Fluorescence Studies on Heavy Meromyosin–Substrate Interaction†

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ABSTRACT: The interaction between heavy meromyosin (HMM) and ATP has been studied by measuring the intrinsic fluorescence of the Trp residues of the protein. The fluorescence changes induced by ATP (17%) were compared to those caused by ADP (5%) and various analogs of ATP. The maximal fluorescence increase (17%) required the presence of Mg^{2+} or Ca^{2+} ions and depended on the structure of the nucleotide. Those structural requirements included: (1) the presence of an amino group on position 6 of the base ring, (2) a triphosphate moiety, and (3) the ability of the enzyme to cleave the phosphate at the γ position. Modification of HMM with *N*-ethylmaleimide abolished its ATPase activity, but not the binding of ATP. The fluorescence changes induced by ATP in this case were much reduced. ATP also causes increased fluorescence with intact myosin and the S-1 fragment. It is proposed that to obtain a large fluorescence change, the enzyme must not only bind its substrate, but also hydrolyze it. Studies of iodide quenching of the Trp fluorescence of HMM suggest that upon addition of ATP 2 out of the approximately 20 Trp residues become inaccessible, whereas only one of them is affected by ADP. It is further suggested that the large effect of ATP on HMM fluorescence is caused by a local conformational change, which is associated with a species formed after the binding of the substrate, presumably the enzyme–product complex.

The important role of ATP in contraction has been established for some time now (Szent-Györgyi, 1951). The details of the interaction of ATP with the contractile proteins, however, are not clear. Enzymatic and nucleotide binding studies indicate that ATP reacts with the globular S-1 subfragment of the myosin molecule (Lowey *et al.*, 1969; Lowey and Luck, 1969). This reaction does not entail large structural changes and the optical rotatory dispersion of myosin and of heavy meromyosin (HMM)[1] remains unaltered by ATP (Gratzer and Lowey, 1969; McCubbin *et al.*, 1967). Nevertheless, several lines of investigation show that ATP induces local changes in conformation in a rather unique manner. The first observation showing a specific effect of ATP on myosin were those of Morita (1967) who found a characteristic absorption change in the tryptophan region of the spectrum. This change was not duplicated by ADP and required the presence of ATP. Once ATP was depleted, the absorption change dropped to the value induced by ADP alone. ATP and ADP also differed in their effect on the fluorescence of ANS bound to myosin (Cheung, 1969). Recently, it was found that the mobility of a spin label combined with a particular thiol group of myosin is increased by ATP (Seidel and Gergely, 1971). The change in electron spin resonance (esr) spectrum specific to ATP persisted as long as ATP was present. The detailed kinetic studies of the presteady state of Lymn and Taylor (1970) and Taylor *et al.* (1970) indicate that the predominant species during ATP hydrolysis is an ADP–myosin complex. In the kinetic scheme proposed by these authors the enzyme–product complex was assumed to be the same as the complex formed on the addition of ADP to myosin or HMM. Seidel and Gergely (1971) pointed out, however, that their esr data and the previous studies of Morita (1967) and of Cheung (1969) indicate that the ADP–myosin complex formed *via* ATP hydrolysis differs from the complex formed from the combination of ADP and myosin. The existence of the two different forms of the ADP complex was also suggested by Viniegra and Morales (1972).

We report here observations on the change of the intrinsic fluorescence of tryptophan residues in HMM, myosin and S-1 subfragment caused by ATP. These fluorescence changes are also specific to ATP and cannot be imitated by ADP alone. Among the factors required for large fluorescence changes is the ability of the enzyme to hydrolyze ATP. Modification of HMM which abolishes the hydrolysis of ATP but not its binding reduces the fluorescence change greatly. Similarly, ATP analogs which are not hydrolyzed by HMM are not much more effective than ADP. The results suggest that ATP affects particular tryptophan residues, and support the notion that the local conformational change induced by ATP is associated with the enzyme–product complex.

Experimental Section

Materials

Myosin was prepared from rabbit muscle as previously described (Szent-Györgyi, 1951). HMM was obtained from myosin by an 8-min digestion at neutral pH at room temperature with trypsin at a ratio of myosin:trypsin, 420:1 (Szent-Györgyi, 1953; Lowey and Cohen, 1962). The S-1 fragment was obtained by treating precipitated myosin for 10 min at room temperature with papain at a myosin:papain ratio of

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[1] Abbreviations used are: AMP-PCP, adenylylmethylenediphosphonate; AMP-PNP, adenylylimidodiphosphate; ANS, 8-anilino-1-naphthalenesulfonate; ATPP, adenosine tetraphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether *N,N'*-tetraacetic acid); HMM, heavy meromyosin; LMM, light meromyosin; P-enolpyruvate, phosphoenopyruvate; S-1, subfragment 1 obtained by papain digestion.

1000:1 (Lowey *et al.*, 1969). The final solution was then dialyzed against a solution of 0.05 M KCl–0.025 M buffer phosphate (pH 7.4) and then concentrated on a UM20 Amicon membrane. The material when tested appeared to be monodispersed in sedimentation studies and had an $s_{20,w}$ = 5.39 S at 1.16% protein concentration (*cf.* Lowey *et al.*, 1969). All the protein preparations were stored in 50% glycerol at −18°. Under these conditions most of the preparations are stable for months, as far as ATPase activity and ability to undergo fluorescence responses are concerned.

Protein concentrations in stock solutions were determined by the Lowry procedure (Lowry *et al.*, 1951) with bovine serum albumin as a standard and by dividing the values by a factor of 1.2 (Szentkiralyi and Oplatka, 1969). The concentrations of protein in the dilute solutions were determined by ultraviolet (uv) absorption, using the following $E_{1\,cm,280}^{1\%}$ values: HMM, 6.33; myosin, 6.35; and S-1, 7.80.

ATP, ADP, AMP, ATPP (93% pure), and GTP were obtained from Sigma Chemical Co. ITP, ADP, AMP, dATP, UTP, CTP, and IDP were from P and L Biochemicals Inc. AMP-PCP was obtained from Miles Laboratories. AMP-PNP (87% pure) was a gift from Dr. R. G. Yount. [^{14}C]ATP (specific activity 32 Ci/mole) was from Schwarz Bio-Research, Inc.

The concentrations of the stock solutions of the nucleotides were obtained from uv absorption spectra (Schwarz BioResearch, Inc., 1954). Several nucleotide triphosphate solutions were also tested for the presence of diphosphates. The concentration of nucleotide diphosphates was determined from the change in absorbance of NADH at 340 nm (Williamson and Corkey, 1969), using the coupled reactions of pyruvate kinase and P-enolpyruvate with lactic dehydrogenase. ATP and AMP-PCP contained less than 0.5% diphosphates; dATP contained 2.8% and ITP 7.6% of nucleotide diphosphate. ADP solutions contained in excess of 90% diphosphate using the same method. All the stock solutions of nucleotides were neutralized and kept frozen at −18°. All the other reagents used were analytical grade. Water was glass redistilled.

Methods

Fluorescence measurements were made at 25 ± 0.5° on a Zeiss ZFM 4C spectrofluorometer with a Zeiss motorized wavelength drive and a Sargent recorder. The fluorescence of a L-tryptophan sample with a similar optical density (within 0.02 OD_{295} unit) was recorded before and after each sample measurement in order to correct for time-dependent fluctuations of the light source. The slit widths on the excitation and emission monochromators were 1.0 and 0.6 mm, respectively. Fluorescence efficiency measurements were made by comparing the fluorescence output of the sample to that of the tryptophan solution under identical conditions. The spectra of both the sample and the standard tryptophan solution were corrected for instrumental variations of sensitivity with wavelength (Lehrer and Fasman, 1965). The fluorescence efficiency of the sample relative to that of tryptophan, R_{Trp} (Cowgill, 1968), was then obtained as follows: $R_{Trp} = h_S/h_T \cdot A_T/A_S$, where h_S and h_T refer to the peak height of the corrected emission spectra of the sample and of tryptophan respectively and A_S and A_T refer to their respective absorbancies, obtained on a Cary 14 spectrophotometer, at the wavelength of excitation. The concentration of protein was between 100 and 300 μg per ml and in most cases about 150 μg/ml. The absorbance of a 150-μg/ml HMM solution at 295 nm, the excitation wavelength in most of the experiments, was 0.024. Fluorescence changes were routinely expressed as the ratio (in per cent) of the change in fluorescence intensity, at the peak of the spectrum, induced by an effector to the absolute intensity measured in the absence of this effector: $\Delta I/I$. These values were obtained both from corrected and uncorrected spectra and agreed within 5%. The reproducibility of a value of $\Delta I/I$ was 1% (in $\Delta I/I$ units), with the same protein sample. Different samples showed a larger variability, generally within 2% (in $\Delta I/I$ units), but in a few cases much lower values of $\Delta I/I$ were obtained, probably due to partial denaturation. In order to normalize the results from various experiments, the fluorescence changes induced by various effectors were compared to that induced by MgATP on the same sample and were expressed as the ratio of their $\Delta I/I$ to that of MgATP. ATP and the other adenine nucleotides do not absorb significantly at the concentrations used (generally 150 μM), at the wavelength of excitation, which in most of the experiments was 295 mm. However, in the case of GTP and CTP, and also when exciting at lower wavelengths in the presence of ATP, corrections for the absorbance of the nucleotides at the wavelength of excitation were applied. Those were estimated by measuring the decrease in fluorescence intensity of the standard tryptophan solution upon addition of the same nucleotide under identical conditions. The fluorescence intensity was also corrected for dilution effects caused by the addition of small aliquots (5–20 μl) of "effectors." These corrections were made according to the percentage of dilution, which did generally not exceed 2–3%. The fluorescence in the presence of the effectors was recorded within 1 or 2 min after their addition. In some experiments, especially when some time-dependent effect was known to occur, fluorescence spectra were recorded at several time intervals, until the intensity remained constant. In cases where a fast reaction occurs, such as in the presence of CaATP, the fluorescence change decayed so rapidly that it was practically impossible to observe the initial value of $\Delta I/I$. In such cases, a nucleotide triphosphate regenerating system was used, consisting of phosphocreatine (815 μM) and creatine phosphokinase (8 μg/ml) (Szent-Györgyi and Prior, 1966).

Most of the experiments were carried out in Tris buffer (20 mM, pH 7.4). In some cases the pH of samples was measured after the fluorescence experiment. Changes in pH were generally less than 0.1 pH unit.

Quenching measurements were made on five solutions of HMM in the presence and in the absence of ATP or ADP with increasing concentrations of KI (0–0.2 M). The ionic strength was kept constant with KCl. The absorbance of each HMM solution was measured and the fluorescence intensity corrected for variations in absorbance at the different iodide concentrations. The fluorescence was also corrected for a small blank fluorescence in the iodide solutions. A small amount of $S_2O_3^{2-}$ (10^{-4} M) was added to the stock solution of KI to prevent I_3^- formation (Lehrer, 1971).

Fluorescence spectra and intensities were measured by exciting at 295 nm, and no correction for iodide absorption was necessary. The results were analyzed according to eq 1 and 2 (Lehrer, 1971), where I_0 and I are the fluorescence in-

$$\frac{I_0}{I} = 1 + K_Q[Q] \tag{1}$$

$$\frac{I_0}{\Delta I_Q} = \frac{1}{[Q]f_a'K_Q'} + \frac{1}{f_a'} \tag{2}$$

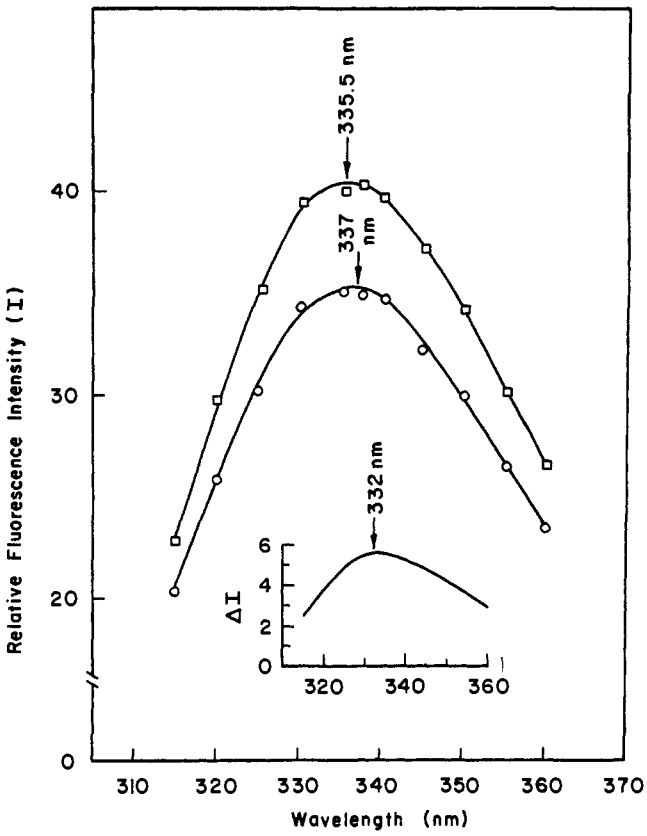

FIGURE 1: Fluorescence spectra of HMM in the absence and in the presence of MgATP. (○) In the absence of MgATP. (□) In the presence of MgATP. Insert: Difference spectrum, b − a. Solvent: Tris, 20 mM, pH 7.4; KCl, 60 mM; HMM, 273 μg/ml; Mg^{2+}, 2 mM; ATP, 158 μM, 25°. Excitation was at 295 nm.

tensity (at the peak) in the absence and in the presence of quencher respectively, K_Q is the Stern–Volmer quenching constant (Stern and Volmer, 1919), [Q] the concentration of the quencher, ΔI_Q the decrease in fluorescence in the presence of the quencher, f_a' is the "effective" fractional accessibility of the fluorophors and K_Q' is their effective quenching constant. In order to check our experimental technique, the K_Q value for L-tryptophan was determined (in 20 mM Tris, pH 7.4, ionic strength 0.20) and the value of 11.5 M^{-1} was obtained in good agreement with values previously obtained under similar conditions (Lehrer, 1971).

Enzyme Activity. The initial velocity of the ATPase reaction was measured by pH-Stat titration with a Radiometer Titrigraph TTT1 at 24°. The conditions of the assay were: pH 7.6; KCl, 28 mM; Ca^{2+}, 10 mM; ATP, 0.5 mM; protein, 100–200 μg in a volume of 10–11 ml, titrant 0.02 or 0.04 N NaOH. The slopes of the straight lines obtained during the first few minutes of the reaction were taken as the steady state rate of ATP hydrolysis. The activity of various HMM samples ranged between 1.3 and 2.1 μmoles of P_i per mg of protein·min, and that of S-1 samples was between 1.0 and 2.7 μmoles of P_i per mg of protein·min.

Protein Modification. Modification of HMM with mersalyl was generally performed by adding the calculated amount of mersalyl to a solution of HMM (molar ratio 32:1) in 60 mM KCl–20 mM Tris buffer (pH 7.4) at 0°. ATPase activity was lost within 3 min after the addition of mersalyl. The reactivation experiment was performed at room temperature by incubating the modified HMM with dithiothreitol for 15 min, at a concentration four to five times higher than that of mersalyl. The solution was then dialyzed for 4 hr in the cold against buffer to remove dithiothreitol and mersalyl–dithiothreitol. Modification of HMM with *N*-ethylmaleimide was performed under similar conditions as with mersalyl, except that the incubation time was 1–2 hr (at 0°).

Binding Studies. Binding of ATP was performed by gel filtration experiments (Hummel and Dryer, 1962) on a 12 × 1 cm column, packed with Sephadex G-25 (coarse). The column was equilibrated in the cold (3–5°) with about 30 ml of a solution of Tris (20 mM, pH 7.7)–60 mM KCl which also contained 5.6 μM [^{14}C]ATP (specific activity 3.2 Ci/mole) and 1.5 mM $MgCl_2$. The experiment was generally started by applying 0.2 ml of 1.16 mg/ml of protein dissolved in the equilibrating solution on the top of the column. The protein was then eluted under pressure (1–2 psi) with the same solution. The rate of flow was 1–2 ml/min. Fractions of 0.42 ml (5 drops) of the eluent were collected manually and the protein concentration was estimated from the absorbance at 280 nm, correcting for the small absorbance of ATP. The protein peak came out with the void volume. From each fraction an aliquot of 0.2 ml was taken out, 6 ml of scintillation liquid [1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene and BioSolve (Beckman) at a ratio 5:1] was added, and the samples were counted in a scintillation counter (Packard TriCarb or Nuclear-Chicago). The concentration of ATP in the various fractions was determined by calibrating the counts per minute values in each experiment with a [^{14}C]ATP solution of known concentration. The amount of ATP in all the fractions under the peak was added up and divided by the total amount of protein in the same fractions to give the value of the number of molecules of ATP bound per molecule of HMM at this particular concentration of ATP.

Results

Relative Fluorescence Efficiency. The emission spectrum of HMM, when excited at 295 nm, has its λ_{max} at 337 nm (Figure 1). The normalized fluorescence emission spectra of HMM show a shift in the emission maximum from 337 to 334 nm when excited at 275 nm instead of 295 nm. The higher fluorescence obtained when exciting at the lower wavelength coupled with the emission shift clearly indicates a component of tyrosine fluorescence peaking below 310 nm, since the λ_{max} of the fluorescence emission of tyrosyl residues in proteins is around 303 nm (Chen *et al.*, 1969). The excitation spectrum of this component had its λ_{max} at 275 nm (not shown). The tyrosine component had an insignificant contribution to the fluorescence emission spectrum of the Trp residues of HMM when exciting at 295 nm. The R_{Trp} (Cowgill, 1968) was found to be 1.03. However, excitation at lower wavelengths resulted in lower values of R_{Trp} (Figure 2). This curve has the shape of an inverted absorption spectrum of a tyrosyl residue, for which λ_{max} is 275 nm (Sober, 1970). Measurement of R_{Trp} of HMM at 337 nm minimizes the contribution of the fluorescence of tyrosine residues and their only effect is to reduce the fluorescence of the Trp residues by absorbing part of the excitation light. Therefore, the experiments were generally conducted using 295 nm as the excitation wavelength (Cowgill, 1968). This excitation wavelength has also the advantage of allowing one to use concentrations of ATP up to at least 300 μM without interference due to absorption effects.

There was virtually no influence of ionic strength upon the R_{Trp} value of HMM (from 60 to 600 mM KCl). The R_{Trp} of

myosin at an ionic strength of 600 mM was 1.16 and the R_{Trp} of the globular head of myosin S-1 was found to be 0.92 at an ionic strength of 60 mM.

Effects of ATP and ADP. MgATP increases the fluorescence of HMM about 17%[2] and also causes a small blue shift (≈1.5 nm) of the emission peak (Figure 1) as previously reported (Werber *et al.*, 1971). This blue shift becomes more evident in the difference spectrum, which has a λ_{max} of 332 nm. Both the shift in wavelength and the increase in quantum yield are indicative of an increase in the nonpolarity of the environment of Trp residues (Van Duuren, 1961). These results were obtained by exciting at 295 nm. Excitation at lower wavelengths (288 and 275 nm) also resulted, after correction for the absorption of ATP at those wavelengths, in similar but somewhat lower increases in the fluorescence of HMM, coupled with a small blue shift. The ATP-induced increase in fluorescence was independent of ATP concentration in the range 5–160 μM. The difference spectrum, however, decreased to the final value of the ADP–HMM complex when the ATP was depleted due to the ATPase activity of HMM. At lower concentrations of ATP the decrease occurred within minutes, at higher ATP concentrations (150 μM) the onset of the decrease was delayed for about 30 min. No attempt was made to measure accurate rates of decay of the difference fluorescence spectrum as was done by Morita in the case of the uv difference absorption spectrum (Morita, 1967). The difference spectrum obtained with MgATP was invariably reduced to 30–35% of the initial value upon the completion of ATP hydrolysis (Table I). The same value was obtained with MgADP,[3] indicating that the low final value observed in the case of MgATP was caused by the formation of a complex of HMM with MgADP.

With Ca^{2+} as the divalent ion, the rate of hydrolysis of ATP by myosin or HMM is approximately 50 times faster than with Mg^{2+} (Lymn and Taylor, 1970) and therefore, even in the presence of 160 μM ATP, the difference spectrum attained its final value within a few minutes. The correct magnitude of the initial difference spectrum induced by CaATP could thus be measured only in the presence of an ATP-regenerating system such as phosphocreatine and creatine kinase. There seemed to be little or no difference between the effects of Ca^{2+} and Mg^{2+} on the ATP induced difference spectrum (Table I). However, a small but significant difference was observed with ADP, possibly due to looser binding of CaADP to myosin (Lowey and Luck, 1969).

In the absence of added metal ions the value of $\Delta I/I$ (the ratio in per cent of the change in fluorescence intensity, at the peak of the spectrum, induced by an effector to the absolute intensity measured in the absence of effectors) induced by ATP was reduced to 61% of that obtained in the presence of Mg^{2+}. The $\Delta I/I$ value induced by ADP in the absence of added metal ions was lower than the value in the presence of Mg^{2+} and comparable to the value in the presence of Ca^{2+}.

[2] The change in absorbance at 295 nm of the Trp residues upon addition of MgATP is about 5%, as calculated from data from difference uv absorption spectroscopy (Morita, 1967). Thus the fluorescence change induced by MgATP is mainly a change in quantum efficiency of the Trp residues.

[3] In experiments with some samples of HMM, higher values of $\Delta I/I$ were seen initially, which decreased within 2–3 min to the actual ADP value. The reason for this effect was not established; it might be due to myokinase activity which is often found in myosin preparations (Lowey and Luck, 1969), but the ADP used did not seem to undergo any significant cleavage in the presence of HMM as checked by the pH-Stat method.

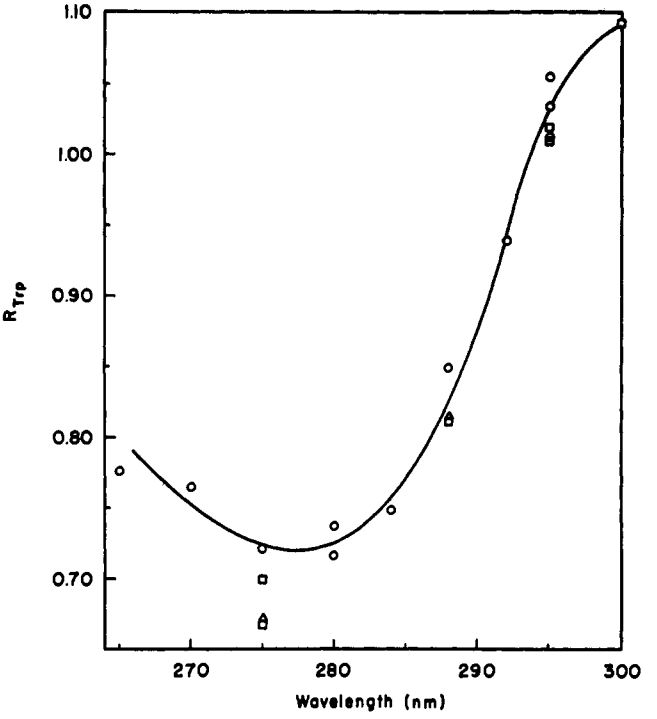

FIGURE 2: Effect of the excitation wavelength, at various ionic strengths, on the R_{Trp} value of HMM. (○) 60 mM KCl, (△) 400 mM KCl, and (□) 600 mM KCl. Solvent: Tris, 20 mM, pH 7.4; HMM, 290 μg/ml, 25°.

There was no influence of the ionic strength from 60 to 600 mM KCl on the $\Delta I/I$ induced by MgATP. However, at high ionic strength the values of $\Delta I/I$ induced by CaATP and MgATP were more stable with time, due to the reduced ATPase activity (Bendall, 1969). The lack of the effect of ionic strength allows a comparison with myosin, which is not

TABLE I: Effects of ATP and ADP on the Fluorescence of HMM.[a]

Addition	$\Delta I/I$ % 1[b]	2[b]	3[b]	4[b]	Effect Rel to MgATP
MgATP	19.0	17.3	16.5	15.7	1.00
Hydrolyzed MgATP[c]	5.5		4.5	4.7	0.31
MgADP	6.2	6.3	4.8	4.3	0.31
CaATP[d]		16.1			0.94
Hydrolyzed CaATP	3.8	4.3		2.6	0.21
CaADP		3.3	3.4	4.0	0.22
ATP[e,f]	11.0	11.5	9.9		0.61
ADP[e]		3.8		3.0	0.21

[a] Divalent metal ion (1–2.5 mM), nucleotide (150 μM), KCl (60 mM), and Tris (20 mM), pH 7.4. [b] Different sets of experiments. [c] At low concentrations of ATP (5 μM). [d] Using an ATP-regenerating system (phosphocreatine + creatine kinase), otherwise lower and fast decreasing values are obtained. [e] No metal ion added. [f] Generally in the presence of EGTA to prevent hydrolysis by traces of Ca^{2+}.

TABLE II: Comparison of ATP and ADP Effects on the Fluorescence of Myosin, HMM and S-1.[a]

Addition	Av $\Delta I/I$ % S-1	Av $\Delta I/I$ % HMM	Av $\Delta I/I$ % Myosin[b]
MgATP	15.5 (1.00)[c]	17.3 (1.00)[c]	10.9 (1.00)[c]
MgADP		4.7 (0.31)	5.2 (0.48)
CaATP	15.7 (1.01)	16.1[d] (0.94)	10.0[d] (0.92)
ATP[e]	7.5 (0.47)	11.5 (0.61)	7.3 (0.67)

[a] Divalent metal ion (1–2.5 mM), nucleotide (150 μM), KCl (60 mM) (except for myosin), and Tris (20 mM), pH 7.4. [b] In 600 mM KCl. [c] Effects relative to MgATP in parentheses. [d] Using an ATP-regenerating system (phosphocreatine + creatine kinase). [e] In the presence of EGTA.

soluble at low ionic strength. The effects of ATP and ADP on myosin (in 600 mM KCl) and on S-1 (in 60 mM KCl) are summarized in Table II and compared to the HMM values. The magnitudes of the difference spectra ($\Delta I/I$) induced by ATP or ADP are not the same in the three myosin fragments, but the effects relative to the MgATP induced changes are comparable for each set.

Effects of Other Substrates and Analogs. The effect of other nucleotides and of several analogs of ATP, in the presence of Mg^{2+}, on the fluorescence of HMM is shown in Table III. Similar effects, not shown, were obtained in the presence of Ca^{2+}, the values being somewhat lower than with Mg^{2+}.

As seen in Table III, CTP induces a similar change to that of ATP, whereas ITP, GTP, and UTP cause smaller effects. The fluorescence change caused by ATPP is similar to that of ATP. The tetraphosphate, which was found to be hydrolyzed 1.7 times faster than ATP, in the presence of Ca^{2+}, induces the maximal effect. It was also observed that two equivalents of base were consumed per molecule of ATPP during its hydrolysis, the product of the reaction being most probably ADP. The fact that the final value of $\Delta I/I$ was 25–30% of the initial value at a low concentration of ATPP (5 μM) is consistent with this conclusion. dATP also causes a similar fluorescence change to that of ATP. This is consistent with the tight binding of dATP to HMM (Szent-Györgyi, 1968), and with our observation that dATP is hydrolyzed at the same rate as ATP (in the presence of Ca^{2+}).

TABLE III: Effect of Various Substrates and Analogs on the Fluorescence of HMM.[a]

Analog	$\Delta I/I$ %[b]	Effect Rel to MgATP
ATP	17.3	1.00
dATP	17.3	1.00
ATPP	17.6	1.02
ADP	5.4	0.31
AMP	0	0
AMP-PCP	2.8	0.16
AMP-PNP	10.0	0.58
ITP	10.4[c]	0.60
IDP	0	0
CTP	18.0[d]	1.04
UTP	4.5[c]	0.26
GTP	4.0[d]	0.23
PP_i	1.6[e]	0.09

[a] Mg^{2+} (2 mM), nucleotide (120–160 μM), KCl (60 mM), and Tris (20 mM), pH 7.4. [b] Averaged values for 10–20 experiments for ADP and ATP, 2 experiments in other cases. [c] Using the triphosphate-regenerating system (phosphocreatine + creatine kinase). [d] Corrected for light absorption (using a Trp solution). [e] At 660 μM PP_i.

TABLE IV: Effect of Modification of SH Groups of HMM on the Activity and on Fluorescence Changes Induced by ATP and ADP.[a]

Protein and Type of Modification	Modifier[b] HMM	Act. (%)	Rel[c] Fluorescence Change upon Addition of MgATP	Rel[c] Fluorescence Change upon Addition of MgADP
HMM		100	1.00	0.31
MSA–HMM	30–32	<4	0	
HMM + DTT, dialyzed	165[d]	68	0.88	
MSA–HMM[e] + DTT, dialyzed[f]	165[d]	60	0.77	
NEM–HMM	10.5	10	0.30	
NEM–HMM	30–32	12	0.29	0.37

[a] CaATPase activity measured in the pH-Stat, at pH 7.6 (see Experimental Section). Conditions for fluorescence experiments as in Table III. [b] Molar ratio. [c] Relative to the effect of MgATP in unmodified HMM. [d] Ratio of dithiothreitol (DTT):HMM. [e] Same as in line 2. [f] Deblocked mersalyl (MSA)–HMM.

ATP analogs, AMP-PCP and AMP-PNP, which bind to but are not cleaved by myosin (Yount *et al.*, 1971), cannot induce the maximal magnitude of the fluorescence change. However, the imido analog causes a much larger effect than the methylene one, in accordance with its stronger binding to myosin, and with its close similarity to ATP in other related effects (Yount *et al.*, 1971; Chaplain and Frommelt, 1968). It is interesting to note that IDP, in contrast to ADP, does not induce any change in fluorescence, probably because it binds very weakly to HMM (Blum, 1955; Kiely and Martonosi, 1969). AMP is without any effect, since it probably binds very weakly or not at all to myosin (Szent-Györgyi, 1968). The very small effect of PP_i, which is known to bind rather tightly to myosin in the presence of Mg^{2+} (Kiely and Martonosi, 1968), emphasizes the importance of the base ring of the nucleotide in inducing the large change in the fluorescence of HMM.

Modification of SH Groups. Using the organic mercurial mersalyl and *N*-ethylmaleimide two different types of modifications were obtained. The ATPase activity of the modified proteins, mersalyl–HMM, was almost abolished (Table IV). As shown in Table IV, no change in fluorescence could

be induced in the case of mersalyl-HMM upon the addition of ADP or ATP. The peak of the fluorescence emission of mersalyl-HMM was shifted to 344–345 nm (uncorrected values) as compared to 342 nm for unmodified HMM and the fluorescence intensity decreased with time. These effects are probably caused by denaturation of the modified protein, leading to a gradual exposure of more Trp residues than in HMM. However, by deblocking mersalyl-HMM with dithiothreitol most of the activity and the ability to undergo fluorescence changes upon interaction with the substrate were restored. In contrast to mersalyl-HMM, *N*-ethylmaleimide-HMM showed a significant fluorescence change in the presence of MgATP. The magnitude of the change was, however, about the same as caused by MgADP. This appeared to be consistent with the notion that, although ATP cannot be hydrolyzed by *N*-ethylmaleimide-HMM, its binding induces a fluorescence change, which is much smaller than in catalytically active HMM.

Binding studies of Mg[^{14}C]ATP by gel filtration (Schliselfeld and Bárány, 1968) on Sephadex G-25 showed that MgATP or MgADP remained bound to *N*-ethylmaleimide-HMM. Under the conditions of the experiment (5–6 μM ATP) about 1.7 moles of nucleotide was bound by a mole of *N*-ethylmaleimide-HMM. On the other hand, as could have been inferred from the fluorescence experiments (see Table IV) no binding of ATP to mersalyl-HMM could be detected. Fluorescence experiments were also performed with the same samples of modified HMM used in the binding studies, utilizing low ATP concentrations (5–6 μM), and gave essentially the same results as shown in Table IV.

Effect of Phosphate Buffer and Other Phosphate Derivatives. Phosphate is known to inhibit the ATPase activity of myosin (Bendall, 1961). Its effect on the fluorescence changes induced upon the addition of MgATP and MgADP to HMM was, therefore, investigated. The fluorescence change was generally smaller in the presence of 20 mM phosphate,[4] but the decrease depended on the nature of the counterion of the buffer and on the order of addition of the buffer (Table V). Sodium phosphate was far more effective than potassium phosphate, causing a reduction of about 60% of the effect of ATP, in the presence of either Mg^{2+} or EGTA, on the fluorescence of HMM or S-1. It completely abolished the effect of ADP. In the case of potassium phosphate, both the effects of ATP and ADP were reduced only 25%. High concentrations of Na^+ are known to inhibit the activity of myosin (Schliselfeld and Bárány, 1968), but in this work, the concentration of Na^+ was only between 20 and 33 mM. In the absence of phosphate, in Tris buffer, the fluorescence change induced by MgATP was the same in 60 mM KCl or NaCl.[5] When the phosphate buffer was added after MgATP, it reduced the fluorescence change about 25% in the presence of Na^+ and not at all in the presence of K^+. On the other hand, the suppression of the effect of ADP by sodium phosphate did not depend upon the order of addition of the buffer. The order of addition of the buffer does thus seem to play a role only when a substrate is being cleaved by HMM. It is also interesting to note that when ATP was added to a solution of HMM in sodium phosphate in the absence of metal ions (but traces of Ca^{2+} might have been present), the fluorescence change was almost completely abolished.

[4] The fluorescence of HMM itself was not affected by the type of buffer used (Tris or phosphate).

[5] The MgADP effect seemed to be even higher in NaCl (0.4 of the effect of MgATP) than in KCl.

TABLE V: Effect of Phosphate upon the Fluorescence Changes Induced in HMM and S-1 by ATP and ADP.[a]

Protein	Addition	Buffer	Order of Addition[b] of Phosphate	Rel Effect[c]
HMM	MgATP	Tris		1.00
		NaP_i	First	0.42
		KP_i	First	0.75
		NaP_i[d]	Last	0.75
		KP_i	Last	0.99
S-1		NaP_i[d]	First	0.38
HMM	ATP[e] or EGTA–ATP	Tris		1.00
	EGTA–ATP	NaP_i[d]	First	0.41
	ATP[e]	NaP_i[d]	First	0.06
HMM	MgADP	Tris		1.00
		NaP_i[d]	First or last	<0.06[f]
		KP_i	First	0.77
NEM:HMM = 10:1	MgADP	NaP_i[d]	First	0

[a] Buffer, 20 mM, pH 7.4; nucleotide, 150 μM, Mg^{2+}, 2 mM; EGTA, 0.3 mM. [b] Before or after the nucleotide. [c] Average values relative to the effects of ATP or ADP on HMM or S-1. [d] Phosphate buffer containing sodium or both sodium and potassium. [e] No metal ion added. [f] Even slightly negative $\Delta I/I$ values in some cases.

Although pyrophosphate (PP_i) induced very little change in the fluorescence of HMM (Table III), it decreased the effect of MgATP. Thus 660 μM of PP_i reduced the effect of ATP (155 μM) by 63%. Similarly, 780 μM of P-enolpyruvate, which is sometimes used with pyruvate kinase as an ATP-regenerating system (Schliselfeld *et al.*, 1970), was found to depress the effect of MgATP on the fluorescence of HMM and S-1 by about 50%. On the other hand, the phosphate donor of our ATP-regenerating system, phosphocreatine, did not by itself affect the fluorescence changes induced by ATP in HMM.

Quenching of Trp Fluorescence of HMM by Iodide Ions. In an attempt to differentiate between the effects of ADP and ATP and also in order to test the hypothesis that Trp residues become buried upon interaction with substrate (Morita, 1967), the method of iodide quenching of protein fluorescence (Lehrer, 1971) was applied to HMM in the absence and in the presence of MgATP and MgADP. Similar information can generally be obtained by the method of solvent perturbation (Herskovitz and Sorensen, 1968). However, no change in the solvent perturbation difference spectrum between free HMM and HMM complexed with ATP could be detected by previous workers (Shimizu *et al.*, 1971a,b).

The Trp fluorescence of HMM, under the three above mentioned conditions, was thus measured at various concentrations of KI (0–0.2 M) and the results were analyzed according to eq 1 and 2. The regular Stern–Volmer plots (eq 1) were all curved but the modified plots (eq 2) yielded straight lines (Figure 3). This and the fact that the difference spectra of HMM, between the KCl solutions and the various iodide

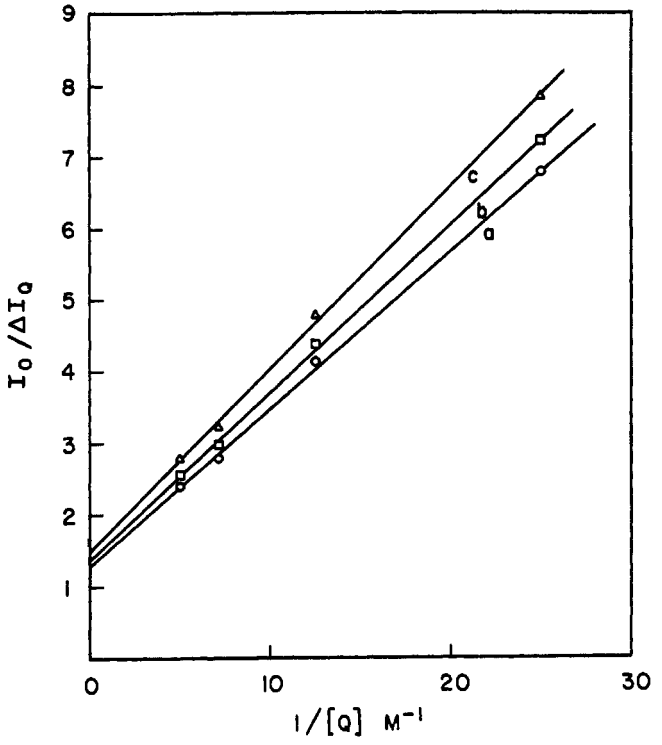

FIGURE 3: Modified Stern–Volmer plots for the effect of iodide ions on HMM fluorescence. $I_0/\Delta I_Q$ is the ratio of the fluorescence in the absence of iodide, I_0 to ΔI_Q, which is the difference in fluoresence due to the presence of iodide ions, [Q] is the concentration of the quencher, iodide. (a, ⊙) HMM alone; (b, □) HMM + MgADP; (c, △) HMM + MgATP. Tris, 20 mM, pH 7.5; KI, 0–0.2 M; KCl added to keep [K$^+$] = 0.21 M; Mg^{2+}, 2 mM; ATP, 167 μM; ADP, 153 μM; HMM, 140 μg/ml, 25°. A least-squares treatment was utilized to plot these curves.

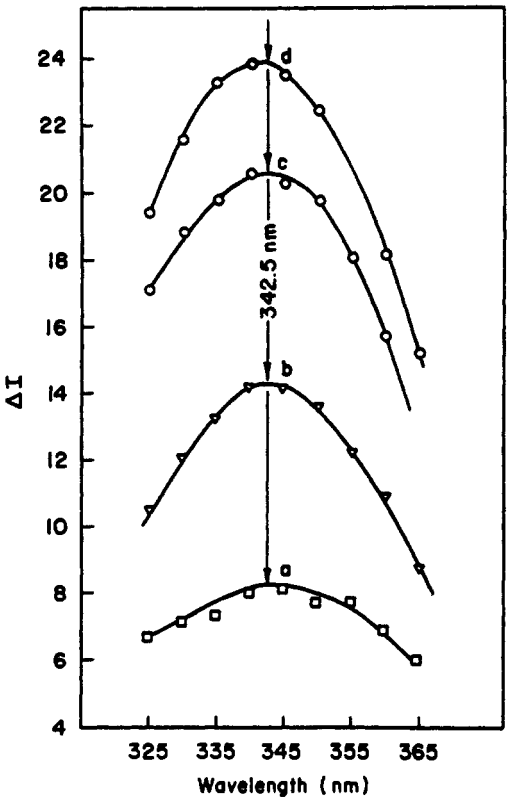

FIGURE 4: Difference fluorescence spectra of HMM perturbed by various concentrations of iodide. (a, □) 0.04 M KI; (b, ▽) 0.08 M KI; (c, ⊙) 0.14 M KI; (d, ⊙) 0.20 M KI. Conditions as in Figure 3. Excitation wavelength, 295 nm.

solutions, have a common λ_{max} = 342.5 nm (Figure 4) are consistent with the interpretation that only one class of Trp was affected (Lehrer, 1971). From the position of λ_{max} one might infer that the accessibility of the quenched Trp is not complete, since this would have caused the λ_{max} of the difference spectra to be closer to 350 nm, which is the λ_{max} of Trp residue fluorescence in denatured proteins (Teale, 1960). The λ_{max} of the fluorescence emission was shifted to 332 nm at the highest iodide concentration used (0.2 M), reflecting the fact that the unquenched Trp residues emit to the blue of the other residues, *i.e.*, that they are in a more apolar environment (Van Duuren, 1961). The results of the plots in Figure 3 are summarized in Table VI. The degree of accessibility, f_a', of the Trp residues was reduced in the presence of ADP or ATP, while the quenching constant K_Q' remained unchanged. Using recent values of the Trp content in HMM (Shimizu *et al.*, 1971a,b), the number of Trp residues that become inaccessible to iodide quenching upon interaction with MgATP can be calculated to be 2, while it is only 1 in the case of MgADP.

TABLE VI: Results of Iodide Quenching of Trp Fluorescence in HMM.[a]

Addition	$\Delta I/I$ %[b]	f_a'[c]	No. of[d] Exposed Trp	K_Q' (M^{-1})[e]
None		0.78	14.8	5.84
MgATP	15.9	0.67	12.6	5.86
MgADP	5.4	0.73	13.9	5.85

[a] Tris buffer (pH 7.4); KCl + KI, 0.21 M (KI from 0 to 0.2 M); Mg, 2 mM; nucleotide, 150 μM. [b] In the absence of iodide ions. [c] f_a', effective fraction of accessible Trp residues. [d] Using a value of 19 Trp per molecule of HMM (Shimizu *et al.*, 1971a,b). [e] K_Q', effective Stern–Volmer quenching constant.

The interpretation that these Trp residues become inaccessible to iodide is further supported by the fact that the absolute fluorescence increase (ΔI) (at constant protein concentration) induced by the nucleotides is independent of iodide concentration whereas the total fluorescence of HMM decreases as a function of iodide concentration. This means that the fluoresence of those Trp residues is increased upon addition of MgATP or MgADP regardless of the presence of iodide ions, *i.e.*, that they become inaccessible. Since the increase of fluorescence is independent of iodide, this ion does not affect the HMM species responsible for the increase of fluorescence.

Discussion

Fluorescence Efficiency. The purpose of measuring the fluorescence efficiency of HMM at various excitation wavelengths was (a) to determine if there is any contribution of the tyrosine residues to the Trp fluorescence due to energy transfer or trivial reabsorption of the emitted fluorescence of Trp resi-

dues (Steinberg, 1971) and (b) to distinguish between different classes of Trp residues, which would display different absorption and fluorescence characteristics (Purkey and Galley, 1970). The normalized fluorescence emission spectra of HMM show a dependence on the excitation wavelength. The emission spectra with excitation at 295 and 275 nm have the peak emission occurring at 337 and 334 nm, respectively. This shift indicates a contribution due to Tyr at lower wavelengths. Part of the excitation light is absorbed by tyrosyl residues, causing the wavelength dependence of the fluorescence efficiency of the Trp residues to assume the shape of an inverted absorption spectrum of a tyrosyl residue (Figure 2). This could be due to the fact that HMM contains 77 Tyr and about 19 Trp residues (Shimizu *et al.*, 1971a,b), and at λ = 275 nm, their contributions to the absorption are more or less equal (Trp, ϵ_M 1900; Tyr, ϵ_M 1420 (Sober, 1970)).

If one assumes, however, that the Tyr residues only reduce the intensity of the excitation light for the Trp residues and do not contribute to their fluorescence due to energy transfer, one should be able to obtain the same values of fluorescence efficiency at the various excitation wavelengths by correcting the R_{Trp} values for the fraction of light absorbed by the Tyr residues. The values obtained are 1.49 (275 nm), 1.38 (280 nm), 1.04 (288 nm), and 1.08 (295 nm). This illustrates that higher corrected R_{Trp} values are obtained at the lower excitation wavelengths, which might indicate that there is some radiationless energy transfer from Tyr to Trp residues, similar to that observed in some other cases (Weinryb and Steiner, 1970; Longworth, 1968). Two different classes of Trp residues may coexist in HMM (see below) as was found for other proteins (Lehrer and Fasman, 1967; Purkey and Galley, 1970; Elkana, 1968) but it would be extremely difficult to distinguish between them by this method because of the contribution of the Tyr to the fluorescence emission of Trp.

Since no ionic strength dependence of the fluorescence of HMM was found, and similar results have been reported for LMM (Cowgill, 1968), it is possible to compare the fluorescence of these proteins obtained at different ionic strengths. Using the published values of the quantum efficiency, R_{Trp} 1.70, for LMM (Cowgill, 1968) the number of Trp residues in this fragment (assuming a molecular weight of 150,000) (Lowey and Cohen, 1962; Lowey *et al.*, 1969) and our value for the fluorescence efficiency of HMM, 1.03, which has 19 Trp residues (Shimizu *et al.*, 1971a,b), one can calculate the quantum efficiency of myosin as follows: $(9 \times 1.70 + 19 \times 1.03)/28 = 1.24$. Our measured value, R_{Trp} 1.16, agrees well with the calculated value. The additivity of the quantum efficiencies of the two fragments of myosin can be interpreted to suggest that there is no detectable interaction between the two moieties of the intact molecule of myosin which carry their respective Trp residues.

Comparison of the Effect of ATP on Myosin, HMM, and S-1. Since myosin, HMM, and S-1 have different quantum efficiencies and a different number of Trp residues, a comparison of the effect of substrate on their fluorescence should take into account all these differences. Such a comparison is shown in Table VII. The difference in fluorescence efficiency, ΔR_{Trp}, upon interaction with substrate is multiplied by $n/2$ (since myosin and HMM consist of two similar chains), where n is the number of Trp residues per molecule of each of those fragments. This gives the average change of fluorescence efficiency, ΔR_{Trp}, per residue (Lehrer and Fasman, 1967) for the 2 Trp whose environment changed upon interaction with substrate (*cf.* Table VI). In the case of S-1 it is assumed that only one Trp residue per molecule is involved. There is almost no difference between myosin and HMM, whereas the value in the case of S-1 is only 77% of that of HMM. This difference might be caused by the fact that the change in the environment of those Trp residues upon interaction with substrate is less pronounced when the 2 globular heads of myosin, S-1, are not on the same molecule, *i.e.*, there exists a certain degree of cooperativity between the two substrate sites, on the two heads of the myosin molecule (Lymn and Taylor, 1970).

TABLE VII: Comparison of the Effect of Substrate on Myosin, HMM, and S-1.

	Protein		
	Myosin	HMM	S-1
R_{Trp}	1.16	1.03	0.92
R_{Trp} (+ATP)[a]	1.242	1.150	1.01
ΔR_{Trp}	0.082	0.121	0.093
Number of Trp residues, n	28[b]	19[c]	2×9[c]
ΔR_{Trp} per residue[d]	1.15	1.08	0.84

[a] Corrected for absorption changes using data from Morita (1967) and Morita and Shimizu (1969). [b] Calculated from the "old" value for the number of Trp in LMM (Lowey and Cohen, 1962) and the recently redetermined value for HMM[c]: 9 + 19 = 28. [c] Shimizu *et al.* (1971a,b). [d] Average fluorescence efficiency change per residue, assuming that two of the n residues contribute to the fluorescence change.

Structural Requirements of Fluorescence Effectors. For a maximal change in fluorescence there are several requirements: the effector must bind to HMM rather tightly, it must be hydrolyzed and the nucleotide diphosphate produced by hydrolysis must also be bound to the enzyme. However, as shown above (Tables II and III), there is no correlation between the rate of hydrolysis of the various substrates and the magnitude of the fluorescence change observed. The ATPase activity of myosin is known to be much lower in the presence of Mg^{2+} than in its absence, or in the presence of Ca^{2+} (Blum, 1955); nevertheless, the magnitude of the fluorescence change induced by ATP in the presence of Mg^{2+} is the same as that in the presence of Ca^{2+}, whereas the change in the absence of metal ions in only 61% of the maximal effect. Similarly, although ITP, UTP, and GTP are hydrolyzed faster in the presence of divalent cations than ATP and CTP (Blum, 1955; Kielley *et al.*, 1956), they are not as effective for induction of the maximal fluorescence change as the nucleotides with an amino group in position 6 of the ring. The order of effectiveness in inducing fluorescence changes, ATP $\approx$ CTP > ITP > UTP > GTP, is very similar to the order found for other properties of the system, such as the binding of the nucleotides, as measured by $1/K_m$ (Blum, 1960; Kielley *et al.*, 1956), the amount of intermediate [^{18}O]phosphate exchange that they catalyze (Swanson and Yount, 1966; Yount and Koshland, 1963), their effect on sulfhydryl reactivity (Yamaguchi and Sekine, 1966) and their effectiveness in supporting contraction (Hasselbach, 1956).

A tight binding of the substrate is very important for bringing about the large change in fluorescence. It is likely that when binding is weak as in the case of IDP only a few HMM sites are occupied. A strong binding of the nucleotide, however, is not the sole requirement, as can be evidenced by the

fact that AMP-PNP, which binds to HMM with K_i values almost identical with those of ATP (Yount *et al.*, 1971), cannot bring about the maximal change in fluorescence (Table III). The same is true for HMM modified with *N*-ethylmaleimide (Table IV), although it is still able to bind ATP with at least the same affinity as unmodified HMM. These results indicate that in order to attain the maximal effect on the fluorescence, cleavage of the triphosphate of the nucleotide must occur. It has been suggested recently by Taylor and coworkers (1970) that the release of products is the rate-determining step in the kinetic scheme for the ATPase reaction of myosin and that the enzyme–product complex is the most populated state during the reaction. In the original scheme it was assumed that there is only one form of the ADP complex which is the same whether obtained *via* ATP or ADP. The scheme, however, requires modification to account for the two forms of ADP complex suggested by Seidel and Gergely (1971) and by Viniegra and Morales (1972). Our results would be accounted for by the following kinetic scheme. The asterisk signifies that

$$E + ATP \rightleftarrows E{:}ATP \longrightarrow E{:}\begin{matrix}{}^{*}ADP \\ P_i\end{matrix} \longrightarrow E + ADP + P_i \rightleftarrows (E{:}ADP + P_i) \quad (3)$$

the enzyme is in a different conformational state which can be achieved only as a result of the cleavage. This state is responsible for the large fluorescence change and is maintained in the enzyme–product complex. It is important to note that there is no correlation between the rate of hydrolysis and the magnitude of the fluorescence effect, *i.e.*, CaATP and MgATP induce the same change in fluorescence, although the hydrolytic rates differ by a factor of about 50, which would agree with an interpretation that there is only a single species of intermediate complex. The final step in parentheses takes place in our scheme only when ATP hydrolysis is completed and corresponds to the lower fluorescent state.

It thus seems that tight binding of the nucleotide diphosphate product is required to conserve the conformational change accompanying hydrolysis. However, binding by itself is not sufficient to cause the maximal fluorescence change (Table I), as it is not attained by adding both products, ADP and P_i together, to a solution of HMM (Table IV). This means that the dissociation step of the enzyme–products complex, the last step in eq 3 (preceding the parenthesis), is not reversible in the sense that once ADP has come off the protein the latter assumes a different conformation and reequilibration with ADP cannot reverse this change. This is consistent with the suggestion that the conformational change has occurred during the cleavage of the substrate.

Effects of Phosphate and Pyrophosphate on the ATP-Induced Fluorescence Changes. The above conclusion is also supported by the dependence of the inhibitory effect of phosphate (Table V) upon its order of addition. When phosphate was added first it decreased the effect of ATP, but not that of ADP, on the fluorescence to a greater extent than when it was added last. This is probably due to the fact that in the latter case the enzyme–product complex has already formed before the addition of phosphate, and the competitive binding of phosphate to the ATP site occurs to a small extent or not at all (in the case of potassium phosphate). This is, therefore, consistent with the assumption that a conformational change has occurred during the hydrolysis of ATP, rendering the enzyme–product complex less accessible than the free enzyme to phosphate anions.

Pyrophosphate was found to be much more efficient than phosphate in reducing the fluorescence change induced by ATP. This effect may be in part due to its rather strong binding to myosin (Kiely and Martonosi, 1968). Pyrophosphate, which is known to activate the ATPase of myosin (Gallop, 1957), might do so by interfering with the binding of ADP rather than ATP. It would thus destabilize the enzyme–product complex $\left(E{:}\begin{matrix}{}^{*}ADP \\ P_i\end{matrix}\right)$ thereby enhancing the overall rate of hydrolysis of ATP by changing the rate-determining step. The rather large effect of P-enolpyruvate in reducing the fluorescence change is probably related to the positioning of the negative charge of the carboxylate ion present in the molecule, in a favorable orientation to become attached to the binding site of the polyphosphate chain of the substrate in HMM, in a similar manner to that of PP_i. This would also be consistent with the observation that creatine phosphate, which has a positive charge on the nonphosphate moiety of the molecule, did not affect the fluorescence change induced by ATP.

Importance of the Binding of Nucleotide Diphosphates. It has been suggested that the differences in rates of hydrolysis between ATP and ITP are due to differences in binding of the products, the nucleotide diphosphates, *i.e.*, in the rate of their desorption from the enzyme (Blum, 1960; Taylor *et al.*, 1970; Kiely and Martonosi, 1969). The observation that IDP does not induce any fluorescence change, while ADP causes such an effect (Table III), is consistent with this suggestion. Similarly, the high steady-state rate for ATP hydrolysis by myosin in the presence of EDTA was attributed to a change in the rate determining step, *i.e.*, to the elimination of the product dissociation step (Lymn and Taylor, 1971), probably due to the suppression of the binding of ADP in the absence of metal ions at high ionic strength (Lowey and Luck, 1969; Kiely and Martonosi, 1969). In agreement with this conclusion ADP has no further effect on the fluorescence of HMM in the presence of EDTA in 0.6 M KCl (Werber *et al.*).[6] At low ionic strength, however, ADP still produced a fluorescence change even in the absence of metal ions (see Table I), although its magnitude was somewhat lower than in the presence of Mg^{2+}. This result agrees with the dependence of the binding of ADP on ionic strength (Kiely and Martonosi, 1969).

The results obtained with IDP and ADP in the absence of metal ions emphasize the importance of the binding of the nucleotide diphosphates for the maximal change in fluorescence to occur, *i.e.*, they stabilize the conformational change incurred during the hydrolysis of nucleotides by myosin. It thus appears that many of the structural requirements of the substrate for the induction of the conformational change, such as the presence of an amino group in position 6 of the ring and the nature of the divalent cation, may be described in relation to the binding of the nucleotide diphosphates in the enzyme–product complex formed during the hydrolysis.

Comparison to Other Methods. The effects of ATP, ADP, and analogs on HMM, myosin, and S-1 have also been followed by related techniques, difference uv absorption spectroscopy (Morita, 1967; Morita and Shimizu, 1969), fluorescence of the probe, ANS (Cheung, 1969), and by esr spectroscopy of spin labels (Seidel and Gergely, 1971). Uv spectroscopy and intrinsic fluorescence are measured on unmodified proteins and offer obvious advantages. A comparison of the results of these two techniques is shown in Table VIII. The comparison shows that larger absolute changes are observed by the fluorescence method. This method also shows greater differences between the effect of ATP and those of ADP and

[6] M. M. Werber, A. G. Szent-Györgyi, and G. D. Fasman, to be published.

TABLE VIII: Comparison of Intrinsic Fluorescence and Difference Ultraviolet Absorption Spectroscopy for Evaluating the Effects of ATP, ADP, and PP_i on HMM and S-1.

Protein	Addition	Intrinsic Fluorescence[a] % Change $\Delta I/I$	Rel Effect	Difference Absorption[b] % Change at 288 ± 1 nm	Rel Effect
HMM	MgATP	17.3	1.00	2.00	1.00
	MgADP	5.4	0.31	1.08	0.54
	$MgPP_i$	1.6	0.09	0.60	0.30
	CaADP	3.8	0.22	0.76	0.38
S-1	MgATP	15.5	1.00	2.60	1.00
	MgADP			1.35	0.52

[a] Conditions: Tris, 20 mM, pH 7.4; KCl, 60 mM; divalent metal ion, 1–2 mM; nucleotide, 150 μM; HMM, 0.15 mg/ml, 25°. [b] Conditions: Tris, 60–70 mM, pH 8.0; KCl, 60–80 mM; Mg^{2+}, 8–10 mM; Ca^{2+}, 1.5 mM; nucleotide, 75 μM; HMM, 1.6–2.2 mg/ml, 25°.

TABLE IX: Separation of the Contributions of Three Classes of Trp to the Fluorescence of HMM.[a]

Addition	$1/f_a'$[b]	I_0[c]	I_1[d]	I_{2+3}[e]	I_3[f]	I_2[g]	$\bar{I}_1$[h]	$\bar{I}_2$[h]	$\bar{I}_3$[h]
None	1.25	145.6	116.5	28.9	0	28.9	9.0	7.2	0
ATP	1.45	168.7	116.3	52.4	23.1	29.3	9.0	7.3	11.5
ADP	1.37	153.5	112.0	41.5	10.0	31.5	8.6	7.9	10.0

[a] Tris buffer, 20 mM, pH 7.5, KCl + KI = 0.21 M, ATP or ADP, 150 μM; Mg^{2+}, 2 mM. All the fluorescence intensity data (I) are expressed in arbitrary units. [b] Intercepts of the modified Stern–Volmer plots (Figure 5). [c] Total fluorescence (in KCl). [d] Fluorescence of class 1 of the Trp residues $I_0/(1/f_a')$. [e] Fluorescence of classes 2 and 3: $I_0 - I_1$. [f] Fluorescence of class 3, *i.e.*, the increase in fluorescence ΔI observed for those Trp. [g] Fluorescence of class 2: $I_{2+3} - I_2$. [h] Averaged fluorescence intensities per one Trp residue.

PP_i. The relative effects of MgADP, CaADP, and $MgPP_i$ which all reflect only binding reactions are larger (by about 0.2) in the absorption data than in the fluorescence data.

Iodide Quenching of Fluorescence. The study on iodide quenching of the fluorescence of HMM revealed that this protein has a very high degree of exposure of its Trp residues, with a f_a' value of 0.80 (see Table VI). The value for lysozyme, for example, varied from 0.38 to 0.66 according to the pH (Lehrer, 1971). This must mean that the structure of the globular heads of myosin, which contain about 18 Trp residues, is fairly loose, at least open enough so as to allow iodide ions to reach 80% of the Trp residues or the majority of the Trp residues lie on the outside of the molecule. A simple explanation of the effects of ADP and ATP (Table VI) assumes that the residues whose fluorescence is increased become inaccessible to iodide ions. The difference between these nucleotides is that ADP influences only one Trp residue while ATP affects two Trp residues. Although the method does not actually allow one to determine the number of residues quenched, because only an effective accessibility fraction, f_a', can be determined, we assume in this discussion that the fluorescence changes can be attributed to specific Trp residues. This assumption is supported by the fact that no change in the K_Q' value is observed upon interaction with ATP. Also consistent with this interpretation is the fact that one can divide the Trp into three classes according to their fluorescence characteristics (see below).

Separation of Contributions of Various Classes of Trp Residues. It is possible to divide the Trp residues of HMM into three classes according to the results of the iodide quenching of the fluorescence. In class 1 are the 13 Trp residues which are accessible to iodide ions even in the presence of ATP; in class 2 belong the 4 Trp residues which are in a nonpolar environment in HMM itself; and class 3 contains the 2 Trp residues which change their environment in the presence of ATP. In Table IX an attempt was made to separate the contributions of those three classes to the total fluorescence, using the data from iodide quenching. The values of I_1 (fourth column) were obtained by dividing the values of I_0 by $1/f_a'$ since in the modified Stern–Volmer equation (eq 2) $I_0/\Delta I_Q$ becomes equal to $1/f_a'$ at infinite concentration of quencher. Thus $I_0/(1/f_a')$ should give the ΔI_Q at infinite concentration of iodide, *i.e.*, all the fluorescence that can be quenched by iodide ions. This is exactly the contribution of the class 1 Trp residues of HMM.

The values in this column are more or less the same in the presence or absence of ADP or ATP. This probably means that the fluorescence of the 2 Trp residues of class 3 was insignificant in the absence of ADP or ATP, since the contribution of class 1 to the fluorescence is the same whether the 2 Trp of class 3 are included in it or not. The contribution of class 3 is thus taken as the absolute increase in fluorescence upon addition of ADP or ATP. Comparison of the data of the last three columns shows that the averaged fluorescence per one Trp residue of class 3 is considerably higher than that of the two other classes. This is consistent with the suggestion that those Trp residues are in a less polar environment in the presence of the nucleotides, which are causing an enhancement of the fluorescence (Table VII) and a shift in the emission wavelength (Van Duuren, 1961) (*cf.* Figure 1).

Molecular Interpretation. ATP changes the fluorescence of both of the Trp residues of class 3. Although the conformational change caused by ATP hydrolysis might involve both of these Trp residues the evidence seems to favor the interpretation that the change is restricted to only one of these Trp residues. This does not imply that 2 moles of ATP cannot be bound per HMM molecule. However, it appears that the fluorescence changes are brought about by one nucleotide residue. The fluorescence of the first Trp residue of class 3 increases as a result of the binding of ATP or ADP. Since this change can be generated from both directions of the kinetic scheme (eq 3), *i.e.*, it is not dependent upon hydrolysis of ATP, and phosphate can compete very efficiently for this site of binding (*cf.* Table V), no conformational change needs to be associated with the increased fluorescence of this residue. An altered polarity of the environment, possibly by the removal of a water molecule which was quenching the fluorescence, would explain the observed effects. A change of this kind may be caused by the stacking of the adenine moiety of the ATP or ADP on this Trp residue. Such a stacking has

been shown to cause about a 15–25% quenching in creatine kinase (Kägi *et al.*, 1971).

The interpretation of the increased fluorescence of the second Trp residue of class 3 is more complex. It appears that when ATP binds (in the presence of a divalent cation, Mg^{2+} or Ca^{2+}) to HMM, it is cleaved very rapidly (Lymn and Taylor, 1971). This step is associated with a local conformational change, causing the second Trp residue of class 3 to move to a more nonpolar environment and the "locking" of the products at the site where they were formed. The main reason for assuming a conformational change is the observation that the increase in the fluorescence of the second Trp residue of class 3 requires ATP hydrolysis and the binding of ATP or ADP is not sufficient to induce it. The fact that the fluorescence change involving the second Trp of class 3 cannot be attained from both directions in this kinetic scheme and that phosphate ions compete much less efficiently for the site in this case (see Table V) lends support to the assumption that a conformational change is associated with the "locking in" of the products. Such a model would be consistent with the interpretation that intermediate exchange in myosin (and HMM) occurs only once for each molecule of ATP cleaved (Swanson and Yount, 1966), whereas medium exchange, which is not dependent upon hydrolysis of ATP, and for which ADP is a better cofactor than ATP (Swanson and Yount, 1965), can occur from all the oxygens of the phosphate. The release of the products from the "locked" conformation depends most certainly on the rate of dissociation of the nucleotide diphosphate from the enzyme which was shown to be about 50 times faster with Ca^{2+} than with Mg^{2+} (Taylor *et al.*, 1970). Since actin has been shown to greatly enhance the rate of dissociation of MgADP from myosin (Lymn and Taylor, 1971), an attractive interpretation would be that the energy derived from the cleavage of ATP and required for contraction is stored in myosin in the form of an enzyme–product complex by virtue of the local conformational change that has taken place at the active site during hydrolysis of the substrate. Actin would then act as an "acceptor" for this energy and thereby accelerate the ATP hydrolysis. PP_i which also activates myosin ATPase (Gallop, 1957) presumably by releasing ADP would be acting thereby as an uncoupler.

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Kinetic Studies on Substrate–Enzyme Interaction in the Adrenal Cholesterol Side-Chain Cleavage System†

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ABSTRACT: A study was made of the kinetics of the oxidative metabolism of (20*S*)-20-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol with adrenocortical heme protein P-450 preparations under various incubation conditions at relatively low substrate concentrations. When the substrate was added last to the complete system, first-order kinetics (within the experimental error) were observed until approximately 10% of the substrate was left; at longer times a decrease in the rate was seen. When the reaction was started by the addition of TPNH after the substrate had been preincubated with the heme protein P-450 system, polyphasic kinetics were observed with a much higher initial rate of substrate disappearance than that observed without equilibration of the substrate with the enzyme system. As a first-order approximation, after equilibration, the early part of the substrate disappearance curve observed could be expressed as a time function with two exponentials. From the parameters of this function could be obtained the first-order (and pseudo-first-order) rate constants of the interaction of the substrate with the enzyme system and of the reaction of the enzyme–substrate complex to irreversibly yield product. This approximation indicated that the latter rate constant was considerably higher than the rates of equilibration. The kinetics of conversion of cholesterol to pregnenolone followed a similar pattern. Under conditions without equilibration, the observed first-order rate constant of substrate disappearance thus appears to represent the overall (slower) pseudo-first-order rate of formation of the enzyme–substrate complex. The access of substrate to the enzyme system appears to be the rate-limiting step in these transformations. The theoretical approach presented may be useful for determining equilibration and reaction rates in other areas of enzymology where equilibration of an enzyme with substrate is possible prior to reaction commencement by the addition of a required cofactor.

In previous studies concerned with the elucidation of the mechanism of the conversion of cholesterol to pregnenolone with adrenocortical preparations, the kinetics of the metabolism of hydroxylated derivatives of cholesterol were studied at low substrate concentrations at which first-order kinetics appeared to hold (Burstein *et al.*, 1970a). In these studies the reaction was always commenced by adding the adrenal enzyme preparation to the substrate in the presence of a TPNH-generating system. In the course of investigations with heme protein P-450 preparations on the relationship between substrate-induced difference spectra of various oxygenated cholesterol derivatives and the enzymatic conversion of cholesterol to pregnenolone (Burstein *et al.*, 1972), it was noticed that the rate at which the spectra developed varied with the particular sterol and preparation studied. Although no definitive correlation between the magnitude (or affinity relationships) of the difference spectra and the enzymatic rates was observed, the rates at which the spectra developed indicated that some interaction between the sterols and the enzyme system was occurring at a measurable rate. This gave us the idea that perhaps the kinetics of substrate metabolism may be different if, prior to reaction commencement, the substrate is allowed to equilibrate with the enzyme preparation. Such an experimental design could be achieved, for example, by preincubating the substrate with the heme protein P-450 supplemented with nonheme iron and flavoprotein, under which conditions no reaction occurs, and by starting the reaction by adding the TPNH-generating system in the presence of oxygen.

This paper describes kinetic studies on the oxidative metabolism of (20*S*)-20-hydroxycholesterol,[1] (22*R*)-22-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol with

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[1] The nomenclature used here conforms with the rules known as the IUPAC–IUB 1967 Revised Tentative Rules for Steroid Nomenclature (*Steroids 13*, 277 (1969)). Thus, the previous terminology for 20α-hydroxycholesterol and (22*R*)-20α,22-dihydroxycholesterol has been changed to (20*S*)-20-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol, respectively.