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INTERACTION OF HEAVY MEROMYOSIN WITH SUBSTRATE

II. RATE OF THE FORMATION OF ATP-INDUCED ULTRAVIOLET DIFFERENCE SPECTRUM OF HEAVY MEROMYOSIN MEASURED BY STOPPED-FLOW METHOD

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SUMMARY

1. The rate of formation of the ultraviolet difference spectrum of heavy meromyosin, induced by ATP, was measured by the stopped-flow method. The initial velocity of formation was proportional to the initial concentration of ATP, indicating a second-order reaction. The second-order rate constant decreased with decrease of $MgCl_2$ and with increase of KCl concentrations, but did not depend on the kind of divalent cation (Mg^{2+} or Ca^{2+}) in the medium.

2. The maximum difference absorbance ΔA_{max} attained after addition of ATP was obtained as a function of initial concentration of ATP. A strong binding of ATP to heavy meromyosin was suggested in the presence of $MgCl_2$. The minimum ATP concentration giving the maximum ΔA_{max} was 2-fold the protein concentration. In the presence of $CaCl_2$, the binding was not as strong.

3. Decay of the difference spectrum was measured. In the presence of $MgCl_2$, k_s calculated using CHANCE's equation agreed well with the maximum velocity of the steady-state ATPase. In the presence of $CaCl_2$, k_s was 3 times larger than the maximum velocity.

4. It is inferred that the difference spectrum accompanies only the Michaelis–Menten complex formed at one of the two sites in heavy meromyosin.

INTRODUCTION

In the preceding papers[1,2], we showed that a difference spectrum of ultraviolet absorption of heavy meromyosin was induced by the addition of ATP or its analogs. The difference spectrum was assumed to be due to a red shift of the absorption bands of some tyrosine and tryptophan chromophores located near the active site of heavy meromyosin and to be a reflection of a substrate-induced local structural change of the protein molecule.

For an understanding of the role of the structural change of the protein molecule in the enzyme action, it seems to be crucial to know what molecular species is associated with the structural change during the process of ATPase reaction. From an

analysis of the decay of the difference spectrum induced by ATP, the change in the local structure of heavy meromyosin has already been estimated to occur while it is present as a Michaelis–Menten complex during the ATPase reaction process[2].

However, it has not been known whether the difference spectrum forms at the same time as the binding of ATP to heavy meromyosin, or whether it forms after some lag period following the binding. In the present paper, to pursue this point, the rate of the formation of the difference spectrum after the addition of ATP was measured using a rapid-flow apparatus with a wide range of ATP concentration or medium composition. In the presence of $MgCl_2$, the minimum concentration of ATP needed to give the maximum change in the difference spectrum was found to be 2-fold the concentration of heavy meromyosin used. The results are compatible with the scheme that there are two ATP binding sites in heavy meromyosin, one is the site for the Michaelis–Menten type ATPase, the other may be for the initial burst of P_i liberation[3]. The difference spectrum forms at the same time with the formation of the *ES* complex at the site for the Michaelis–Menten type ATPase.

EXPERIMENTAL

The ultraviolet difference spectrum was measured with a Hitachi recording spectrophotometer, Type EPS-3T, following the method described previously[2].

The stopped-flow experiments according to the procedure of CHANCE[4] were carried out using an apparatus made of a sensitive spectrophotometer combined with a flow system constructed by NAKAMURA[5]. The rate of formation of the difference spectrum of heavy meromyosin was measured by recording the change of $A_{293\ m\mu}$, where the difference between the difference spectra due to ATP and ADP is the maximum[2].

ATPase activity at the steady state was determined by measuring the time course of P_i liberation. Concentration of P_i was determined using the methods of FISKE–SUBBAROW[6] or of MARTIN–DOTY[7] modified for low P_i contents, depending on the concentration of ATP used.

Preparation and purification of heavy meromyosin have been described[1]. The concentration of heavy meromyosin was determined by measuring $A_{280\ m\mu}$. A value of 6.35 was used as $E^{1\%}_{1\ cm}$ at 280 mμ. Molecular weight of heavy meromyosin used was $3.65 \cdot 10^5$. All the measurements described in this paper were performed in the presence of 20 mM Tris–HCl buffer (pH 8). ATP was purchased from the Sigma Chemical Co.

RESULTS

Formation of the difference spectrum

Formation of the difference spectrum after mixing ATP with heavy meromyosin was followed by the rapid-flow apparatus at a fixed wavelength, 293 mμ. Typical traces of the measurements are shown in Fig. 1. In the presence of $MgCl_2$, as shown in Figs. 1a and 1b, $A_{293\ m\mu}$ reached a maximum value (ΔA_{max}) after a rapid increase of the absorbance, and any decay could not be observed in the same time scale. Each measurement was begun before the decay of the difference absorbance of the former measurement was observed, then the downward deflexion in the spectrophotometric trace was seen at the time of mixing. In the presence of $CaCl_2$, however, an immediate decay followed the increase of the absorbance as shown in Fig. 1c.

The rate of the increase of the absorbance after the addition of ATP depended on the initial concentration of ATP. The initial velocity (v_i) of the increase of the absorbance was estimated from each measurement. In Figs. 2a and 2b, plots of v_i *vs.* initial concentration of ATP are shown. A circle with bar in Fig. 2 is an average value of 3–13 determinations and its standard deviation. Values of v_i are proportional to the initial concentration of ATP, indicating that the formation of the difference spectrum of heavy meromyosin by ATP is of a second-order reaction. The second-order rate constants were determined from the slope of the plot as illustrated in Fig. 2, and are

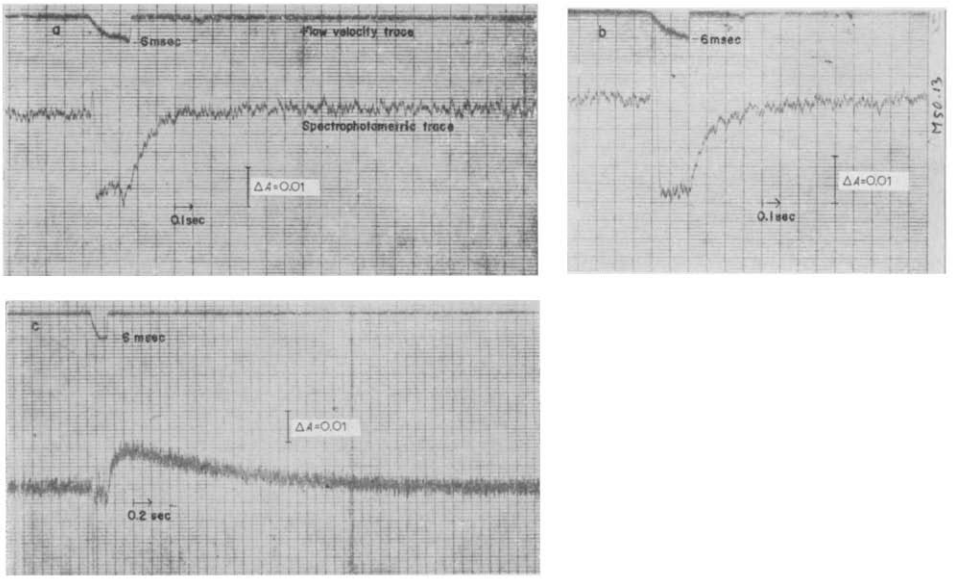

Fig. 1. Traces of the stopped-flow measurement after mixing ATP with heavy meromyosin. a. In the presence of 2.05 mg heavy meromyosin per ml, 1 mM $MgCl_2$ and 0.1 M KCl at 6°. Concentration of ATP was 30.3 μM. b. In the presence of 1.76 mg heavy meromyosin per ml, 1 mM $MgCl_2$ and 0.25 M KCl at 10°. Concentration of ATP was 24.6 μM. c. In the presence of 1.85 mg heavy meromyosin per ml, 2 mM $CaCl_2$ and 0.25 M KCl at 10°. Concentration of ATP was 30.3 μM.

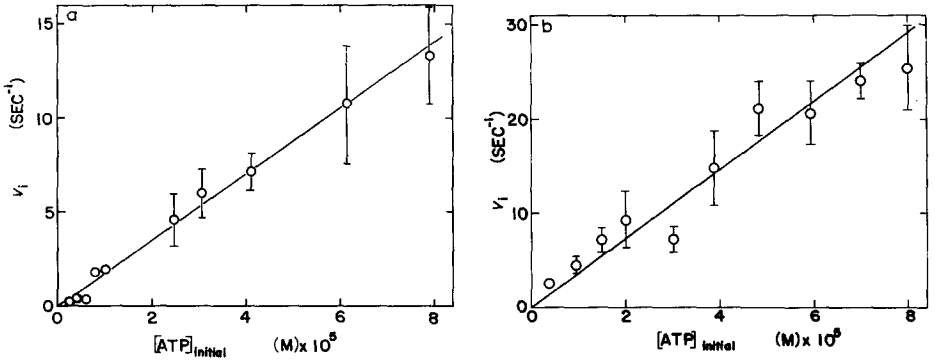

Fig. 2. Dependence of the initial velocity of the formation of the difference spectrum on the initial concentration of ATP. a. In the presence of 1.79 mg heavy meromyosin per ml, 0.2 mM $MgCl_2$ and 0.25 M KCl at 10°. b. In the presence of 1.91 mg heavy meromyosin per ml, 2 mM $CaCl_2$ and 0.25 M KCl at 10°.

shown in Fig. 3 and in Table I. The rate constant increased with increase of $MgCl_2$ concentration and decreased with increase of KCl concentration (Fig. 3). On the other hand, the rate constants did not depend on the kind of divalent cations in the medium, in the presence of either 2 mM $MgCl_2$ or $CaCl_2$ in 0.25 M KCl at 10° (Table I).

Values of ΔA_{max} were determined from each measurement and were shown in Fig. 4 as a function of initial concentration of ATP. In the presence of $MgCl_2$, the curve suggests a strong binding of ATP to heavy meromyosin (Fig. 4a). The minimum

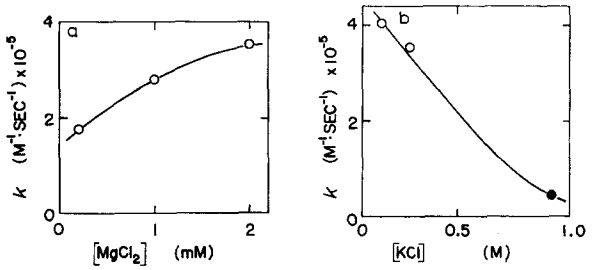

Fig. 3. Effects of ionic conditions on the second-order rate constant. a. Effect of $MgCl_2$ concentration. In the presence of 1.76–1.84 mg heavy meromyosin per ml and 0.25 M KCl at 10°. b. Effect of KCl concentration. In the presence of 1.62–2.08 mg heavy meromyosin per ml at 10°. Concentrations of $MgCl_2$ were 2 mM (○) and 10 mM (●).

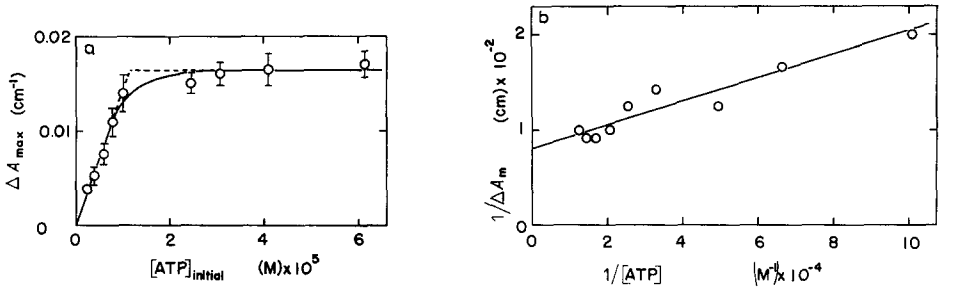

Fig. 4. Dependence of ΔA_{max} on the concentration of ATP. a. In the presence of 2.08 mg heavy meromyosin per ml, 2 mM $MgCl_2$ and 0.1 M KCl at 10°. b. In the presence of 1.91 mg heavy meromyosin per ml, 2 mM $CaCl_2$ and 0.25 M KCl at 10°.

TABLE I

FORMATION OF THE DIFFERENCE SPECTRUM INDUCED BY ATP

Concentration of heavy meromyosin varied from 1.62 to 2.08 mg/ml.

Concn. of KCl (M)	*Concn. of divalent cation (mM)*	*Temp.*	*Second-order rate constant* $(M^{-1} \cdot sec^{-1}) \times 10^{-5}$	*Stoichiometry (n)*
0.1	$MgCl_2$ 1.0	6°	2.5	1.9
0.1	$MgCl_2$ 2.0	10°	4.0	2.0
0.25	$MgCl_2$ 0.2	10°	1.8	2.4
0.25	$MgCl_2$ 1.0	10°	2.8	2.3
0.25	$MgCl_2$ 2.0	10°	3.5	2.2
1.0	$MgCl_2$ 10.0	10°	0.44	—
0.25	$CaCl_2$ 2.0	10°	3.7	—

concentration of ATP needed to give the maximum ΔA_{max} could easily be obtained from the plot as shown in Fig. 4a and was almost 2-fold the concentration of heavy meromyosin used. The stoichiometry (n) obtained in this way under various ionic conditions is listed in Table I. In the presence of $CaCl_2$, on the other hand, data suggested a weaker binding of ATP to heavy meromyosin; these are shown in Fig. 4b as a plot of $1/\Delta A_{max}$ *vs.* $1/[ATP]$ (*cf.* DISCUSSION). The difference molar extinction coefficient at infinite concentration of ATP, ΔE at 293 mμ, calculated from the intercept of the ordinate was 2400 $M^{-1} \cdot cm^{-1}$, which is about 70% of that in the presence of $MgCl_2$.

Decay of the difference spectrum

As has been reported in the previous paper[2], the decay of the difference spectrum induced by ATP in the presence of $MgCl_2$ was slow enough to follow the change by a usual recording spectrophotometer. The value of k_s was calculated from the decay, using the equation originally derived by CHANCE[8]. The value of k_s agreed well with k_2 (= v_{max}/e) obtained from the steady-state ATPase reaction over a wide range of ionic conditions[2]. The relation was reexamined under the same conditions as those of the rapid-flow experiment, using the recording spectrophotometer at a high concentration of ATP compared with that of heavy meromyosin. Values of k_s obtained are listed in Table II, with v_{max}/e determined from the measurement of ATPase reaction. The two values in the same ionic medium agree well with each other.

TABLE II

DECAY OF THE DIFFERENCE SPECTRUM INDUCED BY ATP

For the determination of k_s, concentrations of heavy meromyosin varied from 1.62 to 2.08 mg/ml and of ATP from 0.12 to 0.053 mM. Each k_s value is the average of 2–4 determinations. For v_{max}/e, concentration of heavy meromyosin varied from 0.006 to 0.2 mg/ml.

Concn. of KCl (M)	*Concn. of divalent cation (mM)*	*Temp.*	k_s (sec^{-1})	v_{max}/e (sec^{-1})
0.1	$MgCl_2$ 1.0	6°	0.036	0.028
0.1	$MgCl_2$ 2.0	10°	0.050	0.041
0.25	$MgCl_2$ 0.2	10°	0.043	0.038
0.25	$MgCl_2$ 1.0	10°	0.037	0.035*
0.25	$MgCl_2$ 2.0	10°	0.039	0.040
1.0	$MgCl_2$ 10.0	10°	0.0086	0.0097
0.25	$CaCl_2$ 2.0	10°	13 ± 3	3.7

* Calculated from the initial velocity at 0.11 mM ATP.

We inferred in the previous paper[2] that in the presence of $CaCl_2$, the decay of the difference spectrum induced by ATP was too fast to be followed by the usual recording spectrophotometer. As shown in Fig. 1c, the decay was so fast that it could be followed only by the stopped-flow method. The k_s was calculated by CHANCE's equation, using half-life time obtained from the stopped-flow experiment. The average value of experiments with nine different ATP concentrations was 13 ± 3 sec^{-1} in the presence of 2 mM $CaCl_2$, 0.25 M KCl at 10°. Obviously, this is almost 3 times larger than v_{max}/e, 3.7 sec^{-1}, as shown in Table II.

DISCUSSION

As shown in Table I, in the presence of $MgCl_2$, the minimum concentration of ATP needed to give the maximum change in ΔA_{max} was 2-fold heavy meromyosin concentration. This suggests that 2 moles of ATP are bound per mole of heavy meromyosin. TONOMURA AND MORITA[9] have shown earlier, using the equilibrium dialysis method, that 2 moles of PP_i were bound per mole of myosin, thus indicating two ATP binding sites per mole of myosin. Results reporting the presence of two binding sites have recently been presented by other investigators[10,11], although 3 and 1.5 sites have also been reported[12,13,14].

According to IMAMURA, TADA AND TONOMURA, after the addition of ATP to heavy meromyosin as well as myosin in the presence of $MgCl_2$, 1 mole of P_i is liberated per mole of enzyme in the initial burst before P_i liberation is governed by the steady-state rate. They have proposed a scheme for the reaction of myosin ATPase having two pathways for P_i liberation through a common Michaelis–Menten complex; one leads to P_i liberation of the Michaelis–Menten type, the other to the initial burst of P_i liberation[3]. The difference spectrum of heavy meromyosin induced by ATP was measured in the presence of 1 M KCl, 10 mM $MgCl_2$, which are reported to be the best conditions for obtaining one mole of P_i liberated in the initial burst per mole of heavy meromysin[15]. As shown in Table II, k_s agreed well with v_{max}/e, as was the case under the other conditions. No observable decay corresponding to the P_i liberation in the initial burst was detected. The results seem to suggest that there is no direct correlation between the spectral change in heavy meromyosin induced by ATP and the initial burst of P_i liberation. It may be considered, therefore, that only one out of the two ATP binding sites functions for the Michaelis–Menten type ATPase reaction, and the difference spectrum by ATP or analogs is induced only in this site. The other site may be for the initial burst of P_i liberation.

As shown in Fig. 2, the rate of the formation of the difference spectrum by ATP followed a second-order reaction, under every ionic condition tested. In the presence of $MgCl_2$, k_s agreed with v_{max}/e, as described above and in the previous paper[2], but in the presence of $CaCl_2$, it was about three times larger than v_{max}/e (Table II). On the basis of these results, the following mechanism could be proposed for the ATPase reaction accompanying the spectral change

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES(\mathrm{ATP}) \xrightarrow{k_2} EP(\mathrm{ADP}) \xrightarrow{k_3} E + P_i + \mathrm{ADP} \qquad (1)$$

where ES(ATP) is the Michaelis–Menten complex and (ATP) represents that the complex accompanies the difference spectrum due to ATP. EP(ADP) represents a complex of the enzyme with product(s) and gives the difference spectrum due to ADP.

(i) According to this reaction mechanism, the initial velocity of the formation of the difference spectrum induced by ATP, v_i, is given by the following equation,

$$v_i = k_1[E_0][S_0] \qquad (2)$$

where $[E_0]$ and $[S_0]$ represent the initial concentrations of enzyme and of ATP, respectively. When $[E_0]$ is constant, v_i must be proportional to the initial concentration of ATP. This was fulfilled under every ionic condition. The second-order rate constant obtained, therefore, corresponds to k_1 according to Mechanism 1.

(ii) The value of k_s which was calculated from the decay of the difference spectrum by ATP is given by

$$k_s = k_2 = [S_0]/\int_0^\infty p\,dt = [S_0]/p_{max}\cdot\tau_{1/2} \quad (3)$$

where p represents the concentration of ES(ATP) and p_{max} is the maximum value of p, $\tau_{1/2}$ is the time required for p to fall from p_{max} to one half of p_{max} and $[S_0]$ is the initial concentration of ATP. k_s listed in Table II corresponds to k_2 according to Mechanism 1.

(iii) The steady-state ATPase reaction follows the Michaelis–Menten type and the values of v_{max} and K_m are given by

$$e/v_{max} = 1/k_2 + 1/k_3 \quad (4)$$

$$K_m = (k_2 + k_{-1})/k_1/(1 + k_2/k_3) = K_s/(1 + k_2/k_3) \quad (5)$$

where $K_s = (k_2 + k_{-1})/k_1$. If $k_3 \gg k_2$, then Eqns. 4 and 5 are reduced to

$$e/v_{max} = 1/k_2 \quad (4')$$

$$K_m = (k_2 + k_{-1})/k_1 = K_s \quad (5')$$

In the presence of $MgCl_2$, the k_s (= k_2) agreed well with v_{max}/e as shown in Table II, and Eqn. 4′ is fulfilled. Then k_2 is the rate-determining step. In the presence of $CaCl_2$, however, k_s was significantly larger than v_{max}/e (Table II), and k_3 is the rate-determining step according to Eqn. 4. It is not known in the presence of $CaCl_2$, however, whether 2 moles of ATP are bound per mole of heavy meromyosin or not, and therefore there is no means of estimating true rate constants.

(iv) ΔA_{max} is the concentration of ES(ATP) at $dES(ATP)/dt = 0$; then ΔA_{max} is given by the following equation

$$\Delta A_{max} = e/(K_s/[s] + 1) \quad (6)$$

where $[s]$ represents free concentration of ATP. According to this equation, the plot of $1/\Delta A_{max}$ *vs.* $1/[s]$ must give a straight line. ΔA_{max} at the infinite concentration of ATP and K_s are obtained from the intercept and slope of the line. In the presence of 2 mM $CaCl_2$, the plot followed a straight line, as shown in Fig. 4b. The value of K_s was $1.3\cdot10^{-5}$ M. In the presence of $MgCl_2$, however, the change in ΔA_{max} occurred at a similar concentration of ATP as that of heavy meromyosin (Fig. 4a), and the total concentration of ATP could not be regarded as the free one. The validity of Eqn. 6, therefore, cannot be tested by the double-reciprocal plot. From careful measurement of ATPase reaction at the steady state, K_m was determined as $7.5\cdot10^{-7}$ M by the Lineweaver–Burk plot in the presence of 2 mM $MgCl_2$ and 0.1 M KCl at 10°. Since the values of k_1 and k_2 (v_{max}/e) under the same conditions were $4.0\cdot10^5$ $M^{-1}\cdot sec^{-1}$ and 0.041 sec^{-1}, respectively (as shown in Tables I and II), k_{-1} is calculated to be 0.26 sec^{-1} from Eqn. 5′. This is 6 times larger than k_2. Both K_m and K_s, therefore, are nearly equal to k_{-1}/k_1, the dissociation constant of ES(ATP) complex. The plot ΔA_{max} *vs.* ATP concentration such as shown in Fig. 4a, therefore, could be regarded as the binding curve of ATP to the site in heavy meromyosin accompanying the spectral change.

Then, the shape of the curve of unsaturated portion seems to depend on affinities of ATP to both sites. If the affinity of the site accompanying the spectral change is equal to or higher than the other, ΔA_{max} is almost proportional to total ATP. The

stoichiometry obtained from the minimum concentration of ATP to give the maximum ΔA_{max} is 2 at the same affinities, and it decreases from 2 towards 1 as the affinity of the site accompanying the spectral change becomes greater than the other. If the affinity of the site accompanying the spectral change is lower than the other, the curve may be S-shaped. The shape of the curve could not be estimated correctly because of the low accuracy of ΔA_{max} at low concentrations of ATP. In the previous paper[2], we reported that the stoichiometry of ADP was one per 1 mole of heavy meromyosin at 0.06 M KCl at 25°. Under these conditions, affinity of ADP to the site accompanying the spectral change is probably higher than the other. In the present case, however, both sites seem to bind ATP with a similar affinity, as shown by the fact that the stoichiometry was two, and ΔA_{max} was almost proportional to ATP concentration until it reached the maximum value (Fig. 4a). Assuming an identical affinity, K_s was calculated to be $4 \cdot 10^{-7}$ M from the data of Fig. 4a; this agrees well with K_m ($7.5 \cdot 10^{-7}$ M).

It is therefore concluded that the ultraviolet difference spectrum is formed at the same time as the Michaelis–Menten complex is formed at the site where the apparent steady-state ATPase operates in the presence of $MgCl_2$, and has the same life time as that of the complex.

It may be worthwhile to note that almost the same k_1 was obtained in the presence of $CaCl_2$ and of $MgCl_2$, as shown in Table I. EIGEN AND HAMMES[16] reported that the chelate formation of Ca^{2+} with ATP or other ligands is 10^3 times faster than those of Mg^{2+}. k_1 was $3.6 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ under 0.25 M KCl at 10° and may become several times larger at 25°, which is in the range of the diffusion-controlled formation of enzyme–substrate complex. k_1 markedly depends on KCl concentration, *i.e.*, ionic strength in the medium as shown in Fig. 3b.

The difference spectrum seems to reflect only positional change in the side chains of tyrosine and tryptophan residues in heavy meromyosin molecule. The result that the difference spectrum forms at the same time as the formation of Michaelis–Menten complex indicates that such a structural change in the protein can occur faster than 10^{-3} sec. This is not so surprising considering the fact that helix–coil transition in polypeptide occurs in times as short as 10^{-7} sec (ref. 16).

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