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DOES MYOSIN-SUBSTRATE INTERACTION IN VITRO RESULT IN A DELOCALIZED CONFORMATION CHANGE?

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The effect of substrate on the far UV (185–250 nm) and near UV (250–325 nm) circular dichroism (CD) of myosin and heavy meromyosin (HMM) was studied. The following results were obtained with the addition of ATP (during various conditions of hydrolysis), ADP, and pyrophosphate: (1) no changes were observed in the far UV CD, (2) ATP and ADP perturbed the near UV CD only at spectral regions below 280 nm coinciding with the regions of their optical activity, (3) the optically inactive pyrophosphate caused no change in the near UV CD, and (4) myosin and HMM gave exactly the same results. These results suggest that myosin-substrate interaction in vitro does not result in a delocalized conformational change.

INTRODUCTION

Current models of muscle contraction requiring the myosin "cross bridge" to attach to the thin filament and impart a mechanical thrust invariably assume a conformation change of the myosin molecule (1, 2). Although it is never implicitly stated that this change be a delocalized conformation change, in contrast to a localized conformation change, nevertheless it is implied by the requirements of the models. By the term "delocalized conformation change" we mean any change in the protein geometry which results in the transmission of conformational distortion over great distances from the point of inception. The term "localized conformation change" is used to indicate any change in which the conformational distortion is restricted to the point of inception. In the case of most enzymes this would be areas not much greater than the enzyme's active site. Delocalized conformation change is of great importance in mechanisms of biological function since it provides the means by which information as mechanical free energy can be transferred through and between macromolecules (3). In contrast, localized conformation changes are of importance in mechanisms of chemical reactions where there is a need for localization of energy.

Since the hydrolysis of ATP by myosin is an essential step in the mechanism of muscle contraction, the question posed by the title is an important one. Results of the previous studies on the effects of substrate on the conformation of myosin and heavy meromyosin (HMM) do not provide the necessary information for answering this question (4–10). The conclusion reached by these studies, that there is no net change in the secondary structure of the enzyme during steady state hydrolysis of ATP, neither eliminates nor verifies the possibilities of delocalized conformation change during hydrolysis.

We report circular dichroism (CD) studies of the effects of substrate on the conformation of myosin and HMM which provide the necessary information to answer the title question. The advantage of using CD instead of optical rotatory dispersion (ORD), which was used for such studies in the past, is that one can study the effects of the amide and the aromatic/cystine residue electronic transitions separately (11). Our results show that no significant delocalization of conformation change occurs during in vitro ATP hydrolysis. Therefore the conformational behavior of myosin during this hydrolysis is similar to the characteristic behavior of most enzymes involving only localized conformation change during enzymatic activity.

MATERIALS AND METHODS

ADP, ATP, pyruvate kinase, phosphoenol pyruvate, trypsin, and trypsin inhibitor were purchased from Sigma and used without further purification. Double distilled water was used with the second distillation being performed in all glass still after having passed through a demineralizer. All other chemicals used were of reagent grade.

Rabbit skeletal myosin and HMM were prepared as previously described (12, 13). Protein concentrations were determined by the micro-Kjeldahl nitrogen procedure using a nitrogen factor of 6.2. The ATPase activity of myosin and HMM was determined by the method of Green and Mommaerts (14) with a Radiometer automatic pH-stat system. The assay was conducted at pH 7.0 and 25°C with 0.01 N NaOH as the titrant.

CD and ORD spectra were recorded with a Cary model 60 spectropolarimeter equipped with a model 6003 CD accessory. Instrument calibration, optical cell calibration, temperature control, and experimental procedures have previously been given in detail (15, 16). CD was expressed in terms of mean residue molecular ellipticity, $[\theta]$, for which units are degree square centimeters per decimole. $[\theta]$ was calculated from measured ellipticity by the following:

$$[\theta] = \frac{\theta^{\circ} M}{10\, l\, c_0}$$

where θ° is the measured ellipticity in degrees, M the residue gram molecular weight (here assumed to be 115 for myosin and HMM), l the pathlength in cm, and c_0 the concentration in grams per cm^3. ORD was expressed in terms of mean residue molecular rotation [m], which is given by:

$$[m] = \frac{M\alpha}{dc}$$

where α is the measured rotation in degrees, d the pathlength in decimeters and c the concentration in grams per 100 ml. The units for [m] are the same as for $[\theta]$. ORD spectra were calculated from the experimental CD spectra by evaluation of the Kronig-Kramers integral transform equation with a revised version of a computer program written by Thiery (17, 18). Calculations were carried out with an IBM Systems 370/165 at the Instruction Research Computer Center at The Ohio State University. Details of the computational procedure have been given by Cassim and Yang (16). Absorption spectra were measured by a Cary low UV model 118C double beam recording spectrophotometer with scattered transmission accessory. Details of experimental procedures have been published elsewhere (19).

RESULTS AND DISCUSSION

Far UV Optical Activity Studies (185–250 nm)

The CD of myosin in 0.5 M KF at pH 7.0 and 2°C is reproduced in Fig. 1. Ellipticities below 185 nm have been extrapolated. The manner and validity of such extrapolations have previously been given by Cassim and Yang in detail (16). The spectrum is very similar to the far UV CD of myosin previously reported by several investigators and of partially helical proteins in general (11, 20, 21).

ORD was computed from the CD shown in Fig. 1 by application of the Kronig-Kramers integral transform equation. A typical comparison of the computed ORD and experimentally determined ORD is shown in Fig. 2. The experimental ORD was recorded with an aliquot of the same solution used for CD measurements. It is apparent that in the accessible wavelengths there is an excellent correspondence at every wavelength between calculated and experimental spectra. In fact, the absolute percent deviation between experimental and calculated mean residue molecular rotation at the negative trough

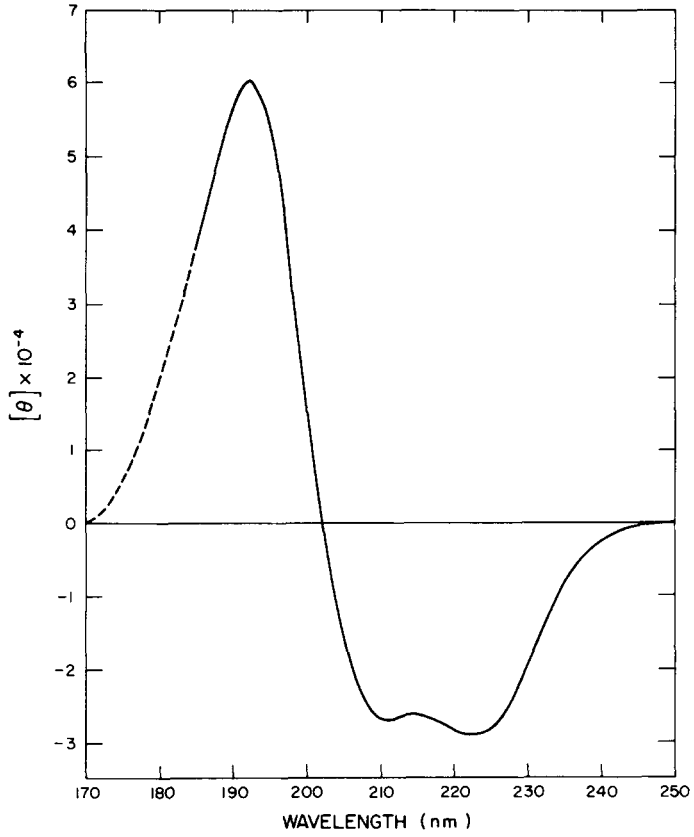

Fig. 1. Circular dichroism of myosin in 0.5 M KF at pH 7.0 and 2.0°C. solid line represents experimental spectrum; dashed line, extrapolations of the positive ellipticity into the inaccessible spectral regions below 185 nm. θ is mean residue molecular ellipticity in degree square centimeters per decimole.

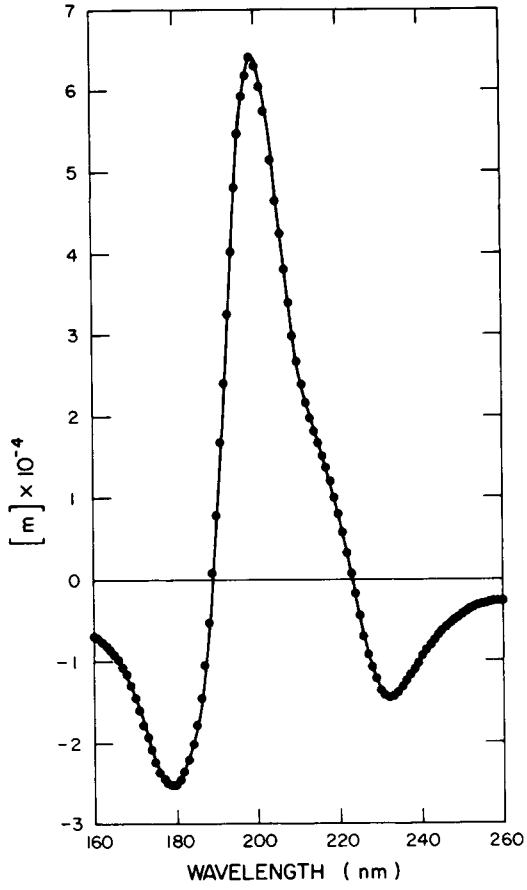

Fig. 2. Comparison of the computed and experimental optical rotatory dispersion of myosin in 0.5 M KF at pH 7.0 and 2.0°C. Solid line represents experimental spectrum; circles represent computed spectrum based upon data shown in Fig. 1 and an application of the Kronig-Kramers transform equation. [m] is mean residue molecular rotation in degree square centimeters per decimole.

(232 nm) and positive peak (199 nm) is 1.5% and 1.6%, respectively. Similar results were obtained with myosin in 0.25 M K_2 HPO_4 at pH 7.0 and 2°C. However, the correspondence between calculated and measured ORD was not as good as observed for the fluoride salt case because of higher absorbance of the phosphate salt which caused some uncertainty in data for lower wavelengths. The same excellent correspondence was observed in similar spectral transformation studies with three helical homopolypeptides (16). However, in several proteins studied similarly, noncorrespondence has been observed in some cases (22). The significance of these spectral transform studies of myosin is: (1) it gives confidence in the accuracy of the experimental data; (2) it shows that simple polypeptides are good spectral analogs of myosin in the far and vacuum UV; (3) it shows that side-chain optical activity in the near UV can be treated separately from the protein backbone amide optical activity in the far UV.

Having demonstrated that the myosin optical activity in the far UV can be measured with the same experimental certainty as the optical activity of simple polypeptides in the same spectral region, studies of the effects of substrate and inhibitor on the

optical activity of myosin were made. Since most of the present knowledge of myosin ATPase activity in vitro is based on studies in a high ionic strength environment of chloride salts at room temperature, our substrate interaction studies of myosin were carried out under these conditions. Of course, the relatively high absorption of nucleotides and chloride salts limited meaningful studies to about 200 nm. No changes were observed in the spectra with protein concentrations which varied from 0.147 mg/ml to 1.49 mg/ml, $MgCl_2$ concentration from 1 mM to 6 mM, and ATP concentration from 10 μM to 182 μM. The state of the ATP hydrolysis during these studies was determined by following the ATPase activity in the pH-state. No observable changes in spectra were observed whether ATP hydrolysis was in the steady state or ATP hydrolysis was completed or if ADP was added or if pyrophosphate was added. The effects of ATP could not be distinguished from the uncertainty of the measurements themselves which is about ± 0.1% at the 222 nm CD extremum. Similar results were obtained when myosin was replaced by HMM in all of the experiments.

The optical activity of proteins in the far UV has been shown to depend mainly on the interaction between the electronic transitions of the amide groups in the protein backbone. Therefore optical activity in this spectral region is highly sensitive to any perturbation which alters the dissymmetric environment of these amide transitions. It offers the most sensitive means of detecting secondary structure changes of proteins in solutions. Since the optical activity in this spectral region is rather insensitive to tertiary and quaternary structure changes, it provides in principle, a means of discriminating between these and the secondary structural changes. Therefore, it is reasonable to consider a significant change in far UV optical activity of a protein to be indicative of a change in its secondary structure. However, the finding of no change in optical activity upon perturbation cannot be rigorously interpreted as no change in secondary structure, but only that there has been no net change in the secondary structure. The probability that each change in secondary structure may be accompanied by a compensating secondary structural change which result in complete negation of spectral changes is not very high. Nevertheless, the probability is of sufficient significance that for conclusions of high confidence such changes must be ruled out by additional supporting evidence. In the past there has been a tendency to disregard this possibility (10).

Near UV Optical Activity Studies (250–325 nm)

To gain additional information concerning possible conformation changes during enzyme activity of myosin one may extend CD studies to the near UV region. CD of proteins in this spectral region has been attributed in part to interactions of the local protein environment with aromatic amino acid side chains (tryptophan, tyrosine, and phenylalanine) and disulfide groups (cystine) and to interactions between these groups (11). Electronic transitions of these groups may reflect features of both the secondary and tertiary structures and perhaps the quaternary structures as well.

Both the absorption and CD spectra of myosin in the near UV exhibit a very complex spectral pattern due to many overlapping bands, as shown in Fig. 3. A similar near UV CD spectrum for myosin has recently been reported (23). However, several peaks and inflection points found in absorption spectrum at 300.5, 292, 284, 278, 274, 269, and 259 nm (Fig. 3a) can be correlated with extrema found in the CD spectrum at 299.5, 291, 283, 278, 272, 265, and 259 nm (Fig. 3b). No attempts were made to resolve the spectra into Gaussian components because no unique solution is possible with such complex spectra. A very similar spectrum was observed for HMM. However, the mean residue ellipticities

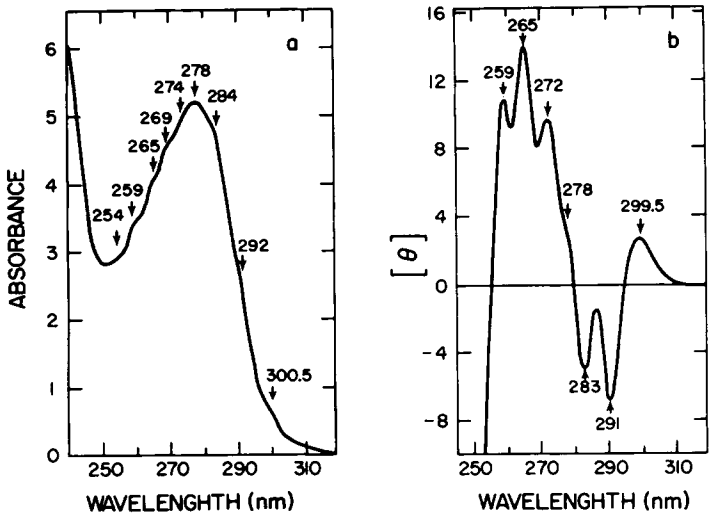

Fig. 3. (a). Absorption spectrum of myosin in the near UV in 0.5 M KCl, 30 mM Tris-HCl at pH 7.0 and 25°C. Absorbance is given as the optical density of a 1% protein solution with 1 cm path length. (b). CD of myosin in the near UV with the identical conditions used for absorption spectrum. [θ] is mean residue molecular ellipticity in degree square centimeters per decimole.

of the aromatic residues of myosin and HMM in the near UV (26–210 per aromatic residue), as shown in Table I, are less than the values (500–1500 per aromatic residue) previously reported for several proteins (11). We have no simple explanation for this difference at the present.

The following observations were noted from the near UV DC studies of myosin-substrate and HMM-substrate interaction under various experimental conditions (ATP in steady state hydrolysis, ATP allowed to hydrolyze completely to ADP in situ, addition of ADP, ATP plus ATP regenerating system with ATP being activity regenerated, ATP plus ATP regenerating system with ATP allowed to hydrolyze completely to ADP in situ, and addition of pyrophosphate):

(1) The CD is unaltered at wavelengths greater than 280 nm under all experimental conditions of substrate addition.

(2) The CD is slightly perturbed at wavelengths less than 280 nm, when ATP is added under steady-state conditions or ATP is allowed to hydrolyze to ADP in situ with and without ATP regenerating system or if ADP is added, resulting in slight increases in intensities of extrema with no significant change in the positions. This change in spectrum is only apparent when a zeroth order correction is made for the significant intrinsic optical activity of ATP and ADP in this region by subtracting the intrinsic CD of the nucleotide from CD of the myosin plus nucleotide.

(3) The CD is slightly perturbed at wavelengths less than 280 nm, when ATP is added with an ATP regenerating system and ATP is actively being regenerated, resulting in a very slight decrease in intensities of extrema with no significant change in their positions. This change is apparent with and without the correction for the intrinsic optical activity of ATP and ADP and in fact is greater without it.

(4) The CD is unaltered at wavelengths less than 280 nm with the addition of pyrophosphate. Details are given in Table I and Fig. 4.

TABLE I. The Effect of Substrate on the Near UV Circular Dichroism of Myosin and Heavy Meromyosin at pH 7.0 and 25°C

MYOSIN Wavelength of extrema in nm	Mean residue molecular ellipticity[1]			
	Without ATP generating system[2]		With ATP generating system[3]	
	No nucleotide	ATP or ADP	No nucleotide	ATP
299.5	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
291	−6.9 ± 0.2	−6.9 ± 0.2	−6.9 ± 0.2	−6.9 ± 0.1
283	−5.1 ± 0.3	−5.1 ± 0.3	−5.1 ± 0.3	−5.1 ± 0.3
278	3.7 ± 0.5	5.7 ± 0.6	3.7 ± 0.5	3.2 ± 0.6
272	9.8 ± 0.4	13.5 ± 0.6	9.8 ± 0.4	8.4 ± 0.5
265	14.0 ± 0.4	17.8 ± 0.8	14.0 ± 0.4	12.2 ± 0.7
259	11.1 ± 0.3	14.6 ± 0.4	11.1 ± 0.3	10.1 ± 0.4
HEAVY MEROMYOSIN Wavelength of extrema in nm	Mean residue molecular ellipticity[1]			
	Without ATP generating system[4]		With ATP generating system[5]	
	No nucleotide	ATP or ADP	No nucleotide	ATP
299.5	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
291	−8.2 ± 0.2	−8.2 ± 0.2	−8.2 ± 0.2	−8.2 ± 0.2
283	−7.0 ± 0.3	−7.0 ± 0.3	−7.0 ± 0.3	−7.0 ± 0.3
278	0.4 ± 0.5	1.0 ± 0.6	0.4 ± 0.5	0.2 ± 0.6
272	4.9 ± 0.5	8.6 ± 0.8	4.9 ± 0.5	3.5 ± 0.6
265	7.4 ± 0.4	11.2 ± 1.2	7.4 ± 0.4	5.1 ± 0.8
259	4.9 ± 0.3	8.4 ± 0.5	4.9 ± 0.3	4.5 ± 0.4

[1] In degree square centimeters per decimole.
[2] 0.5 M KCl, 30 mM Tris-HCl, 6.7 mM $MgCl_2$, protein concentration 4.95 mg/ml, and nucleotide concentration 73 μM.
[3] 0.5 M KCl, 12 mM Tris-HCl, 1 mM $MgCl_2$, 30 μg/ml pyruvate kinase, 4 mM phosphoenol pyruvate, protein concentration 2.66 mg/ml, and ATP concentration 43 μM.
[4] 0.01 M K_2HPO_3, 1 mM $MgCl_2$, protein concentration 4.46 mg/ml, and nucleotide concentration 259 μM.
[5] 0.5 M KCl, 26 mM Tris-HCl, 1 mM $MgCl_2$, 30 μg/ml pyruvate kinase, 4 mM phosphoenol pyruvate, protein concentration 39 mg/ml, and ATP concentration 40μM.
Note: (1) Ellipticities are given on a per residue basis. To convert to a per aromatic/cystine residue basis myosin and HMM values should be multiplied by a factor of about 15 and 13, respectively. (2) Zeroth order correction was made for the intrinsic optical activity of ATP and ADP by subtracting the intrinsic CD of the nucleotide. If no correction is made column two would be identical to column one. (3) 10 hr after adding ATP to myosin or HMM plus ATP regenerating system, when the regenerating system has run down and ATP is completely hydrolyzed, results shown in column four become identical to results of column two.

Two important findings emerge from these results. First, the optically active substrate, ATP, perturbs the near UV spectrum only at the spectral regions below 280 nm coinciding with the regions of its optical activity. The significance of this finding follows from the fact that the CD spectra of proteins in the near UV reflect the environments and interactions of the aromatic/cystine residues of the protein. The CD bands due to these residues are found at various wavelengths both above and below 280 nm. If conformational distortions resulting from nucleotide interaction caused displacement of only a few side chains, then this might not sufficiently alter the environment of enough aromatic/cystine residues to produce a measurable change in the CD spectrum which is a

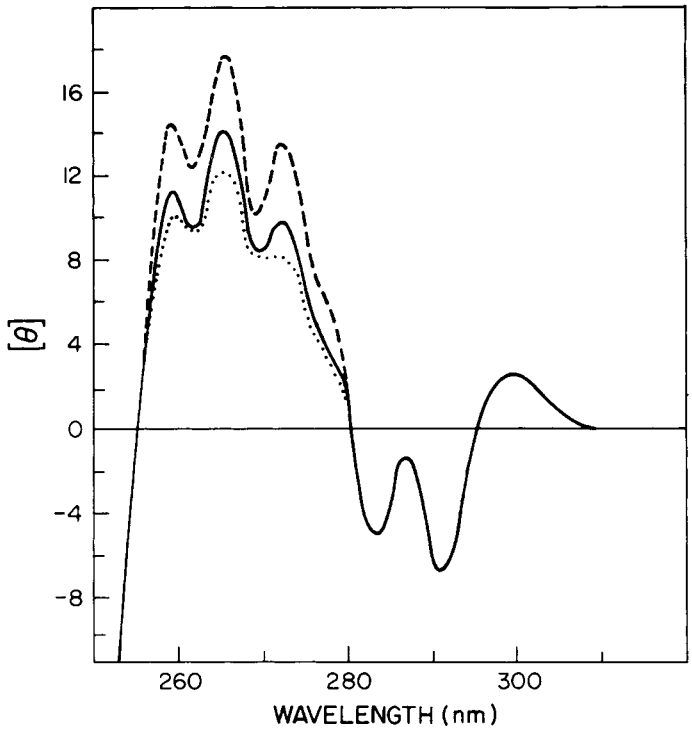

Fig. 4. The effect of a ATP regenerating system on the near UV CD of myosin. Solid line represents myosin in 0.5 M KCl, 12 mM Tris-HCl, 1 mM $MgCl_2$, 30 μg/ml pyruvate kinase, 4 mM phosphoenol pyruvate at pH 7.0 and 25°C, myosin concentration 2.66 mg/ml; dotted line, after adding 42.5 μM ATP; dashed line, 10 hr after adding ATP when pyruvate kinase-phosphoenol pyruvate system has run down and ATP is completely hydrolyzed (note: this curve is the same as the ones obtained for ATP during steady-state hydrolysis or ADP from completed ATP hydrolysis or added ADP, all without the ATP regenerating system). Zeroth order correction was made for the intrinsic optical activity of ATP and ADP by subtracting the intrinsic CD of the nucleotide from the CD of myosin plus nucleotide.

sum of the contributions from all such residues. On the other hand, if this conformational distortion spreads over a much larger portion of the peptide chain or chains, then the environment of a much larger number of such residues should be altered and significant changes in near UV CD would be expected both above and below 280 nm.

The ATPase active sites of myosin are located in the head portion of the molecule. Myosin contains about 280 aromatic/cystine residues per molecule, as indicated by its amino acid composition. More than 80% of these residues are located in the head portion of the molecule and make up approximately 11% of the total number of residues found there. (This is apparently the reason that there was no difference in results when HMM was substituted for myosin in any of the studies.) Assuming that these residues are randomly distributed in the myosin head, one would expect a significant CD change in the near UV both above and below 280 nm if the conformational distortion resulting from the action of the ATP at the active site was transmitted over a major portion of the head. Since no CD changes were observed above 280 nm, this possibility does not seem very likely. Additional support against this possibility is given by the second important finding.

The second important finding was that the optically inactive pyrophosphate, which is a competitive inhibitor of ATP, caused no CD change and that ATP and ADP caused CD changes only at the wavelengths of their own optical activity. This suggests that the observed CD changes are a result of possible π-π^* resonance interactions between the optically active nucleotides and some aromatic residue or residues of the protein and are not the results of large local conformational changes of the protein per se. This possibility is strengthened by the published observation that 6-throinosine triphosphate, a modified nucleotide, perturbs the HMM CD spectrum in the region of its intrinsic optical activity at wavelengths greater than 280 nm and not those below 280 nm as occurred with ATP and ADP (23).

In view of the above discussion let us consider the question, can the possibilities of compensating secondary structure changes during myosin-substrate interaction be ruled out with high confidence? The answer is yes, considering (1) that there has been no spectral change in the near UV over the entire spectral range which can be ascribed to conformation changes; (2) the low probability that changes in both secondary structure and aromatic/cystine residue environment can simultaneously cause CD changes that cancel each other; and, (3) recent observations that small changes (1–7%) in the far UV CD of myosin and HMM, upon temperature, pH, and solvent perturbation are accompanied by significant overall changes in the CD in the near UV (22, 24).

Now for the title question itself, does myosin-substrate interaction in vitro result in a delocalized conformation change? First let us put the distinction between delocalized and localized conformation changes into perspective. By the term "delocalized conformation change" we mean any change in protein molecular geometry which has resulted in the transmission of mechanical free energy over large distances in the protein. Such conformation changes may or may not involve large changes in the protein's secondary structure and shape. In contrast, the term "localized conformation" change is used to indicate any change which does not result in the transmission of mechanical free energy over large distances. These conformational distortions may be restricted to areas no larger than the enzyme's active sites. In the past, the term "large conformation changes" has been used by writers to indicate changes involving large secondary structure changes and overall shape changes of the molecules. It is apparent that large conformation changes are specialized forms of delocalized conformation changes. We feel that the term "delocalized conformation change" is a more useful term since large changes in the secondary structure of proteins are not commonly observed under normal biological conditions. For example, lysozyme and certain heme proteins are known to undergo tertiary and quaternary structure changes without significant second structure changes (25, 26). Delocalization of conformation change is definitely involved in these cases. However, most enzymatic action probably involves localized conformation changes. One must bear in mind that protein interactions with other proteins or with small molecules in which specificity is apparent must involve some kind of conformation change. Therefore, local conformation changes are inherent in every protein interaction, whereas delocalized conformation changes are unique.

Recent studies of myosin-substrate interaction utilizing a number of techniques (electron spin resonance, fluorometry, and difference spectrophotometry) which are highly sensitive to localized conformation changes seemingly have detected such changes (27–34). However, there is no evidence, of course, relating these localized conformation changes directly with the contractile mechanism. It seems reasonable that these changes are essential to the enzymatic function of myosin. But, on the other hand, the very

nature of the contractile phenomenon seems to suggest that the transmission of mechanical free energy by means of delocalized conformation change be an essential part of the contractile mechanism. Although the most probable interpretation of the present CD studies (it is apparent that no unequivocal conclusion is possible or should be inferred from indirect evidence such as spectra) leads to a negative answer to the title question, its significance should not be minimized. Isolated myosin may act as a well-behaved enzyme even in solution. However, when it is isolated from the highly ordered structure of muscle, it may have lost some of those unique characteristics which are essential to its in situ central role in the contractile mechanism. Further experimentation especially under in situ conditions is necessary before this problem can be resolved.

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