thiols of Myosin on Its Spectral and Actin-Binding Properties†

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ABSTRACT: The circular dichroic and fluorescent spectral properties of the myosin head (subfragment I (SFI)) modified by covalently bridging the two essential thiol groups have been examined. CD spectra of SFI with the two thiols linked through reaction with a bifunctional reagent, *N,N'-p*-phenylenedimaleimide, show enhancement of the 282-nm minimum similar to that observed for the long-lived kinetic intermediate (Mg**MgADP·P_i) formed during the ATP cleavage reaction. No significant perturbation of the CD band at 282 nm is seen on blocking both thiol groups with the monofunctional reagent *N*-ethylmaleimide. The fluorescence emission maximum also shifts to lower wavelengths following covalent bridging (from 343 to 340 nm), but no change in fluorescent intensity has been detected. Formation of the covalent bridge completely inhibits interaction of the modified protein with F-actin. These results suggest that the local conformational state of the polypeptide chain formed on bridging the two thiol groups exhibits certain similarities with the state produced following binding of MgATP to native myosin.

Recent stopped-flow kinetic studies have provided a detailed reaction sequence for cleavage of MgATP by myosin. The reaction takes place in a series of steps (at least seven) which lead ultimately to release of P_i and MgADP. These different states have been characterized by a variety of optical techniques employing rapid and steady-state analyses by fluorescence (Werber et al., 1972; Mandelkow and Mandelkow, 1973a,b; Bagshaw et al., 1974), uv difference spectroscopy (Morita, 1967), circular dichroism (Murphy, 1974), and ESR spectroscopy (Seidel and Gergely, 1973; Stone, 1973). A primary feature of the MgATP–myosin interaction is the existence of a relatively long-lived intermediate, designated M**MgADP·P_i (Bagshaw et al., 1974), which is responsible for the very low turnover rate of the substrate. The conformational state M**MgADP·P_i is observed only in the presence of MgATP and is not detected when the products of hydrolysis, P_i and MgADP, or nonhydrolyzable analogues of the substrate, e.g., MgAMP-PNP or MgAMPPCP, are mixed with myosin. To account for the unique character of this conformation and for the slow rate of its decay to the subsequent products, it has been proposed that MgATP forms a stable cyclic ternary complex with the two essential thiol sites within each head of myosin, SH_1 and SH_2 (Burke et al., 1973; Reisler et al., 1974a).

If formation of the unique conformation of the M**MgADP·P_i state involves a structural transition which brings the SH_1 and SH_2 groups into close steric proximity, and if these two groups could be covalently "trapped" in this conformation, it may be reasoned then, that the optical properties of the polypeptide chain in this state might resemble the long-lived intermediate even in the *absence* of the substrate.

In the study to be described below, we have determined the circular dichroism and fluorescence spectra of subfragment I modified by covalently linking the two essential thiols with the bifunctional reagent *N,N'-p*-phenylenedimaleimide. Our experiments indicate that the CD properties of the covalently formed ring structure and the

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M**MgADP·P_i intermediate are indeed similar. Fluorescence studies demonstrate that comparable shifts in the wavelengths of maximum fluorescence emission are also displayed by both structures.

It was also of interest to see whether covalent stabilization of the ring structure would itself preclude binding of myosin to actin in the absence of MgATP. Our results indicate that this is indeed the case. Additionally, it appears that formation of the ring occurs in the absence of any gross change in the three dimensional geometry of the subfragment I molecule. Hydrodynamic studies employing the differential velocity sedimentation method show no detectable difference in the shape of the myosin head in the M**MgADP·P_i and unliganded conformations.

Materials and Methods

Glass distilled water was used throughout and inorganic salts and reagents were of analytical grade. *N*-Ethylmaleimide was the product of Eastman Kodak Co. (Rochester, N.Y.) and *N,N'-p*-phenylenedimaleimide (pPDM[1]) was obtained from The Aldrich Chemical Co. (Milwaukee, Wis.). The preparation of myosin has been described elsewhere (Godfrey and Harrington, 1970). Subfragment I (SFI) was prepared by reacting myosin (1.75%) in the form of a precipitated slurry in ammonium acetate (0.2 M, pH 7.0) for 5 and 7 min at 15 °C with papain (0.03 mg/ml myosin). Actin free of the regulatory proteins tropomyosin and troponin was prepared according to the method of Bailin and Bárány (1972).

The preparations of subfragment I, modified by selective reaction of either the SH_1 or SH_2 sulfhydryl groups with NEM or by coupling these two groups covalently with the bifunctional reagent *N,N'-p*-phenylenedimaleimide, have been described elsewhere (Reisler et al., 1974a,b). We have recently found that, by employing low ionic strengths and low temperatures ($\mu < 0.05$ M, T 5 °C; Schaub et al. (1975)), the bridging reaction between the SH_1 and SH_2 groups of myosin (or subfragment I) can be achieved with stoichiometric amounts of pPDM (1 mol of pPDM/mol of myosin "head"). In this situation, sodium dodecyl sulfate gel electrophoretic analyses show no evidence of interchain cross-linking and the time course of ATPase activity changes is very similar to that reported previously (Reisler et al., 1974b). The employed stoichiometry of 1:1 for pPDM to myosin "head" and the lack of interchain cross-linking establish that the two essential thiol groups are bridged on each head. Protein concentrations were determined by the biuret method.

Conformational Studies. A Cary Model 60 spectropolarimeter with auxillary CD attachment was utilized to obtain circular dichroic spectra. Instrument calibration was checked with a standard solution of camphor -d_{10}-sulfonic acid. Solutions were placed in a cell of 10-mm path length and measurements were made at 25 °C. Protein concentrations were 2.0 or 2.5 mg/ml. The concentration of MgATP was 5×10^{-5} M. The contribution of free nucleotide to these spectra was subtracted where appropriate and results are presented as molar ellipticities, based on a molecular weight of subfragment I of 1.15×10^5 g/mol.

Circular dichroism spectra were measured over the wavelength range 250–310 nm. In this range the band occurring in the 290–299 nm region can be assigned mainly to tryptophyl residues, whereas the band in the region 280–285 nm may have contributions from both tryptophyl and tyrosyl residues. Wavelength spectra between 250 and 280 nm include contributions from nucleotides as well as changes stemming from the polypeptide chain conformation (see Murphy, 1974, and references therein for a discussion of these assignments). An Aminco spectrofluorimeter equipped with a recorder was employed for fluorescence measurements using the same ionic conditions as those employed by Werber et al. (1972). The protein concentration in these studies was 0.14–0.16 mg/ml and that of the nucleotide was 0.2×10^{-4} M. These measurements were all made at 25 °C in a buffer solution consisting of 0.5 M KCl, 0.05 M Tris-HCl at pH 7.9. We employed a modified BASIC computer program kindly provided by Dr. Robert P. Detoma to smooth the experimental data and compute the derivative ($dI/d\lambda$) of the fluorescence intensity vs. wavelength profile. The derivative data allow for easy and accurate assignment of the emission maxima. Incident light of 295 nm was employed for fluorescence excitation (Werber et al., 1972), limiting the emission spectrum to contributions from the tryptophyl residues of subfragment I. Relative fluorescence intensities were calculated with respect to that of native subfragment I with appropriate corrections for dilutions in the case of added ligands.

Differential sedimentation velocity experiments were conducted at 25 °C with a Spinco Model E ultracentrifuge equipped with interference optics and a uv scanner. Runs monitored by the scanner at 290 nm were made at 56 000 rpm in 12-mm double sector cells. The solvent limb of the cell was filled with a solution of subfragment I at a concentration of 1 mg/ml. The solution limb contained the same concentration of subfragment I and, in addition, MgATP at a concentration of 2.5×10^{-4} or 5×10^{-4} M. When interference optics were employed, the speed was 40 000 rpm and the concentrations of protein and nucleotide in this case were 5 mg/ml and 10^{-3} M, respectively.

Actin-Binding Studies. We have employed turbidity and viscosity measurements to detect interaction between actin and native or modified myosins. The protein concentration in these studies was 1 mg/ml for both actin and myosin. Turbidity measurements of the isolated components and the mixtures were performed at 5 °C at 350 nm in matched semi-micro quartz cuvettes employing a Beckman DU spectrophotometer. Viscosity measurements of actin, myosin, and mixtures of these two proteins were made with a modified Ostwald viscometer at low shear (average shear gradient of 158 s^{-1}) supported in a thermostated water bath (5.9 ± 0.01 °C). Measurements of the outflow time (130 s for solvent) were made on 2.0-ml samples and were repeated four to five times on each solution. These results were averaged and converted into relative viscosities, η_{rel}. The viscosity of the mixtures (actin and myosin) was compared with the viscosity expected for a mixture of two noninteracting components A and B (see Szent-Györgyi, 1951), where

$$\log \eta_{rel}(A + B) = \log \eta_{rel}(A) + \log \eta_{rel}(B)$$

The solvent employed in all cases was 0.5 M KCl, 0.005 M Tris, pH 7.9.

Results and Discussion

Circular Dichroism and Fluorescence Spectral Changes Associated with Ring Formation. Murphy (1974) has recently demonstrated that the circular dichroic spectrum of

[1] Abbreviations used: SFI, subfragment I; NEM, *N*-ethylmaleimide; pPDM, *N,N'-p*-phenylenedimaleimide; SH_1 and SH_2, the two essential thiol sites within each head of myosin; HMM, heavy meromyosin.

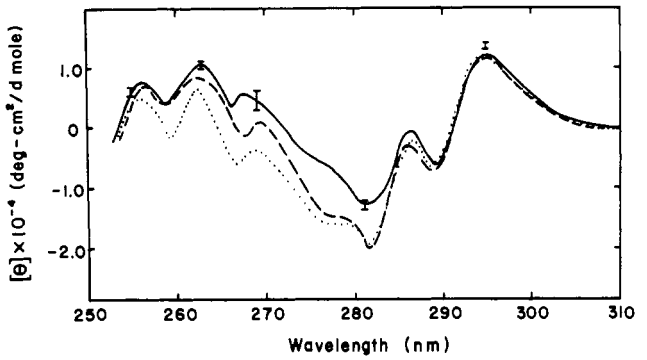

FIGURE 1: Circular dichroic spectra for SFI (—), SFI**MgADP·P_i (- - -), and pPDM SFI in the absence of MgATP (. . .). See Materials and Methods for experimental conditions. Bars (I) represent amplitude of noise about mean position.

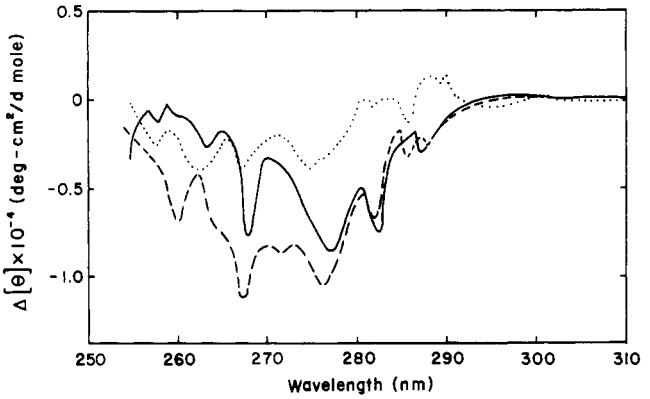

FIGURE 2: Circular dichroic difference spectra for pPDM SFI (- - -), SH_1NEM·SH_2 NEM SFI (. . .), and SFI**MgADP·P_i (—) vs. unliganded unmodified SFI.

HMM[1] shows distinct changes on addition of MgATP, the most characteristic feature being a marked enhancement of the 282-nm dichroic band. This change has been assigned to formation of the steady-state complex (HMM**MgADP·P_i) since it does not occur on binding of MgAMP-PNP or MgADP, i.e., on formation of the pre- and post-steady-state complexes. The changes induced in the CD spectrum of SFI by MgATP binding are shown in Figure 1 and can be more precisely displayed as a CD difference spectrum as shown in Figure 2. The binding of MgATP to SFI results in the formation of three distinct minima at 268, 276, and 282 nm.

Since we have postulated that the steady-state intermediate is a cyclic structure involving interaction between MgATP and the two essential sulfhydryl groups, it was of interest to compare its spectrum with that obtained following covalent ring closure of these groups with the bifunctional reagent *N,N'-p*-phenylenedimaleimide (pPDM) (Reisler et al., 1974b). It was conceivable that, if by covalently binding these two essential thiols the tertiary structure of SFI is locked into a conformation inducible by binding MgATP, the CD spectrum of the covalent ring structure should in the absence of MgATP be similar to that of the native steady-state complex formed in the presence of MgATP. An examination of the spectra shown in Figures 1 and 2 is in accord with this proposal. The presence of the difference minima at 267, 276, and 282 nm for the covalently bridged SFI suggests a conformation of the polypeptide chain similar to that of the steady-state complex (Figure 2). The minima observed at 267 and 276 nm appear to

Table I: Perturbation of 282-nm Circular Dichroic Band Induced by Ligand Addition or Modification.[a]

Protein	Ligand	θ_{282}(system)/θ_{282}(SFI)
SFI		1.0
SFI	MgATP	1.5
pPDM SFI		1.5
SH_1 NEM-SH_2 NEM SFI		1.0

[a] The magnitude of the 282-nm CD band for unliganded, native subfragment I has been assigned the value 1.0 as the basis of reference.

Table II: Effect of Ligand Binding and Modification on Fluorescence Properties of Subfragment I.

Protein	Ligand	λ_{max}	I_{340}(system)/I_{340}(SFI)
SFI		343	1.00
SFI	MgATP	340	1.17
pPDM SFI		340	1.00
SH_1 NEM-SH_2 NEM SFI		342	1.00

arise primarily from local perturbations in the polypeptide conformation, rather than from the nucleotide, since they are observed with pPDM SFI in the absence of nucleotides. That the observed spectral changes result from the steric restraints imposed by the ring is strongly suggested by the fact that modification of both thiol groups with the monofunctional reagent NEM produces minimal spectral perturbation in the range of 280–290 nm (see Figure 2). The results of the CD measurements are summarized in Table I, where the relative change in the 282-nm dichroic band has been employed to estimate conformational perturbation.

The work of Werber et al. (1972) has shown that the conformation of myosin (or subfragment I) in the presence of MgATP and CaATP is characterized by two features of the fluorescence emission spectrum: the wavelength of the emission maximum of the steady-state intermediate is shifted to lower wavelengths by about 3 nm in comparison with that of myosin alone and the fluorescence intensity increases significantly (about 17%) compared with that of the control. We have utilized these features to monitor changes in the conformation of subfragment I accompanying covalent bridge formation between the two essential sulfhydryl groups.

The results presented in Table II show that the emission maximum of the *N,N'-p*-phenylenedimaleimide-treated subfragment I shifts to a lower wavelength (340 nm) compared with that found for the NEM-treated subfragment I (342 nm) or for unmodified subfragment I (343 nm) suggesting that the local environment around the fluorescence chromophore is perturbed by bridging the SH_1 and SH_2 groups. The fact that a similar wavelength shift is seen when MgATP is added to unmodified subfragment I (Table II, see also Werber et al., 1972) again points to structural similarity between the steady-state intermediate and the covalently bridged subfragment I. However, formation of the covalent bridge does not lead to an increase in the intensity of fluorescence emission as is found in the case of the steady-state intermediate (Table II). The reason for this is not clear at the moment but it is not expected that covalent

Table III: Turbidity and Viscosity Data Showing the Effect of Myosin Modification on Actin-Binding Capacity.

System	Σ Components	Mixture	Mixture + MgATP
A. Turbidity[a]			
Actin + myosin	0.310	0.390	0.326
Actin + pPDM myosin	0.312	0.309	0.305
Actin + SH_1 NEM-SH_2 NEM myosin	0.309	0.383	0.314
B. Viscosity[b]			
Actin + myosin	0.28	0.71	0.25
Actin + pPDM myosin	0.28	0.27	0.27
Actin + SH_1 NEM-SH_2 NEM myosin	0.28	0.52	0.23

[a] Absorbance 350 nm. [b] Log η_{rel}.

bridging would provide an environment for the fluorescent chromophores identical with that obtained when the Mg-nucleotide complex is present in the active-site cleft. Addition of MgATP to the covalently bridged subfragment I is not accompanied by further spectral perturbations, indicating either that MgATP does not bind to a covalently bridged myosin head or if it does bind such binding proceeds without a detectable effect on its CD or fluorescence properties.

Actin-Binding Studies. The binding of SFI to actin is known to be extremely sensitive to the conformational states of the SFI entity and the transitions between these states occurring during the hydrolysis of MgATP. For example, binding of MgATP to the myosin component of the actomyosin complex results in extremely rapid dissociation of the protein-protein complex. Present evidence indicates that both the isomerization step and the cleavage step occur on the dissociated myosin head (Lymn and Taylor, 1971; Bagshaw et al., 1974). Since actin activates myosin Mg-ATPase, it must recombine with myosin at some subsequent stage of the hydrolytic cycle. (For a detailed account of the conformational states of myosin existing in the hydrolysis sequence, see Bagshaw et al. (1974).) If the activating effect of actin results from opening of the proposed "ring" complex, thereby releasing the conformation of the M**MgADP·P_i state and allowing for the rapid release of the hydrolysis products, we may inquire whether, in a situation where the "ring" *cannot* be opened, this state would preclude interaction of SFI with actin.

The interaction of actin and myosin can be conveniently monitored by absorbance readings at 350 nm (Lymn and Taylor, 1971; Fraser et al., 1975). In the absence of interactions the expected turbidities for mixtures of actin and myosin will be given by the sum of their respective contributions (column 2, Table III). For actomyosin, acto-pPDM myosin, and acto-SH_1NEM·SH_2NEM myosin this value is 0.310 ± 0.002. The turbidity value (shown in column 3, Table III) for the native actomyosin solution in the absence of MgATP is significantly higher (0.390) and is indicative of interaction between the two proteins. The addition of MgATP to this system causes a decrease in the turbidity to a value of 0.326 (column 4, Table III) consistent with a dissociation of the actomyosin complex. These results may be contrasted with that obtained for the acto-pPDM myosin system (row 2, Table III). In this case the turbidity in the absence of MgATP (0.309) is essentially identical with the value expected for noninteracting systems (0.312; compare columns 2 and 3, row 2, Table III). Thus, as might be expected, the addition of MgATP to this material does not lead to any significant change in turbidity. To investigate the possibility that the loss of actin binding arises from simple blocking of the two essential thiol groups we have examined the interaction of SH_1NEM·SH_2NEM myosin and actin. These results are presented in row 3 of Table III and demonstrate that, in the absence of MgATP, SH_1NEM·SH_2NEM myosin binds almost as effectively to actin as native myosin.

We have also employed viscosity as a measure of the interaction between actin and myosin and the results of this study are summarized in Table III. In the absence of MgATP both actomyosin (row 1) and acto-SH_1NEM·SH_2NEM myosin (row 3) exhibit log η_{rel} values which greatly exceed the values computed for the respective noninteracting situations. On the other hand, acto-pPDM myosin (row 2) in the absence of MgATP has a value of log η_{rel} closely similar to that expected for the noninteracting situation. Thus when MgATP is absent both native myosin and SH_1NEM·SH_2NEM modified myosin strongly interact with actin, whereas pPDM myosin shows no tendency to interact. This conclusion is supported by the viscosity data obtained for these systems in the presence of MgATP. In this case log η_{rel} for both the actomyosin and acto-SH_1NEM·SH_2NEM myosin decrease significantly whereas no effect is observed in the case of acto-pPDM myosin (column 4, Table III).

The above turbidity and viscosity data indicate that covalently bridged pPDM myosin is unable to interact with actin in the absence of MgATP. Since interaction with actin is not precluded by blocking the thiol pair with monofunctional NEM, we can rule out the possibility that simple blocking of these thiols alone prevents interaction with actin. Similar results have also been obtained by Lamed et al. (1976). They found that pPDM bridged SFI does not bind to actin columns, whereas the SH_1NEM·SH_2NEM-modified protein retains its ability to bind to actin. Although we cannot at present rule out the possibility that the phenyl ring prevents interaction by steric interference, our results suggest that the binding of actin and the opening of the ring may be coupled events and that one process cannot occur without the other.

Effect of Ring Formation on the Hydrodynamic Properties of Subfragment I. Since the steady-state intermediate exists in a distinct conformational state it is important to determine whether this state is also associated with a gross alteration in the shape of the subfragment I molecule. Such a shape change might result in an alteration of the friction factor and thus a change in the sedimentation coefficient of the complex. Earlier physiochemical and hydrodynamic studies (Gratzer and Lowey, 1969; Godfrey and Harrington, 1970) failed to show any significant change in the secondary structure or shape of the myosin molecule in the presence of MgATP. Since it might be argued that a conformational transition within a local segment of myosin may produce too small an effect on the friction factor of the intact native molecule to be detected, we have searched for a shift in the sedimentation coefficient of subfragment I on binding MgATP. In these studies, we have employed the sensitive differential sedimentation technique (see Methods). Solvent conditions and temperature were also chosen (0.5 M KCl, 25 °C) to ensure that the predominating

species in solution during the sedimentation experiments was the long-lived intermediate (SFI**MgADP·P_i). The results, spanning a protein concentration range of 1 to 5 mg/ml show, however, no measurable difference in sedimentation coefficient between the two species SFI and SFI**MgADP·P_i as measured either by the scanning or the Rayleigh interference optical system of the ultracentrifuge. Thus, although the data derived from the CD and fluorescence studies are consistent with the suggestion that the long-lived intermediate results from formation of a cyclic complex between the two essential thiol groups, generation of this conformational state is apparently not accompanied by a detectable change in the shape of the molecule. The tendency of pPDM-bridged subfragment I to aggregate slowly on standing rendered this material unsuitable for differential velocity sedimentation experiments.

Conclusions

The results of the present study suggest that formation of the steady-state intermediate following binding of MgATP to myosin (M**MgADP·P_i) may involve a local structural transition in SFI such that the two essential thiol groups SH_1 and SH_2 are brought into close spatial proximity. It is known that when the protein binds MgADP, this thiol pair is approximately 12–14 Å apart since in this situation they can be cross-linked by the reagent *N,N'-p*-phenylenedimaleimide of this cross-linking span (Reisler et al., 1974b; see also Materials and Methods). A comparison of the circular dichroic and fluorescence properties of subfragment I containing the pPDM bridge with that of the steady-state intermediate shows interesting conformational similarities. Thus the circular dichroic difference spectra of these species with respect to unliganded, native subfragment I show the presence of three minima at 267, 276, and 282 nm, the latter corresponding to the major spectral change attributed by Murphy (1974) to formation of the steady-state conformation. With respect to the fluorescence spectra, both species show a common bathochromic shift of about 3 nm compared with unliganded native subfragment I, although in this case no fluorescence enhancement is seen for the pPDM bridged protein. The reason for this is unclear at present.

The evidence that the bridging of the sulfhydryl groups is responsible for these conformational similarities and not simply the blocking of these groups is provided by the observation that, on specific modification of both SH_1 and SH_2 by the monofunctional reagent NEM, the protein exhibits no changes attributable to the steady-state conformation or to the pPDM state (Figure 2, Tables I and II). Furthermore, the fact that both turbidity and viscosity data (Table III) show that, in the absence of MgATP, pPDM-bridged myosin does not interact with actin suggests that by restricting the two essential sulfhydryl groups to a 12–14 Å separation the conformational constraints prevent interaction with actin.

Our hydrodynamic studies employing differential velocity sedimentation methods show no detectable change in the sedimentation coefficient of the SFI molecule on adopting the steady-state conformation. We may therefore conclude that, although there is evidence suggesting that the conformation of the steady-state complex M**MgADP·P_i is one in which the essential thiol pair is constrained to a separation distance of 12–14 Å, the attainment of this geometry is not accompanied by a measurable change in the shape of the molecule.

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