that the masking originally observed when the polymer is in water is due to an ice-like hydration lattice, then it follows naturally that the hydrogen-bond breaking power of urea[17] would manifest itself by disrupting the hydration lattice of the polymer. Such an action would account immediately for the upward shift in pK_a of the polyvinylpyrrolidone conjugate.

Since denaturation of this synthetic polymer cannot involve any disruption of intramolecular hydrogen bonds, the question arises whether it is necessary or valid to assume that denaturation of protein molecules always involves a disruption of intramolecular hydrogen bonds. The primary step with protein too might be instead a perturbation of solvent-(macromolecular) solute interactions.

Acknowledgment.—This investigation was assisted by a grant from the National Science Foundation.

(17) It has been suggested by W. Kauzmann [*Adv. Protein Chem.*, **14**, 1 (1959)] that urea disrupts hydrophobic bonds. Such a postulate, however, represents a substantial departure from the generally-accepted view that the action of urea depends on its ability to break hydrogen bonds. Hydrophobic bonds, as usually pictured, are not based on direct hydrogen-bonding. Urea does indeed form inclusion complexes with hydrocarbons, as mentioned by Kauzmann, but only if they are straight-chain aliphatic molecules (not branched nor aromatic) with a length of six or more carbon atoms [E. Bengen and W. Schlenk, Jr., *Experientia*, **5**, 200 (1949)]; hydrophobic amino acid side chains do not fulfill these requirements.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE AND THE RESEARCH INSTITUTE FOR CATALYSIS, HOKKAIDO UNIVERSITY, SAPPORO, JAPAN]

Kinetic Analysis of the Pyrophosphate–Myosin B System by the Use of the Light-scattering Method

BY FUMI MORITA AND YUJI TONOMURA

RECEIVED DECEMBER 24, 1959

For the explanation of the change produced by pyrophosphate (PP) in the shape of myosin B (M) in 0.6 M KCl solution, the following mechanism was postulated; $M + PP \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} MPP$ (1), $MPP \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} M^*PP$ (2), where the asterisk indicates the changed state of myosin B. All the experimental results obtained by the use of the light-scattering method were in accord with this mechanism. The dissociation constant of the over-all reaction K and the forward rate constant of step 2 k_2, were determined from the decrements at equilibrium and at the transient states, respectively, in the light scattered by myosin B caused by PP. In the presence of Ca^{++} in concentrations higher than 0.3 mM, the dissociation constant of step 1 K_1 could be determined according to the transient method and that of step 2 K_2 could be calculated from the values of K and K_1. The mean values of K_1 and K_2 of the seven preparations tested were 1.1×10^{-3} and 5.2×10^{-2} M, respectively, in the presence of Ca^{++} and at pH 7.9, $D = 82.56$ and at 5°. The steady-state velocity for the reverse direction was measured by the addition of inorganic pyrophosphatase at the equilibrium of the reaction between myosin B and PP. The ratio of the velocity of the reverse reaction to that of the forward one agreed well with the dissociation constant. The enthalpy change (ΔH) of each step was calculated according to the Arrhenius equation; the mean values of ΔH of the steps 1 and 2 are −9.2 and −11 kcal./mole, respectively. The change in electrostatic free energy could be calculated from the dependence of K_1 and K_2 on the dielectric constant of the medium. The values of 11 and −44 kcal./mole were obtained for the steps 1 and 2, respectively. The entropy changes of the steps 1 and 2 were evaluated from the changes in free energy and enthalpy, and the values of −19 and −33 cal./mole deg. have been obtained, respectively. K_2 was nearly independent of pH, while K_1 decreased remarkably at pH above 8. The molecular mechanism of the change in the shape of myosin B was discussed on the basis of the results obtained.

Introduction

The molecular mechanism of the change in the size and shape of myosin B caused by adenosinetriphosphate (ATP) or pyrophosphate (PP) has not been clear until recently, in spite of much effort made by several workers.[1–3] However, it was shown by the use of the light-scattering method and the ultracentrifugal analysis[4,5] that the main components of myosin B become elongated by the addition of ATP or PP. The results obtained by the equilibrium dialysis and the light-scattering method indicated that the myosin B molecule contains one mole of the binding site for PP per 5.6×10^5 g. and that the intensity of the light scattered decreases with an almost constant value, every time one PP molecule is bound to one site of myosin B, each site being equal in its intrinsic affinity for PP.[6]

On the basis of these results the molecular kinetic mechanism for the interaction between myosin B and PP were investigated by the use of the light-scattering method. One of the present authors[7] previously studied the transient light-scattering change of myosin B following the addition of ATP. However, the change in light-scattering used to take place so fast that the present technique of mixing seemed to be somewhat unsatisfactory and the correction on account of ATP hydrolysis was required. Myosin B does not hydrolyze PP. Change in the light scattered proceeds much slower after the addition of PP than after the addition ATP. Therefore, by the use of the transient light-scattering method, the effect on the shape of myosin B can be investigated more quantitatively in the case of PP than in the case of ATP.

(1) J. J. Blum and M. F. Morales, *Arch. Biochem. Biophys.*, **43**, 208 (1953).

(2) A. Weber, *Biochim. Biophys. Acta*, **19**, 345 (1956).

(3) J. Gergely, *J. Biol. Chem.*, **220**, 917 (1956); J. Gergely and H. Kohler, "Conference on the Chemistry of Muscular Contraction," Igakushoin, Tokyo, 1957, p. 14.

(4) M. F. Gellert, P. H. Von Hippel, H. K. Schachman and M. F. Morales, THIS JOURNAL, **81**, 1384 (1959).

(5) T. Nihei and Y. Tonomura, *J. Biochem.*, **46**, 1355 (1959).

(6) Y. Tonomura and F. Morita, *ibid.*, **46**, 1367 (1959).

(7) Y. Tonomura, S. Watanabe and K. Yagi, *ibid.*, **40**, 27 (1952).

Experimental

Myosin B solutions were prepared from minced rabbit skeletal muscle by extraction for 24 hr. with a 6-fold weight of Weber–Edsall solution. The extracts were then purified by repeating four times precipitation and resolution at ionic strength of 0.2 and 0.6, respectively. The product was dissolved in 0.6 *M* KCl, clarified by centrifugation at 23,000 g. for 30 min. and stored in a refrigerator at 0° until used. Crystalline inorganic pyrophosphatase (PPase) was obtained through the courtesy of Dr. M. Kunitz of the Rockefeller institute, New York.

Measurements of the scattering of light were carried out at the angle of 90° from the incident beam in the apparatus previously described.[8] Currents from the photomultiplier were measured by a micromicroammeter (Ohkura, Model AM 102) or recorded by a Sanborn Twin Viso Recorder (Model 60–1300 B).

The experimental procedures were: aliquots of the stock solution of myosin B were diluted with buffer (usually mixture of 0.6 *M* KCl and 0.01 *M* tris-(hydroxymethyl)-amino-ethanemaleate at *p*H 7.9, containing a certain quantity of Ca^{++} or Mg^{++}) to a final protein concentration of about 0.5 mg./ml. It was previously reported[4,5] that the second virial coefficient of myosin B solution is essentially zero. In fact, in the range of 0.2–2 mg. protein/ml., the results were found to be independent of the concentration of the protein. Ten ml. of the protein solution were transfered to the rectangular cell and a certain amount of PP was blown out into the solution using a pipette with its tip cut rectangularly to its axis. Organic solvents were diluted previously and mixed with the protein solution 5 min. before the start of the reaction. The experiments were carried out at 5°, unless otherwise stated.

The optical property of the PP-myosin B system varied to some extent from one preparation to another, particularly in the presence of Mg^{++}.[9] Therefore, in the present experiments, the kinetic and the thermodynamic constants were measured on as many preparations as possible and the properties common to all the data were taken as valid.

Quantitative Treatment of the Mechanism.—It was shown by the method of light-scattering and the ultracentrifugal separation that PP elongates the majority of 24-hr. extracted myosin B, without changing the molecular weight of myosin B.[5] Furthermore, it was observed that the relation between the degree of change of light-scattering (Δ/Δ_c) and the concentration of PP, [PP], was almost independent of the angle of light scattered.[6] Here Δ and Δ_c are defined by

$$\Delta = I_0 - I_d$$

$$\Delta_c = I_0 - I_\infty$$

where I_0 and I_d are the intensities of light-scattering before and after the addition of some amount of PP, respectively, and I_∞ after the addition of sufficient amount of PP. Accordingly, it was concluded that in the case of 24 hr. extracted myosin B, the decrease in light-scattering at 90° is due decisively to the elongation of the myosin B molecule. From the comparison of the extent of the binding of PP with the degree of the change in light-scattering of myosin B, it was concluded that 5.6×10^5 g. of myosin B contains one mole of the binding site available to PP-binding, that the interaction between the sites is negligible and that the intensity of light scattered by myosin B is decreased by an almost constant value every time one PP molecule combines to one binding site of myosin B.[6]

Basing on these conclusions, the following reaction sequence has been postulated for explaining the change by PP in the light- scattering of myosin B

$$M + PP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MPP \qquad (1)$$

$$MPP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M^*PP \qquad (2)$$

where M is the unit of myosin B which behaves almost independent of each other in respect to the change of light-scattering and the binding of PP, the asterisk indicating the change of myosin B. Now, assuming that the light-scattering due to the intermediate complex, MPP, is practically identical to that due to M, then, at the equilibrium of the reactions 1 and 2, the relation between the decrease of light-scattering and [PP] is given by

$$\frac{[M^*PP]}{\epsilon} = \frac{\Delta}{\Delta_m} = \frac{1/(1 + K_2)}{1 + K/[PP]} \qquad (3)$$

where

$$K = K_1K_2/(1 + K_2),\ K_1 = k_{-1}/k_1 \text{ and } K_2 = k_{-2}/k_2$$

$$\frac{[M^*PP]}{[MPP] + [M^*PP]} = \frac{\Delta_c}{\Delta_m} = \frac{1}{1 + K_2} \qquad (4)$$

and

$$\frac{[MPP] + [M^*PP]}{\epsilon} = \frac{\Delta}{\Delta_c} = \frac{1}{1 + K/[PP]} = \langle \nu \rangle \qquad (5)$$

where ϵ is the total concentration of the unit of myosin B and Δ_m is defined by

$$\Delta_m = I_0 - I_{\infty,\ K_2 << 1}$$

where $I_{\infty,K_2<<1}$ denotes I_∞ which is observed when $K_2 \ll 1$. The experiment of equilibrium dialysis[6] has already demonstrated the relation 5, where $\langle \nu \rangle$ denotes the average occupation of the site by PP.

During the early stage where the reverse reaction of the step 2 can be neglected, the equations obtained for the course of time of the change in the shape of myosin B are

$$\frac{d[M^*PP]}{dt} = k_2[MPP]$$

and at the steady state

$$k_1[M][PP] = (k_{-1} + k_2)[MPP]$$

$$\frac{d[M^*PP]/\epsilon}{dt} = \frac{k_2}{1 + K_b/(PP)}(1 - [M^*PP]/\epsilon)$$

where

$$K_b = (k_{-1} + k_2)/k_1$$

Therefore, the course of time of the decrease in light-scattering is given by

$$\ln (1 - \Delta_t/\Delta_m) = -\vec{k}\, t \qquad (6)$$

$$\vec{k} = \frac{k_2}{1 + K_b/[PP]} \qquad (7)$$

where $\Delta_t = I_0 - I_t$ and I_t is the intensity of light-scattering at time t after the addition of PP.

Therefore, when the data are plotted in terms of $\ln (1 - \Delta_t/\Delta_m)$ *vs.* t, the apparent rate constant for the forward direction $\vec{k}$ can be evaluated from the inclination of the line. The values of k_2 and K_b are determined by the usual manner from the straight line of $1/\vec{k}$ *vs.* $1/[PP]$. Especially, when $k_2 \ll k_{-1}$, K_b can be reduced to K_1, and K_2 calculated from the values of K and K_1.

For the course of time of the reverse reaction after the addition of a sufficient amount of PPase to the myosin B elongated beforehand by PP, the equations obtained are

$$-d[M^*PP]/dt = k_{-2}[M^*PP] - k_2[MPP]$$

and at the steady state

$$(k_{-1} + k_2)[MPP] = k_{-2}[M^*PP]$$

$$-\frac{d[M^*PP]}{dt} = \frac{k_{-1}k_{-2}}{(k_{-1} + k_2)}[M^*PP]$$

Therefore, the equations obtained for the course of time of the increase of the light-scattering after the addition of PPase are

$$-\ln \Delta_t/\Delta_s = \overleftarrow{k}\, t \qquad (8)$$

$$\overleftarrow{k} = k_{-1}k_{-2}/(k_{-1} + k_2) \qquad (9)$$

Here Δ_s and Δ_t are defined by

$$\Delta_s = I_0 - I_s$$

$$\Delta_t = I_0 - I_t$$

(8) H. Matsumiya, F. Morita, S. Kitagawa, K. Yagi and Y. Tonomura, *J. Biol. Chem.*, **44**, 347 (1957).

(9) Y. Tonomura, F. Morita and K. Yagi, *J. Phys. Chem.*, **61**, 605 (1957).

where I_s and I_t are the intensities of light-scattering at time zero and t after the addition of PPase.

It is apparent from eq. 7 and 9 that when $K_b \gg$ [PP], $\vec{k}/$[PP] is reduced to $k_1k_2/(k_{-1} + k_2)$ and $\overleftarrow{k}$ [PP]$/\vec{k}$ is equal to K_1K_2.

Results

Effects of Calcium.—It has already been reported[9] that the relation between Δ/Δ_c and [PP] is given by eq. 5 in the presence of Ca^{++} and the dissociation constant K is independent of [Ca^{++}]. As shown in Fig. 1, K of the preparation used was also independent of [Ca^{++}] in the range of 0.1 to 0.7 mM.

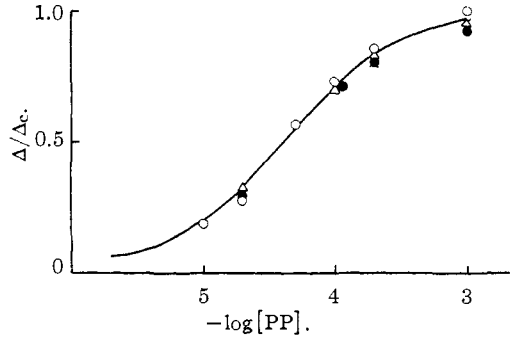

Fig. 1.—Effect of concentration of Ca^{++} upon the decrease in light-scattering of myosin B in the presence of PP, at equilibrium; pH 7.9; 0.6 M KCl; D, 82.56; 5°; prep. no 4. ○, 0.5 mM; ×, 0.3 mM; △ 0.1 mM; ●, 50 μM of Ca^{++}. The solid line represents $\Delta/\Delta_c = 1/\left(1+\frac{7.6 \times 10^{-5}}{[PP]}\right)$

As is illustrated in Fig. 2, during the early stage of the reaction, the values of $\ln(1 - \Delta_t/\Delta_m)$ were linear with the time within the experimental error.

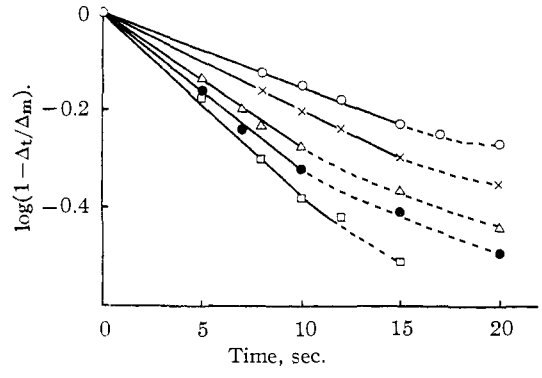

Fig. 2.—Decrease in light-scattering of myosin B after addition of PP; pH 7.9; 0.6 M KCl; 0.5 mM Ca^{++}; D, 82.56; 5°; prep. no. 4. ○, 0.2 mM; ×, 0.3 mM; △, 0.5 mM; ●, 0.7 mM; □, 2 mM of PP.

The reciprocal inclination of these lines was plotted in Fig. 3 in terms of $1/\vec{k}$ *vs.* 1/[PP], and the straight line was obtained in each concentration of Ca^{++}. The values of K_b and k_2 were evaluated from eq. 7. K_b was nearly independent of [Ca^{++}], while k_2 increased with the increase of [Ca^{++}]. For example, for prep. no. 4 and at dielectric constant, D = 82.56, k_2 increased from 0.10 to 0.18 sec.$^{-1}$ with the increase of Ca^{++} from 0.3 to 0.7 mM, while K_b was 4.4×10^{-4} M irrespective of [Ca^{++}].

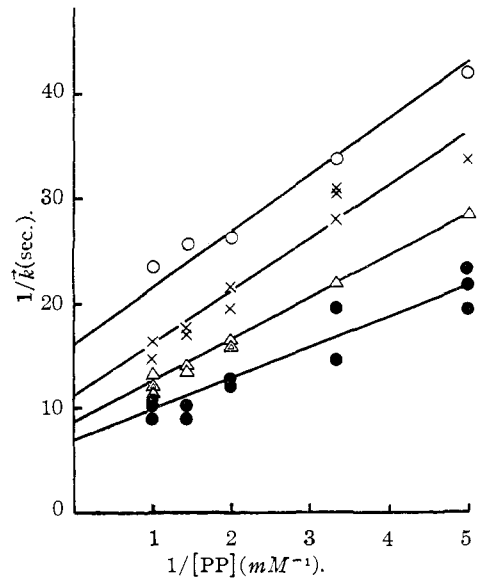

Fig. 3.—Reciprocal apparent rate constants for the decrease in light-scattering in the presence of Ca^{++} as a function of 1/[PP]; pH 7.9; 0.6 M KCl; D, 82.56; 5°; prep. no. 4. ○, 0.1 mM; ×, 0.3 mM; △, 0.5 mM; ●, 0.7 mM of Ca^{++}.

For prep. no. 5 and at D = 85.37, k_2 increased from 0.014 to 0.032 sec.$^{-1}$ with the increase of Ca^{++} from 0.1 to 0.7 mM, while K_b was constant (3.6×10^{-4} M), independent of [Ca^{++}]. These results indicate that in the presence of Ca^{++} at concentrations higher than 0.3 mM, $k_{-1} \gg k_2$ and K_b can be reduced to K_1. The values of K, K_1 and k_2 of several preparations are listed in Table I together with the values of K_2 which can be evaluated from K and K_1.

TABLE I

DISSOCIATION AND RATE CONSTANTS OF THE LIGHT-SCATTERING CHANGE OF MYOSIN B BY PP

Experiments at pH 7.9, with 0.6 M KCl, at D = 82.56 and 5°

Prep. no.	[Ca^{++}], mM	K × 10^5, M	K_1 × 10^4, M	K_2 × 10^2	k_2 × 10^2, sec.$^{-1}$	k_{-2} × 10^2, sec.$^{-1}$
2	0.5	5.4	13	4.6	14	6.4
4	.5	4.2	4.4	10	18	18
5	.5	3.1	5.7	6.2	10	6.2
6	.5	4.0	12	3.6	9	3.2
7	.5	2.7	7.0	4.0	15	6.0
8	.4	3.4	6.3	5.7	8	4.5
9	.56	4.7	29	2.3	25	5.7

Effect of Magnesium.—In the presence of Mg^{++}, Δ/Δ_c depends on [Mg^{++}] and on [PP] in a complicated manner. Moreover the relation Δ/Δ_c *vs.* [PP] varies largely from one preparation to another.[9] However, Δ_c is usually equal to Δ_m which is observed, when the dielectric constant is sufficiently low and in the presence of Ca^{++} (see below). Therefore, it may be concluded that, in the presence of Mg^{++}, $K_2 \ll 1$ and K is equal to K_1K_2.

The relation of $\vec{k}$ to [PP] also varies largely from one preparation to another and with [Mg^{++}]. For example, in the presence of 0.1 mM of Mg^{++} and at D = 86.30, pH 7.9, 5°, the plot of Δ/Δ_c *vs.*

[PP] of prep. no. 7 followed eq. 5 and K was 2.6×10^{-5} M. The relation of $\vec{k}$ to [PP] fitted eq. 7 and the value of K_b was led to 4.8×10^{-4} M and that of k_2 to 0.15 sec.$^{-1}$. This value of k_2 is much larger than that of the same preparation which is obtainable in the presence of Ca^{++} (0.013 sec.$^{-1}$). In the presence of 10 mM of Mg^{++}, the plot of Δ/Δ_c *vs.* [PP] was, however, given by a dissociation curve of the second order, and the plot of $1/\vec{k}$ *vs.* $1/[PP]^2$ was found to be linear (see Fig. 4). This indicates that in this case the binding step is of the second order with respect to [PP].[9]

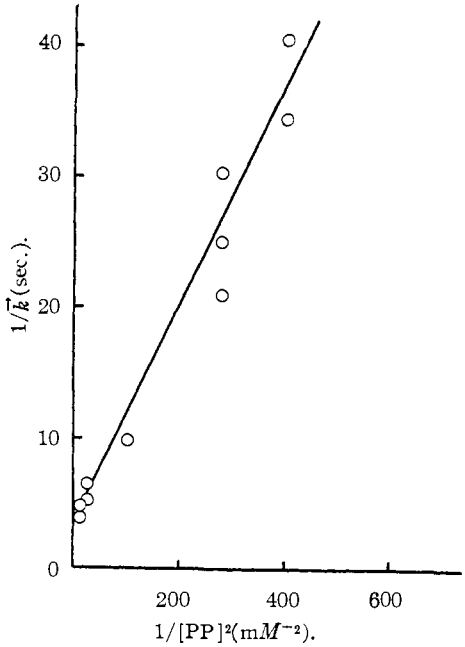

Fig. 4.—Reciprocal apparent rate constants for the decrease in light-scattering in the presence of Mg^{++} as function of $1/[PP]^2$; *p*H 7.9; 0.6 M KCl; 10 mM Mg^{++}; D, 86.30; 5°; prep. no. 7.

For prep. no. 11, the plot of Δ/Δ_c *vs.* [PP] followed eq. 5 in the presence of Mg^{++}, in the range of 0.1 to 20 mM. K decreased with increase in the concentration of Mg^{++}. It was 3.4×10^{-6} M in the presence of 10 mM of Mg^{++}. On the other hand, $\vec{k}$ was proportional to [PP] at every concentration of Mg^{++} and $\vec{k}/[PP]$ was 9.4×10^2 M^{-1} sec.$^{-1}$ at 10 mM of Mg^{++}. Thus, for this preparation, it may be concluded that $K_b \gg$ [PP].

Velocity for the Reverse Direction.—The velocity for the reverse reaction can be measured when PP is hydrolyzed so rapidly by the addition of PPase that the hydrolysis of PP is not rate-determining for the increase of light-scattering from the reduced state.

Ten μM of PP were added to 0.6 M KCl solution of prep. no. 11 in the presence of 10 mM of Mg^{++} and at *p*H 7.9 and 5°. After the reaction between PP and myosin B reached its equilibrium, PP was hydrolyzed by addition of 0.05 mg./ml. of PPase.[10] The course of time of the increase in light-scattering fitted well eq. 8 and the apparent rate constant for the reverse reaction, $\overleftarrow{k}$, was 3.2×10^{-3} sec.$^{-1}$ (Fig. 5). As described above, for prep. no. 11, $K_b \gg$ [PP] and $\vec{k}/[PP]$ was 9.4×10^2 M^{-1} sec.$^{-1}$. Therefore, the ratio of $\overleftarrow{k}$ to $\vec{k}/[PP]$ was 3.4×10^{-6} M which agreed exactly with the value of K ($= K_1K_2$), 3.4×10^{-6} M, as experimentally determined from the relation of Δ/Δ_c to [PP]. The agreement of K with $\overleftarrow{k}[PP]/\vec{k}$ was also observed in the case of prep. no. 10. The same conclusion has already been drawn for the ATP-myosin B system in the presence of EDTA.[11]

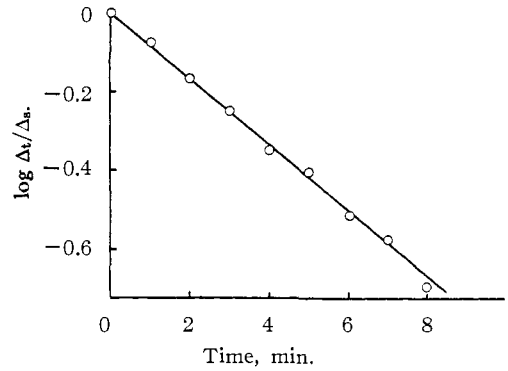

Fig. 5.—Increase in light-scattering after addition of PPase to the reduced state of scattered intensity; *p*H 7.9; 0.6 M KCl; 10 mM Mg^{++}; 5° ; prep. no. 11. 0.05 mg./ml. of PPase is added after intensity of light-scattering is reduced by addition of 10 μM of PP.

Effect of *p*H.—As described above, when the concentration of Ca^{++} was higher than 0.3 mM, both K_1 and K_2 were independent of [Ca^{++}]. Thus, in the presence of a sufficient concentration of Ca^{++} the intrinsic properties of the dissociation constants of the system saturated by Ca^{++} could be obtained.

The effects of *p*H on K_1 and K_2 were investigated over the range of *p*H from 6.0 to 9.6, because it was previously demonstrated[5] that the intensity of the light scattered at every angle was independent of *p*H over the above range. Fig. 6 shows clearly that K_2 is nearly independent of *p*H, while K_1 decreases at *p*H above 8 and increases at *p*H below 6.5. This increase of K_1 at the acidic side may be due to the reaction, $CaPP^{-2} + H^+ \rightleftarrows PP^{-3} + Ca^{+2}$.[12]

Effect of Temperature.—One example of the effect of temperature upon the dissociation and the rate constants is shown in detail in Table II. The values of enthalpy change of the steps 1 and 2 were calculated according to the Arrhenius equation and summarized in Table III. The mean values for three preparations were −9.2 and −11 kcal./mole for the first and the second steps, respectively.

(10) The activity of PPase was 3.2 mg. P/min./mg. under the present experimental condition and the velocity of the increase in light-scattering was not affected by the decrease in concentration of PPase to 0.04 mg./ml. Furthermore, the light-scattering due to PPase was found to be negligibly smaller than that due to myosin B.

(11) Y. Tonomura, H. Matsumiya and S. Kitagawa, *Biochim. Biophys. Acta*, **24**, 568 (1957).

(12) J. I. Watters and S. M. Lambert, THIS JOURNAL, **81**, 3201 (1959).

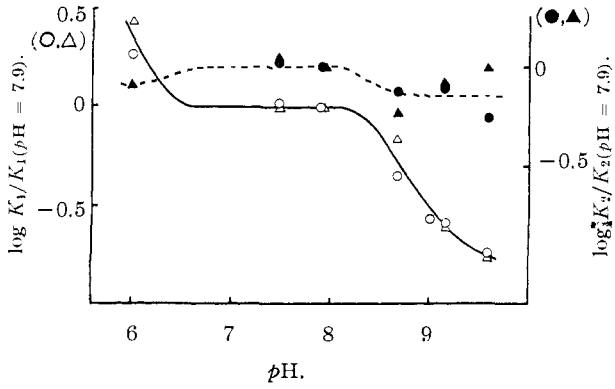

Fig. 6.—Relative values of the dissociation constants of the steps 1 and 2 as function of pH; 0.54 M KCl; 0.4 mM Ca^{++} for prep. no. 8 and 0.6 M KCl, 0.56 mM Ca^{++} for prep. no. 9; D, 82.56, 5°. ○,● represent prep. no. 9 and △,▲ prep. no. 8.

Effect of the Dielectric Constant.—The dissociation and the rate constants were markedly influenced by the dielectric constant of the medium (D). The data from one preparation are given in detail in Table IV and plotted in Fig. 7 in terms of

TABLE II

EFFECT OF TEMPERATURE UPON DISSOCIATION AND RATE CONSTANTS OF THE LIGHT-SCATTERING CHANGE OF MYOSIN B (PREP. NO. 5) CAUSED BY PP

Experiments at pH 7.9, with 0.6 M KCl, 0.5 mM Ca^{++} and 4% dioxane

Temp., °C.	K × 10^5, M	K_1 × 10^4, M	K_2 × 10^2	k_2 × 10^2, sec.$^{-1}$	k_{-2} × 10^3, sec.$^{-1}$
5	3.1	5.7	6.2	10	6.2
19	18	14	15	8	12

TABLE III

THERMODYNAMIC FUNCTIONS OF ELEMENTARY STEPS OF THE LIGHT-SCATTERING CHANGE OF MYOSIN B BY PP

Experiments at pH 7.9, with 0.6 M KCl, 0.5 mM Ca^{++} at D = 82.56 and 5°

Prep. no.	ΔF_{ele} kcal./mole	ΔF, kcal./mole	ΔH, kcal./mole	ΔS, cal./mole deg.
		Step 1		
2	12	−3.7	− 9.7	−22
5	12	−4.1	−10.0	−21
6	9	−3.8	− 8.1	−15
		Step 2		
2	−44	−1.7	−12	−37
5	−39	−1.5	−10	−31
6	−48	−1.8	−11	−33

log K *vs.* $1/D$. These results clearly show that log K_1 and log K_2 are linear against $1/D$ and that the inclination of these lines is not changed by adding either dioxane or acetone to reduce D and that K_1 and k_2 increase, while K and especially K_2 decrease with the decrease in D.

As shown in Table IV, Δ_c approached gradually to its limiting value, Δ_m, as D decreased. The values of $1/(1 + K_2)$ were in a good accordance with the values of Δ_c/Δ_m observed. This makes the assumption probable that the light-scattering due to MPP is practically identical to that due to M (see eq. 4).

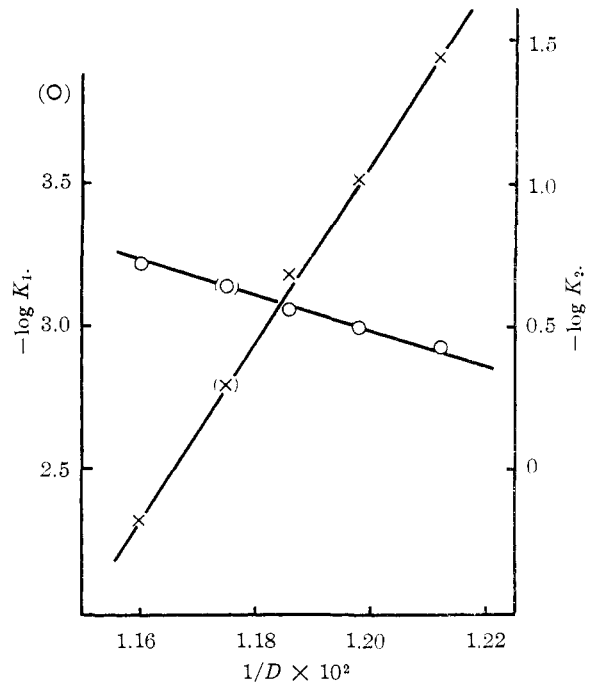

Fig. 7.—Dissociation constants of the steps 1 and 2 as function of $1/D$; pH 7.9; 0.6 M KCl; 0.5 mM Ca^{++}; 5°; prep. no. 6. ○, K_1; ×, K_2. D is decreased by addition of acetone or dioxane. The bracket represents the result obtained in acetone–water mixture.

Discussion

The mechanism of reaction proposed here corresponds with that[7] previously employed to explain the change in the light-scattering of myosin B caused by ATP. In the PP-myosin B system described in this report, all the equations derived from the mechanism accord well with the results obtained at varying pH, temperatures and the dielectric constants.

TABLE IV

EFFECT OF DIELECTRIC CONSTANT UPON DISSOCIATION AND RATE CONSTANTS OF THE LIGHT-SCATTERING CHANGE OF MYOSIN B (PREP. NO. 6) CAUSED BY PP

Experiments at pH 7.9, with 0.6 M KCl, 0.5 mM Ca^{++} and at 5°

Organic solvent added	D[a]	K × 10^5, M	K_1 × 10^4, M	K_2 × 10^2	k_2 × 10^2, sec.$^{-1}$	k_{-2} × 10^3, sec.$^{-1}$	$\Delta c/\Delta m$, %	$1/(1 + K_2)$, %
None	86.30	36	6.0	150	0.84	13	44	40
Acetone	85.20	24	7.2	51	1.9	9.7	70	66
Dioxane	84.30	15	8.9	21	2.9	6.1	86	83
Dioxane	83.50	8.7	10	9.5	5.0	4.8	96	92
Dioxane	82.56	4.0	12	3.6	9.0	3.2	100	97

[a] The values of D are determined from the data by G. Akerlöf, THIS JOURNAL, **54**, 4125 (1932), for acetone–water mixture and by G. Akerlöf and O. A. Short, *ibid.*, **58**, 1243 (1936), for dioxane–water mixture.

As clearly shown in eq. 5, the dissociation constant of the over-all reaction in the PP-myosin B system is a function of the dissociation constants of the binding step and of the step of the change in shape of the protein. Karush[13] and Lumry and Eyring[14] already pointed out that, when a protein

(13) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(14) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954).

can adapt its configuration to accommodate the bound molecule, the thermodynamic change of the binding reaction will include that associated with the change in the configuration. In the present study, the configurational change of the protein could be directly followed up by the use of the light-scattering method and the thermodynamic changes due to the configurational change could be separated from that associated with the binding step.

Several thermodynamic functions of the steps 1 and 2 are summarized in Table III. The change in electrostatic free energy, denoted by ΔF_{ele}, is calculated from

$$RT \ln K = A/D_{eff} + \Delta F_{n.ele}$$
$$\partial \ln K/\partial (1/D_{eff}) = A/RT$$

where A/D_{eff} is a term of change in electrostatic free energy, $\Delta F_{n.ele}$ is a term of change in non-electrostatic free energy and D_{eff} signifies the dielectric constant effective to the reaction. In general, the bulk dielectric constant of the medium (D) differs from D_{eff}, but as will be mentioned in the appendix, it is theoretically possible to some extent that in the present study ΔF_{ele} can be evaluated from the inclination of the plot of log K_1 or log K_2 *vs.* $1/D$.

In step 1, the change in electrostatic free energy is considerably large and positive, *viz.* 11 kcal./mole. This implies that the binding of PP is not promoted by its electrostatic attraction to myosin B, but PP combines with myosin B by a covalent linkage, breaking down intramolecular electrostatic bond of the protein. Pertinent to this point, the three following informations are already available: (i) PP combines with myosin A but not with F-actin,[15] (ii) the binding of PP to myosin A is inhibited by *p*-chloromercuribenzoate,[15] (iii) one mole of myosin A contains about 2 moles of Ca^{++} bound tightly to the protein[16,17] which are released completely following the addition of *p*-chloromercuribenzoate to myosin A.[17] Therefore, PP seems to combine by chelation with the intrinsic Ca^{++} tightly bound to the sulfhydryl group of myosin A. The marked decrease of K_1 at basic *p*H suggests that the intramolecular bond, which is broken by the binding of PP, may be a linkage in which an amino group has a share.

(15) J. Gergely, A. Martonosi and M. A. Gouvea, "Sulfur in Proteins," Academic Press, Inc., New York, N. Y., 1959, p. 297.

(16) W. Hasselbach, *Biochim. Biophys. Acta*, **25**, 562 (1957).

(17) S. Kitagawa, unpublished observations.

The shape change of the step 2 is related to the distinctly large decrease in electrostatic repulsion (−44 kcal./mole) and decrease of entropy (−33 cal./mole deg.). This may support Morales and Botts' theory[18] that entropy and electrostatic repulsion are the important factors that determine the length of myosin B. However, the electrostatic repulsion for the elongation of myosin B may not depend on the charge of PP, because Salyrgan[19] which has no net charge produces the same effect on the shape of myosin B as PP does,[5] and the amount of the bound PP necessary to the change of myosin B is only 1 mole per 5.6×10^5 g. of protein.[6] The independence of K_2 of *p*H from 6.0 to 9.6 indicates that the electrostatic repulsion of the step 2 may be dominantly attributable to that existing between the carboxyl groups of the protein.

Appendix

For explaining the present reaction the two following models may be assumed. First, it is probable that the protein is separated by an infinite plane from the medium and a charge e_1 in the medium of the neighborhood of the protein is fixed at distance a from the plane boundary. Using the method of image, Schellman[20] has calculated the work required (W) to bring another charge e_2 to distance d from the first charge. When the two charges are on a line perpendicular to the interface, W is given by

$$W = \frac{1}{D}\left\{\frac{e_1e_2}{d} + \left(\frac{D - D_p}{D + D_p}\right)\left(\frac{e_2^2}{4(a + d)} + \frac{e_1e_2}{(2a + d)}\right)\right\}$$

where D and D_p are the dielectric constants of the medium and the protein, respectively.

Second, assuming that, in the medium, two charges e_1 and e_2 are fixed at distance a from the plane boundary and the charges are separated by distance d from one another, and applying the same method as Schellman's, the free energy of the electrostatic interaction between the two charges will be evaluated by

$$W = \frac{1}{D}\left\{\frac{e_1e_2}{d} + \left(\frac{D - D_p}{D + D_p}\right)\left(\frac{e_2^2}{4a} + \frac{e_1e_2}{d^2 + 4a^2}\right)\right\}$$

The reasonable estimate for D_p is 5 and D is changed from 82.56 to 86.30 in the present experiments. Therefore, $(D - D_p)/(D + D_p)$ is nearly equal to one, and in either case, W is almost proportional to $1/D$. Thus ΔF_{ele} may be evaluated from $\partial \ln K/\partial (1/D)$, using the bulk dielectric constant of the medium.

(18) M. F. Morales and J. Botts, *Arch. Biochem. Biophys.*, **37**, 283 (1952).

(19) Salyrgan = Salicyl-(λ-hydroxymercuri-β-methoxypropyl)-amide-O-acetate.

(20) J. A. Schellman, *J. Phys. Chem.*, **57**, 472 (1953).