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ELECTRIC BIREFRINGENCE OF MYOSIN SUBFRAGMENTS

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SUMMARY

Electric birefringence measurements have been made on aqueous solutions of myosin subfragments, heavy meromyosin, subfragments 1 and 2 (S-1 and S-2). All of these showed positive electric birefringence. Heavy meromyosin and S-2 showed a large intrinsic Kerr constant. From the analysis of the build up and decay process of the birefringence, the contribution of the slow induced dipole moment was concluded in heavy meromyosin and S-2, although the existence of the permanent dipole moment was not completely excluded. The decay process of the birefringence of heavy meromyosin was found to consist of two components; the fast one of which had a relaxation time of the same order as that of S-1. This is probably due to the presence of a flexible hinge in heavy meromyosin.

INTRODUCTION

Electric properties of muscle proteins may play an important role in the contraction mechanism. Investigations along this line, however, are very few. Previously, one of the authors studied the electric birefringence of F-actin and showed the existence of a large electric dipole moment, which was cancelled on interaction with heavy meromyosin [1, 2]. Minakata investigated the dielectric dispersion of G-actin and showed that it has a permanent dipole moment [3].

This paper reports the result of an electric birefringence study on myosin fragments, heavy meromyosin and subfragments S-1 and S-2. Heavy meromyosin and S-2 show strong electric birefringence due to large dipoles. The relaxation process of birefringence gives a reasonable estimation of molecular dimensions; particularly intramolecular flexibility of heavy meromyosin.

MATERIALS AND METHODS

Myosin was prepared from rabbit skeletal muscle by the method of Szent-Györgi [4]. The preparation of heavy meromyosin was made following the method of Young et al. [5]. Gel chromatography on Sephadex G-200 and the sedimentation pattern in 0.06 M KCl gave a single peak. S-1 was prepared from myosin by digestion

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with soluble papain according to Tada et al. [6]. S-1 was stored until use in stock solution and was purified by Sephadex G-200 gel filtration. S-2 was prepared by the method of Lowey from the alcohol precipitant of the water-soluble fraction of papain-digested myosin [7].

The optical system for the electric birefringence measurement was the same as used previously [1]. although the light source was replaced by a 500 W Ushio high pressure xenon lamp. Pulse generators of two different types were used. For application to long molecules, a generator was constructed by using power vacuum tubes, Toshiba 1T-21P×2, which produces a pulse of max. 600 V×2 ms on a load of 100 Ω. The rise and decay time of 10–90 % of the pulse were about 0.2 μs and 0.4 μs, respectively. For application to small molecules, another generator was constructed by using a hot cathod hydrogen thyratron tube, Toshiba 3G-49P, which produces a pulse of max. 12.5 kV×2 μs or 4 μs on a load of 50 Ω [8]. The rise and decay time of 10–90 % of the pulse were about 20 ns and 40 ns, respectively. Kerr cells of three types were prepared; of which the gap and the length of the electrodes were 0.15 cm and 0.98 cm; 0.050 cm and 0.98 cm; 0.14 cm and 5.0 cm. The optical signal was detected by a photomultiplier, Toshiba MS-9SY, of which the output, passing through a cathode follower, was displayed on an oscilloscope, Iwasaki SS-5503. The saturation effect of the photomultiplier current was corrected based on the measurement of birefringence of urea solutions at high electric fields [9].

The specific Kerr constant is defined as

$$K/c = (\lambda_0/2\pi ncl)\,(\delta/E^2)_{E\to 0}$$

where δ is the steady state value of the optical phase retardation (in radians) in which the contribution of the solvent is subtracted, if necessary, n is the refractive index of the solution, λ_0 is the mean wave length of the incident light in vacuum (in cm) [10], which is $4.4 \cdot 10^{-5}$ cm in the present experiment, l is the length of the electrodes (in cm), c is the concentration of the solute (in g/ml) and E is the applied field strength (in cgs, esu). The intrinsic Kerr constant is defined as the extrapolated value of the specific constant to zero concentration.

The decay process of birefringence was photographed on a 35 mm film; the pattern was traced on graph paper being smoothed by hand and, if necessary, the numerical data from the curve were analysed by computer (FACOM 230-60, Nagoya University).

All measurements were made at room temperature. The sedimentation velocity study was carried out with a Spinco Model E analytical ultracentrifuge. The partial specific volume for all subfragments was taken as 0.728 cc/g [11].

RESULTS AND ANALYSES

(1) *Heavy meromyosin*

(*a*) *The Kerr constant.* A typical oscillogram of electric birefringence of a heavy meromyosin solution is shown in Fig. 1. The sign of birefringence was positive. The dependence of the steady-state value of retardation in the electric field at various protein concentrations is shown in Fig. 2. Some deviation appeared from the linear dependence on the square of the field strength. Therefore, the quadratic equation was determined by the least-squares method and the specific Kerr constant was obtained

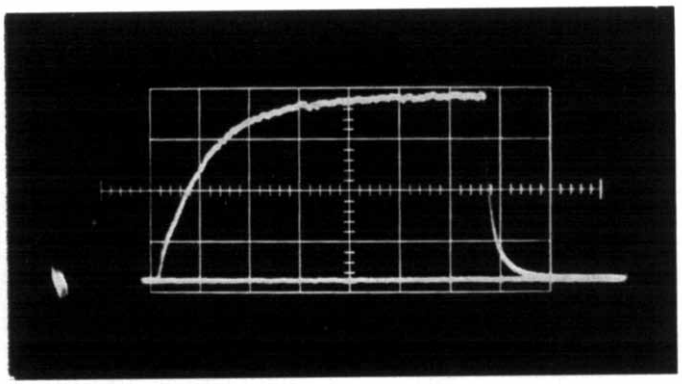
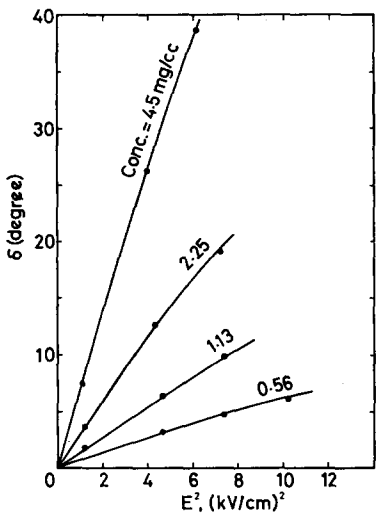


Fig. 1. Oscillogram of the electric birefringence of heavy meromyosin. Sweep velocity: 20 μs/division, protein concentration: 0.43 mg/ml, 0.2 mM Tris · HCl (pH 8) and field strength: 3.8 kV/cm.



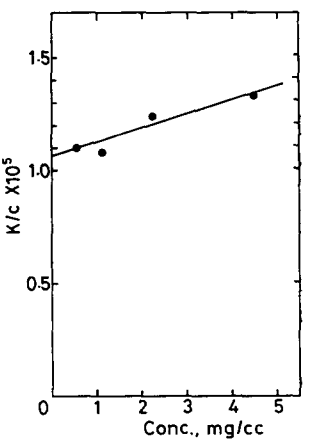


Fig. 2. Electric field dependence of the phase retardation in steady state for heavy meromyosin with various concentrations. 0.5 mM Tris · HCl (pH 7) and electrode length: 0.98 cm.

Fig. 3. Concentration dependence of the specific Kerr constant for heavy meromyosin. Experimental conditions are the same as in Fig. 2.

from the linear term of it. As shown in Fig. 3, the specific Kerr constant slightly decreases with decreasing concentration. Extrapolation gives the intrinsic Kerr constant of $(1.07\pm0.05)\cdot 10^{-5}$. This value of the Kerr constant was not stable when heavy meromyosin was stored at low salt concentrations.

(*b*) *Relaxation.* The relaxation process of birefringence after cutting off the field did not show a single exponential decay. The decay curve was analysed based on various types of relaxation time distribution. Then, the best fit was found when a sum of two exponential decays was applied; that is,

$$\delta = a_1\exp(-t/\tau_1)+a_2\exp(-t/\tau_2).$$

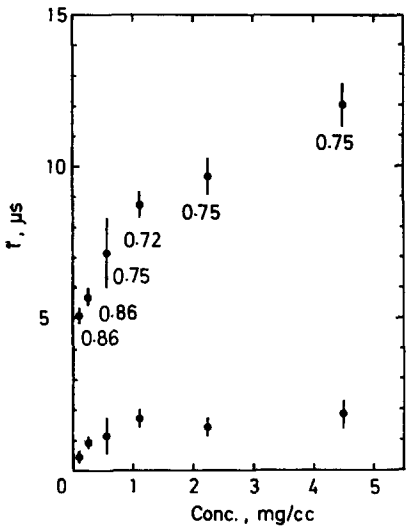


Fig. 4. Concentration dependence of the relaxation times for heavy meromyosin. Procedure for the analysis is described in the text. Bars indicate the error regions and the number in the figure indicates the fraction of the slow components. Experimental conditions are the same as in Fig. 2.

The least-squares fit for each curve of birefringence decay gave the result shown in Fig. 4. The numbers by the data points give the fractions of the slow component. The relaxation time of the slow component τ_2 decreases with decreasing concentration from about 10 μs to 5 μs. The extrapolated value 5 μs gives a molecular length of about 55 nm assuming the rod of the axial ratio of 10, which is reasonable for the dimension of heavy meromyosin molecule [7]. The relaxation time of fast component τ_1 also decreases with decreasing concentration for about 2 μs to 0.5 μs or lower.

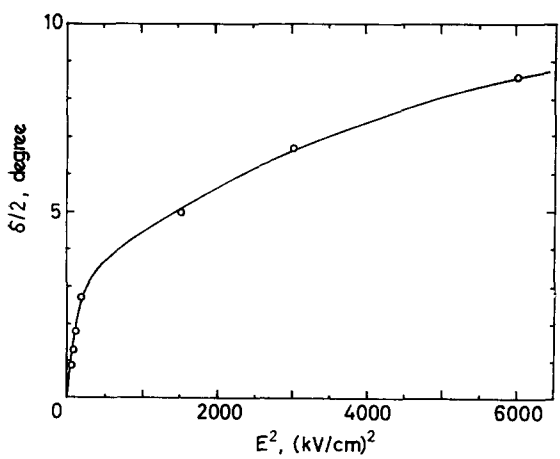


Fig. 5. Dependence of the steady state birefringence for heavy meromyosin on field strength varied up to the high field region. Protein concentration: 0.24 mg/ml, 0.5 mM Tris · HCl (pH 8) and electrode length: 0.98 cm.

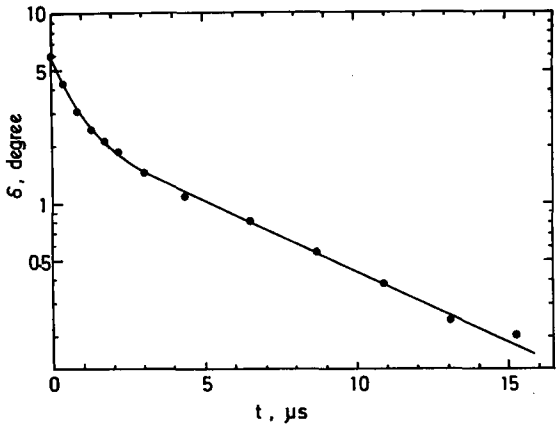


Fig. 6. Time course of the relaxation of the birefringence for heavy meromyosin oriented by an application of high field. Protein concentration: 0.82 mg/ml, 5 mM Tris · HCl (pH 8.6), field strength: 24.8 kV/cm and electrode length: 0.98 cm.

(*c*) *High field.* Fig. 5 shows the steady-state birefringence at high field strengths. The birefringence increases with the square of the field strength in two phases, suggesting that there are two components, one is easily oriented by low field, and the other is oriented by higher fields. Fig. 6 shows the decay curve at a high field. It is composed of two exponential decays given by the equation;

$$\delta = (3.7 \pm 0.1)\exp(-t/(0.70 \pm 0.03)) + (2.4 \pm 0.1)\exp(-t/(5.9 \pm 0.3))$$

where t is expressed in μs. The relaxation times of two components are of the same orders as those found at low field. The fraction of the fast component, however, is larger than that at low field. This may be due to that the orientation of the fast component was enhanced by the application of high fields.

(*d*) *The fast component.* To see if the fast component was due to a molecular species other than heavy meromyosin, the homogeneity of heavy meromyosin in the sample solution was examined by sedimentation and chromatography. As described previously, the chromatography on Sephadex G-200 gave a single peak. The sedimentation pattern in 0.06 M KCl also gave a single peak, of which the sedimentation constant (7.0 S) was very close to that reported for heavy meromyosin [7]. The sedimentation was also carried out at low salt concentrations where the electric birefringence was measured. The pattern showed a tail or a small peak (about 20 % in the total area) behind the major peak. The sedimentation constant of the minor component also increases with decreasing concentration, tending to about 5.4 S. This component is not free of contamination of heavy meromyosin, but must be some part of heavy meromyosin dissociated in the absence of salts. It might be the halfmer of heavy meromyosin. The total amount of this minor component, however, was too small to explain the fast component observed in the decay process of the electric birefringence by the application of high field considering the intrinsic Kerr constants of myosin subfragments (see Table II). Therefore, it is unlikely that it came from small molecules other than heavy meromyosin; it was probably due to intramolecular flexibility of heavy meromyosin. Actually, the relaxation time of the fast component

is of the same order as that of the subfragment S-1, as will be shown later.

(*e*) *Dipole moment.* The orientation of heavy meromyosin by the electric field is due either to a permanent dipole or to an induced dipole or both. The oscillogram shows that the building up process of birefringence is much slower than the decay process. This suggests the contribution of a permanent dipole or a slowly induced dipole. For the estimation of these dipoles, the standard procedure developed by Tinoco was applied [12]. Let the birefringence in the rising process, in the decay process and in the steady state be represented by $\delta_r(t)$, $\delta_d(t)$ and δ_s, respectively, where t is the time after application of the field or after cutting off the field. Define the quantity H as

$$H = \int_0^\infty ((\delta_s - \delta_r(t) - \delta_d(t))/\delta_s)\mathrm{d}t$$

which represents the difference between the rising and the decay process, having the dimension of time. If the orientation is totally due to an instantly induced dipole, this quantity H is equal to zero. If the orientation is caused by combination of a permanent dipole and an instantly induced dipole, the ratio of contributions of these dipoles β is given by

$$\beta = \mu^2/(\alpha_3 - \alpha_1)kT$$

where μ is the permanent dipole moment and α_3 and α_1 are induced polarizabilities along the major axis and the minor axis; $(\alpha_3 - \alpha_1)$ is the anisotropy of the polarizability. The ratio β is related to the quantity H by the equation:

$$\beta = 1/(3\langle\tau\rangle/H - 1)$$

where $\langle\tau\rangle$ is the mean relaxation time of the birefringence decay; i.e., $\langle\tau\rangle = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$. On the other hand, if the orientation is due to induced dipoles having finite relaxation (induction) times, the same quantity H becomes equal to the mean relaxation (induction) time of the dipoles; that is,

$\langle\tau^i\rangle = H$, where $\langle\tau^i\rangle = (a_1\tau_1{}^i + a_2\tau_2{}^i)/(a_1 + a_2)$

$\tau_1{}^i$ and $\tau_2{}^i$ are the relaxation times of the induced dipoles for the two components.

Table I gives the value of H obtained from the oscillogram at three different concentrations of heavy meromyosin. It is larger than the mean relaxation time of the birefringence decay; that is, the time for induction of dipoles is longer than the time for rotation of the molecule. The calculated value of the ratio $H/3\langle\tau\rangle$ is larger than unity at low concentrations of heavy meromyosin. This means that the rising process

TABLE I

CONCENTRATION DEPENDENCE OF H AND $\langle\tau\rangle$ FOR HEAVY MEROMYOSIN

Experimental conditions are the same as in Fig. 2.

Concn (mg/ml)	H (μs)	$\langle\tau\rangle$ (μs)	$H/3\langle\tau\rangle$
1.13	13.0	6.8	0.64
0.28	16.3	5.0	1.08
0.14	16.2	4.4	1.22

was slower than that expected from the orientation of a permanent dipole.

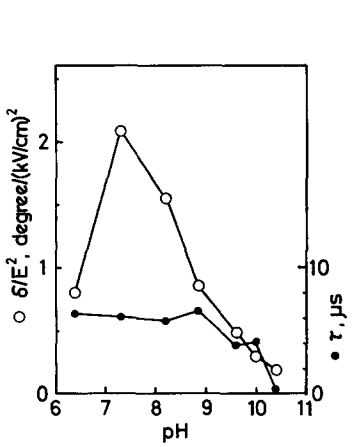
The intrinsic Kerr constant is related to the value of the apparent dipole moment by the equation [12]

$$(K/c)_{c\to 0} = (2\pi\bar{V}/15n^2)\,(g_3-g_1)\mu^2_{app}/k^2T^2$$

where g_3-g_1 is the optical anisotropy factor of the solute molecule and $\bar{V}$ is the partial specific volume of the solute. μ_{app} is the permanent dipole moment which gives the same orientation as the solute molecule. This apparent moment includes contributions of the two components. Assuming the refractive index increment $dn/dc = 0.208$ cc/g [13] and the axial ratio of 10, g_3-g_1 was estimated to be 0.0140. From the value of the intrinsic Kerr constant $1.10 \cdot 10^{-5}$, the dipole moment of heavy meromyosin was found to be about 2600 Debye units.

(*f*) *Effect of pH.* Fig. 7 shows the pH dependence of the specific Kerr constant and the relaxation time. The Kerr constant increased with increasing pH from 6 to 7.5 and decreased with pH from 7.5 to 9.0. The relaxation time did not change with pH in this range. That is, the molecular shape was maintained but the charge distribution on the molecule was changed. The ionizable histidine and amino groups may be responsive to the change of the Kerr constant with pH. The addition of simple salts, for example, KCl to 15 mM decreased the Kerr constant; but the effect was so small that the change of pH was not attributable to the change of the ion concentration in the buffer solution.

(*g*) *Effect of ATP.* When ATP was added to a solution of heavy meromyosin, the electric birefringence was remarkably reduced. The decay curve of birefringence in the presence of ATP indicated that the fast component became more predominant than in its absence. This effect of ATP remained even when ATP was split into ADP and inorganic phosphate by the ATPase activity of heavy meromyosin. The binding of ATP decreased the dipole moment and made the molecule more flexible.



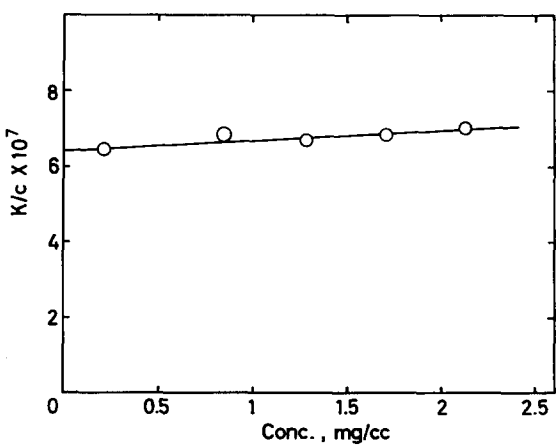


Fig. 7. pH dependence of the electric birefringence and the relaxation time of slow component for heavy meromyosin. Protein concentration: 0.214 mg/ml, field strength: 2.6–3.9 kV/cm and electrode length: 5.0 cm.

Fig. 8. Concentration dependence of the specific Kerr constant for S-1. 1 mM Tris · HCl (pH 8).

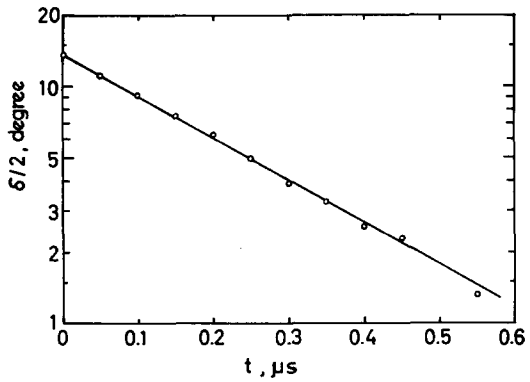


Fig. 9. Decay process of birefringence for S-1. Protein concentration: 1.07 mg/ml, 0.25 mM $CaCl_2$, 10 mM Tris · HCl (pH 8.0), field strength: 13.7 kV/cm and electrode length: 0.98 cm.

(2) *Subfragment S-1*

The specific Kerr constant of the subfragment S-1 is shown in Fig. 8 as a function of concentration. The extrapolation to zero concentration gave the intrinsic Kerr constant of $(6.5 \pm 0.3) \cdot 10^{-7}$, although the value fluctuated depending on the preparation. The decay of birefringence was well expressed by a single exponential curve, as shown in Fig. 9. The relaxation time was about 0.25 μs, which is consistent with a prolate model of the axial ratio of 7 and the length of 24 nm with the anhydrated molecule of molecular weight [7] of $1.15 \cdot 10^5$. The comparison of the rising curve and the decay curve suggested the contribution of a permanent dipole or a slowly induced dipole. The value of H was about 0.6 μs. The apparent dipole moment was estimated to be about 630 Debye units using the same optical anisotropy factor as the one for heavy meromyosin.

Fig. 10 gives the pH dependence of the Kerr constant and the relaxation time of S-1. The Kerr constant decreased with increasing pH but the relaxation time did not change. The addition of ATP to S-1 decreased the Kerr constant markedly, keeping the relaxation time constant.

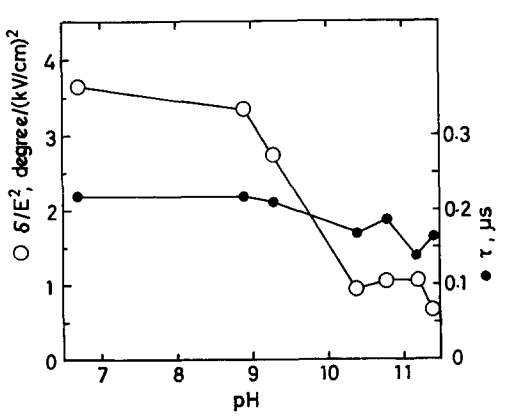
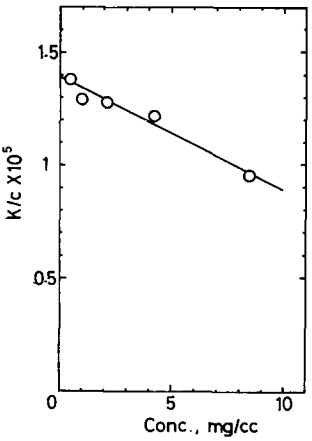


Fig. 10. pH dependence of the electric birefringence and the relaxation time for S-2. Protein concentration: 0.59 mg/ml, field strength: 10–13 kV/cm and electrode length: 0.98 cm.



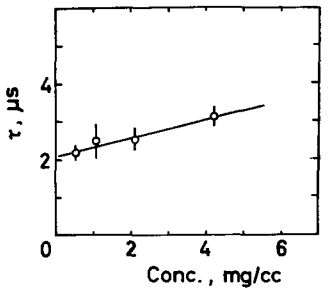


Fig. 11. Concentration dependence of the specific Kerr constant for S-2. 0.5 mM Tris · HCl (pH 8).

Fig. 12. Concentration dependence of the relaxation time of the fast component for S-2. Bar indicates the error region. 0.5 mM Tris · HCl (pH 8) and field strength: 2–3 kV/cm.

(3) *Subfragment S-2*

The specific Kerr constant of the subfragment S-2 is shown in Fig. 11 as a function of concentration; the extrapolation gave the intrinsic Kerr constant of about $1.40 \cdot 10^{-5}$. The decay curve of birefringence consisted of a major fast component and a minor slow component. The relaxation time of the fast one was about 2.0 μs, which slightly decreased with decreasing concentration as shown in Fig. 12. This value corresponds to a rod model of the axial ratio of 10 and the length of 40 nm. Subtracting the part for the minor component from the (total) intrinsic Kerr constant given in Fig. 11, the intrinsic Kerr constant of S-2 was estimated to $1.2 \cdot 10^{-5}$; and the apparent dipole moment was calculated to about 2800 Debye units using the same procedure as in S-1. The value of H was about 15 μs.

These results on heavy meromyosin, S-1 and S-2 are summarized in Table II.

TABLE II

INTRINSIC KERR CONSTANTS, RELAXATION TIMES, END-TO-END LENGTH $2a$, H AND $\langle\tau\rangle$ FOR HEAVY MEROMYOSIN, S-1 AND S-2

Protein	$10^6 \times (K/c)_{c\to 0}$	τ (μs)	$2a$ (nm)	H^e (μs)	$\langle\tau\rangle^e$ (μs)	μ_{app} (D)
Heavy meromyosin	11.0±0.5	5.0+0.7	55[b]	16.2	4.4	2600
S-1	0.5 - 2[a]	0.25	24[c]	0.63	0.26	560–1100
S-2	12.0	2.0	40[d]	9.2	2.6	2800

a, Variation depending on preparation was shown; b, calculated from the longer relaxation time assuming the rod of the axial ratio 10; c, prolate of the axial ratio 7 was assumed; d, rod of the axial ratio 10 was assumed; e, the sample concentrations for heavy meromyosin, S-1 and S-2 were 0.14, 1.07 and 0.53 mg/ml, respectively.

DISCUSSION

All myosin subfragments, especially heavy meromyosin and S-2, gave large positive electric birefringence; that is, they have large dipoles along the major axis. The S-1 molecule has a smaller intrinsic Kerr constant as compared with that of the S-2 molecule, which may be due to the electrical anisotropy of S-1 which is small. The decay curve of the birefringence of heavy meromyosin consisted of two components; the fast component is likely due to a flexible part of the heavy meromyosin molecule. The contribution of this component to birefringence increased at high fields. The relaxation time of disorientation was of the same order as or a little larger than that of the subfragment S-1. This is consistent with the model of heavy meromyosin in which the S-1 part is connected with S-2 through a perfect flexible hinge to form heavy meromyosin. It is still true, however, that the mere existence of two different relaxation times in heavy meromyosin, one of which is reasonably close to that of one of the subfragments is insufficient by itself to draw the conclusion associating the flexibility of heavy meromyosin with that of a "perfect hinge" to one of its subfragments. A theoretical foundation that describes the decay process of the orientation of the perfect hinge molecule may be required. Nevertheless, the data is quite compatible with the data of Mendelson et al. [14]. They gave the relaxation time of the same order (0.40 μs) which was attributed to the movement of the S-1 part by the fluorescence depolarization measurement. Therefore, this interpretation is quite probably correct. The Kerr constant of heavy meromyosin is expected to be the mean of the constants of S-1 and S-2. This was found experimentally to be the case.

The orientation of heavy meromyosin and subfragments was all interpreted to be due to slow induced dipoles. The mean relaxation time of induction was a little larger than the relaxation time of disorientation. The dielectric dispersion measurement by Minakata [15] also showed that heavy meromyosin has an induced dipole, of which the mean relaxation time was estimated to be 23 μs, nearly equal to that obtained here. The dispersion profile was well explained by the counter ion fluctuation theory [16], although the contribution of the permanent dipole can not be completely excluded. The observed relaxation time of induction of the dipole was, however, much larger than that expected if counter ions can move along the major axis with the diffusion constant in the free state. The movement of small ions is not very free on the heavy meromyosin molecule. This is the point to be investigated further.

Finally it must be pointed out that in the striated muscle myosin forms thick filaments, from which the heavy meromyosin part makes a cross bridge to thin filaments. Since myosin has a large net charge, a strong electric field is formed around the thick filament. If heavy meromyosin and subfragments have large (induced) dipoles, their orientation may be more stable in the direction of the field; that is, perpendicular to the thick filament. Thus, the electric property of heavy meromyosin and subfragments may be important in the construction of the muscle structure.

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REFERENCES

1 Kobayasi, S., Asai, H. and Oosawa, F. (1964) Biochim. Biophys. Acta 88, 528
2 Kobayasi, S. (1964) Biochim. Biophys. Acta 88, 541
3 Minakata, A. (1966) Biochim. Biophys. Acta 126, 570
4 Szent-Györgyi, A. G. (1953) Arch. Biochem. Biophys. 42, 305
5 Young, D. M., Himmelfarb, S. and Harrington, W. F. (1964) J. Biol. Chem. 239, 2822
6 Tada, M., Bailin, G., Bárány, K. and Bárány, M. (1969) Biochemistry 8, 4842
7 Lowey, S., Slayter, H. S., Weeds, A. G. and Baker, H. (1969) J. Mol. Biol. 42, 1
8 Krause, S. and O'Konski, C. T. (1959) J. Am. Chem. Soc. 81, 5082
9 Kobayasi, S. (1973) Seibutu-buturi 13, 1
10 O'Konski, C. T. and Haltner, A. J. (1957) J. Am. Chem. Soc. 79, 5634
11 Parrish, R. G. and Mommaerts, W. F. H. M. (1954) J. Biol. Chem. 209, 901
12 Tinoco, Jr, I. and Yamaoka, K. (1959) J. Phys. Chem. 63, 423
13 Holtzer, A. and Lowey, S. (1959) J. Am. Chem. Soc. 81, 1370
14 Mendelson, R. A., Morales, M. F. and Botts, J. (1973) Biochemistry 12, 2250
15 Minakata, A. and Kobayasi, S. (1973) Biopolymers 12, 2623
16 Oosawa, F. (1970) Biopolymers 9, 677