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Flexibility of myosin in pyrophosphate and NaCl solutions

An electric birefringence study

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Abstract. The orientational relaxation time of myosin has been reported as 38 μs when measured in pyrophosphate media at elevated pH (Hvidt et al. 1984) and as 17 μs when measured in 0.3 *M* KCl at pH 7.3 (Bernengo and Cardinaud 1982). This discrepancy, which is reexamined in the present report, suggests that in KCl solution the rod portion of the myosin molecule is bent with an average angle close to 110°, whereas in pyrophosphate at elevated pH it assumes a nearly straight and rigid conformation. Electric birefringence shows that the amount of dimeric and polymeric species in pyrophosphate media at pH's 8.0 and 8.5 is certainly greater than usually thought. In these media, relaxation times can be measured correctly at pH 9.0. A comparative analysis of the influence of protein concentration, field strength, medium composition and concentration, pH and temperature showed that a high relaxation time is associated with the presence of pyrophosphate and that the myosin tail is significantly stiffened in the presence of this anion.

Key words: Myosin – Flexibility – Electric birefringence

Introduction

In the sliding filament models of muscle contraction it is currently accepted that a hinge within the rod portion of myosin, probably close to or in the proteolytically sensitive junction between HMM and LMM, plays an important role in enabling each cross-bridge to generate the same force, regardless of filament spacing (e.g. Huxley 1969; Huxley and Simmons 1971; Harrington 1971).

A number of observations favour the existence of a hinge in the rod (Highsmith et al. 1977; Tsong et al. 1979; Swenson and Ritchie 1980; Bernengo and Cardinaud 1982; Lu and Wang 1985; Rodgers and Harrington 1987). Other results, however, fail to support this hypothesis (Harvey and Cheung 1977; Rosser et al. 1978; Hvidt et al. 1984), and the problem of a freely bending rod has not been completely resolved (Harvey and Cheung 1982). A sharp bend seen in electron micrographs (Elliot and Offer 1978; Takahashi 1978; Walker et al. 1985) is often taken as evidence that the bend is, indeed, present in solution. However, a number of molecules appear not to be bent and it is difficult to distinguish between two possibilities: *1*) the same proportion of bent and straight molecules seen in electron micrographs exists in the solution; *2*) the stress due to fixing procedures converts some of the molecules (which are otherwise all bent or all straight) into the other form.

Electric birefringence gives an averaged representation of a population of myosin molecules in solution, with the advantage that they may be expected to exhibit dynamic properties closer to those assumed in vivo. An interesting discrepancy between reported myosin orientational relaxation times is now being reexamined. A relaxation time of 38 μs was observed in pyrophosphate (PPi) media at elevated pH (Hvidt et al. 1984) whereas an earlier value of 17 μs was measured in 0.3 *M* KCl, pH 7.3 (Bernengo and Cardinaud 1982). Interpreted with available theoretical models (Garcia de la Torre and Bloomfield 1981), these experimental relaxation times lead to the conclusion that in KCl solution the rod portion of myosin is bent with an average angle close to 110°, whereas myosin in PPi at elevated pH assumes a nearly straight and rigid conformation. In contrast, available results on the rod conformation under the present sets of conditions show that very close values are obtained (28 μs, Hvidt et al. 1984; 24 μs, Cardinaud and Bernengo 1985).

The present report is a comparative analysis of the influence of such parameters as protein concentration, field strength, medium composition and concentration, pH and temperature in these two kinds of media and shows that a high $\tau_{20,w}$ value is associated with the presence of PPi.

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Experimental

Proteins

Myosin was extracted from the back muscle of rabbit as described earlier (Cardinaud 1979), with three precipitation cycles at low ionic strength, and was further purified as described in our previous electric birefringence studies (Bernengo and Cardinaud 1982). The fraction precipitated between 40 and 45% ammonium sulfate saturation (adjusted to pH 6.75) was dissolved in 0.177 (v/w) 3 *M* KCL and diluted to the stock solution concentration (usually 10–12 mg/ml) with the appropriate filtered solvent. The solution was dialyzed overnight against the same solvent and then clarified by centrifugation (100 000 *g* for 30 min). All solutions were prepared with deionized and glass-distilled water with reagents of the best grade available. All operations were carried out at 2–4 °C.

Sample preparations. To obtain preparations of the best optical quality, the samples were dissolved and subsequently diluted with filtered (Millipore filters type VM 50 nm) buffers. All samples were brought to the desired concentration and were centrifuged at 100 000 *g* for 2 h just before measurement, then transferred directly from the centrifuge tube to the Kerr cell. All measurements were made within 4 days of myosin preparation.

Protein analyses

SDS PAGE and Urea-PAGE were used to check the purity and characteristics of all samples under conditions described previously. The samples were shown to be less than 4% phosphorylated by Urea-PAGE (Cardinaud 1986). Myosin concentration was determined before and after measurements either by the Folin-Lowry technique or by absorbance at 280/330 nm using $E_{(1\%/280)}=5.55$ (Godfrey and Harrington 1970).

Electric birefringence measurements

The apparatus used for these experiments has already been described (Bernengo et al. 1973) and the simplified limiting low field theory can be found in textbooks (Fredericq and Houssier 1973).

The specific Kerr constant is defined as:

$$K_{sp}=\frac{\Delta n_0}{n c \bar{v} E} \tag{1}$$

where Δn_0 is the steady state birefringence, c is the specific concentration, $\bar{v}$ is the partial specific volume and E is the applied electric field.

On a molecular basis, K_{sp} can be expressed in terms of optical and electrical polarizabilities: Δg and $\Delta\alpha_E$

$$K_{sp}=\frac{2\pi}{15 n^2 K T}\cdot \Delta g \cdot \Delta\alpha_E (r+1) \tag{2}$$

in which r is the ratio of permanent and fast induced moment contributions to the molecular rotation:

$$r=\frac{\mu^2}{\Delta\alpha_E K T}. \tag{3}$$

When the applied field is cut off, the decay can be represented by a sum of exponential terms of the form:

$$\Delta n(t)=\Delta n_0 \cdot \sum_i a_i \cdot e^{-t/\tau_i}. \tag{4}$$

The rise of the birefringence is a more complicated function. We give here the equation for the simple case of a single relaxation time,

$$\Delta n_r(t)=1-\frac{3r}{2(r+1)}\cdot e^{-t/3\tau}+\frac{r-2}{2(r+1)}\cdot e^{-t/\tau}. \tag{5}$$

Results

Sample characteristics. Myosin, prepared as described, had the same electrophoretic charateristics, i.e. a slight C-protein contamination (less than 2%) with standard light chain ratio: LC1:LC2:LC3 = 1.35:2.00:0.65 (±0.03).

Electric birefringence measurements. To establish a reference set of conditions, myosin was studied first in 0.3 *M* KCl, 0.02 *M* PO_4, pH 7.3 (solvent R). Owing to the high conductivity of this solution the experiments were carried out at 11 °C. This low temperature condition is more suitable for the study of long relaxation processes since the conductivity of the solution is lower at this temperature than at higher temperature and longer pulses can be used for the same heat production inside the cell.

A typical signal (rise and decay) is shown in Fig. 1 A for myosin in solvent R at a concentration of 2 mg/ml with an applied field of 600 V/cm. Its characteristics are identical to those previously recorded under very similar conditions (Bernengo and Cardinaud 1982). The steady state birefringence in this solvent is plotted in Fig. 2 versus concentration at different applied fields and again previous results were reproduced here. Linear variations are observed up to a myosin concentration of 4 mg/ml showing that no molecular interaction leading to saturation or a decrease in polarizability can be detected in the concentration range studied. Specific Kerr constant values (Table 1), calculated according to (1), show that the Kerr

Table 1. Specific Kerr constant (K_{sp} in $cm^2 \cdot V^{-2} \cdot 10^{12}$) at different applied fields. Myosin concentration range 0.5 to 6 mg/ml in solvent *R* and 0.5 to 1 mg/ml in PPi. These values are the average of K_{sp} values for the selected myosin concentrations (see text for concentration effects)

Solv. \ *E*(V/cm)	300	400	480	580
solv. *R* pH 7.3, 11 °C	23.8±1.6	23.3±2.0	25.0±1.5	
PPi 10 m*M* pH 9.0, 24.4 °C	73.0±6		73.0±7	70.0±6

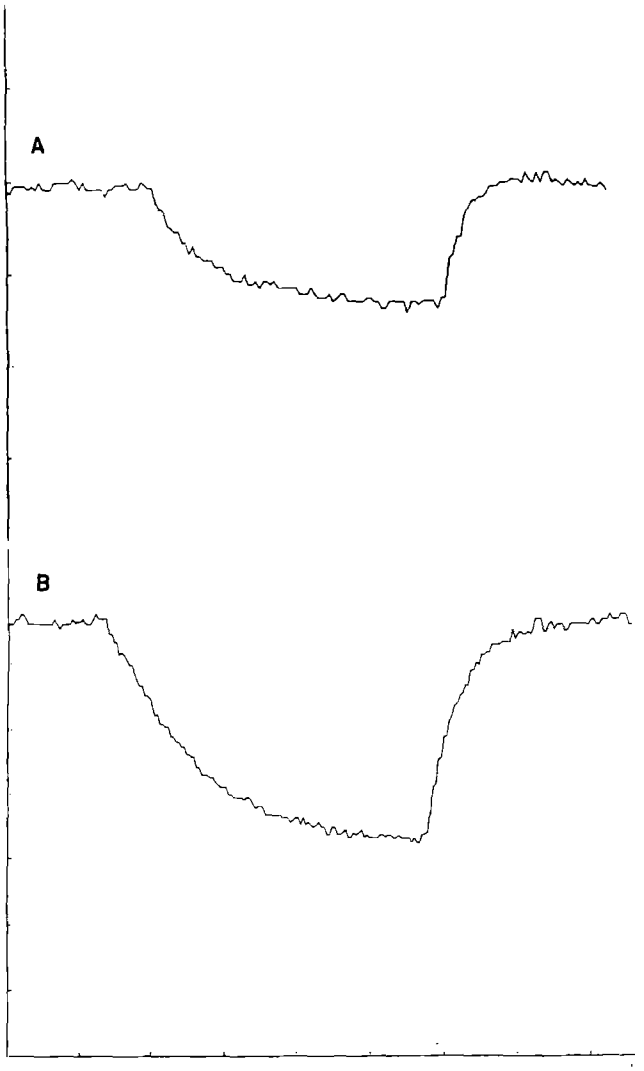

Fig. 1. Typical birefringence signals. *A*: in solvent *R*; temperature 11 °C. Myosin concentration 2 mg/ml; applied electric field 600 V/cm; steady state birefringence 2×10^{-8}. *B*: in PPi 10 m*M*, pH 8.5; temperature 11 °C. Myosin concentration 0.5 mg/ml; applied electric field 500 V/cm; steady state birefringence: 3×10^{-8}

law is followed over the entire applied field range, except for a slight deviation at 680 V/cm for concentrations above 3 mg/ml.

The same study was made with myosin in various PPi solutions: 10 m*M* PPi, pH 8.0; 5 m*M* PPi, pH 8.5; 10 m*M* PPi, pH 8.5; 10 m*M* PPi, pH 9.0 at the same temperature, 11 °C. Owing to the lower conductivity of the solution at this temperature, good signals were much more easily obtained (a typical signal is reproduced in Fig. 1 B), but saturation effects appeared with high concentrations and high field and limited the condition-overlap zone between solvent R and PPi solvents. Plots of Δn_0 vs myosin concentration (in 5 m*M* PPi, pH 8.5, recorded at 11 °C) for different field strengths (Fig. 3) show that values are consistent with the Kerr law only within a limited cncentration range. K_{sp} values were calculated from Δn_0 obtained by averaging the results of four to six independent pulses, the solution being changed after three pulses. In the media used (10 m*M* PPi, pH 8.0; 5 m*M* PPi, pH 8.5; 10 m*M* PPi, pH 8.5 and 10 m*M* PPi, pH 9.0 at 11°) these K_{sp} values were found to decrease with field strength even in the concentration range (below 0.75 mg/ml) where Δn_0 was proportional to concentration (not shown). However the Kerr law was followed within a reasonable concentration range in 10 m*M* PPi, pH 9.0 at 24.4° as seen in Table 1.

Relaxation times

The decay time in PPi solvents were studied in the same concentration and field strength ranges as in solvent R. In all our results the decays could be fitted with a monoexponential function at least as accurately as with a bi-exponential function. The quality of this fit was evaluated as the standard relative deviation (SRD) between calculated and experimental data points. In our case SRD is:

$$\sigma_r = \frac{1}{N-1} \cdot \sqrt{\sum_{i=1}^{N} \left(\frac{(\Delta n_{exp})_i - (\Delta n_{calc})_i}{(\Delta n_{exp})_i} \right)^2}$$

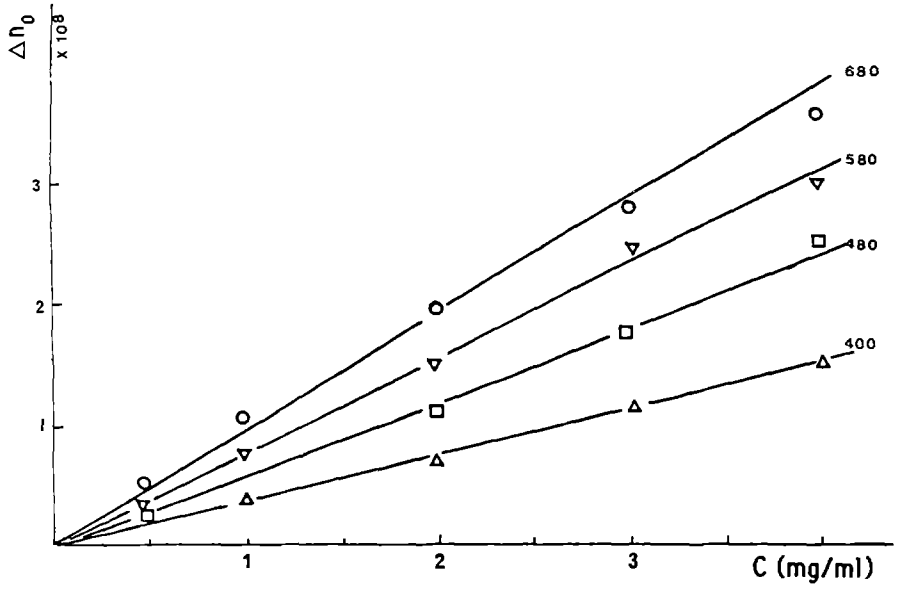

Fig. 2. Steady state electric birefringence of myosin in solvent *R* at several applied fields (V/cm). Temperature 11 °C

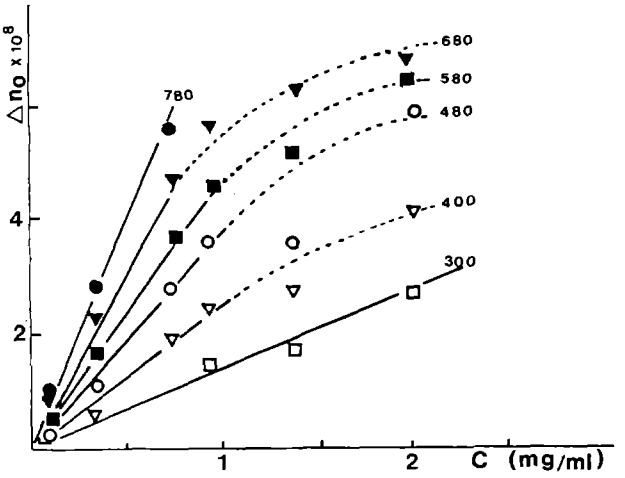

Fig. 3. Steady state electric birefringence of myosin in 5 m*M* PPi, pH 8.5 at several applied fields (V/cm). Temperature 11 °C

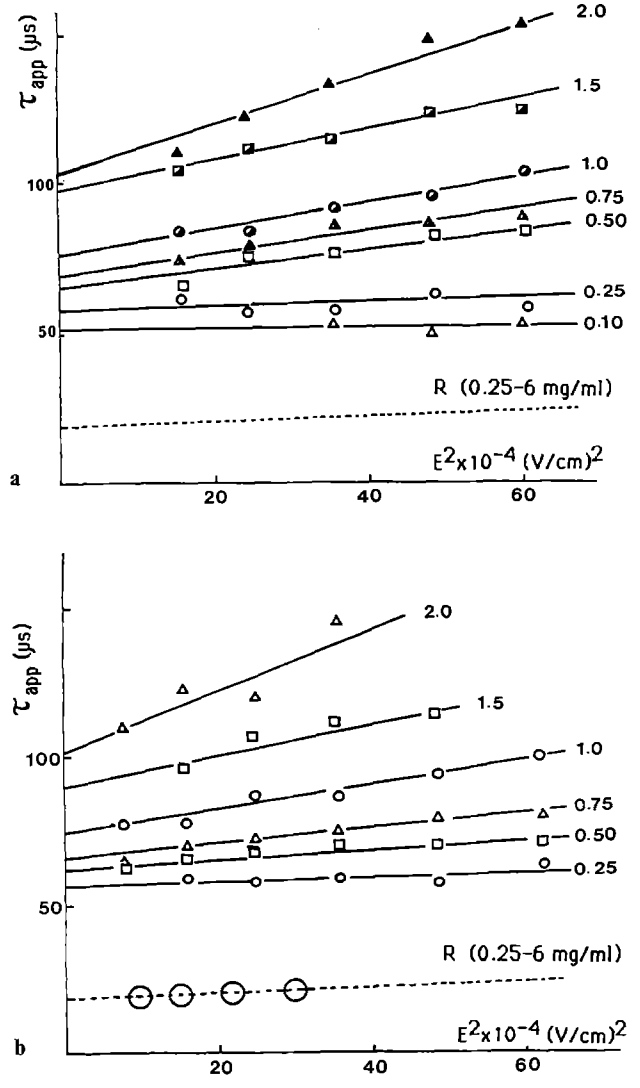

Fig. 4a, b. Apparent relaxation time dependence with applied field for selected myosin concentrations (0.1 to 2.0 mg/ml). **a** in 10 m*M* PPi, pH 8.5; **b** in 10 m*M* PPi, pH 9.0. Temperature 11 °C, myosin concentration in mg/ml. The broken line is for myosin in solvent *R*; changing concentration in the range 0.25–6 mg/ml had a negligible effect

Typical examples of this relaxation time dependence with applied field in 10 m*M* PPi, pH 8.5 and in 10 m*M* PPi, pH 9.0 are presented in Fig. 4 a and b. The apparent linear dependence slope, as well as the intercept at zero field strength, varied significantly with myosin concentration as seen in Table 2 for a number of experiments. At 24.4 °C, 10 m*M* PPi, pH 9.0 (not shown) the slope became practically independent of concentration up to 1 mg/ml. A comparison of results obtained under these various conditions indicates that the conformation of the relaxing species was dependent on pH, temperature and the concentration of PPi and myosin. When $\tau_{E=0}$ values (intercept at zero field strength) were plotted against concentration, it became apparent (Fig. 5) that all the curves extrapolated to the same relaxation time for $C=0$ ($\tau_{11°C}=46$ (± 2) μs; $\tau_{24.4°C}=34$ (± 2) μs) in all PPi media tested. Furthermore, no intrinsic temperature effect could be detected since standard rotational relaxation times ($\tau_{20°,w}$) were 37.0 (± 2) μs and 37.5 (± 2) μs, respectively. These values differ markedly from the value of 17 (± 2) μs obtained in 0.3 *M* NaCl, pH 7.3 as reported here and in previous publications.

Polarization mechanism

In buffer R, myosin molecules were essentially polarized through a permanent dipole moment, confirming our previous results. Birefringence signals, such as those shown in Fig. 1 a, have a rise which can be fitted, according to (5), by a single rotational relaxation time combined with a ratio r greater than 10 in all cases.

PPi solutions exhibited an induced moment polarization which increased strongly with myosin concentration. The concentration dependence of K_{sp} is a clear indication that in PPi media (at least at low temperature) more than one orientation mechanism contributes to the birefringence rise. This excludes a quantitative treatment by use of (5). Only at low myosin concentrations, where a single monomeric species becomes predominant, is $r_{app}>5$ and the $\Delta n_T/\Delta n_P$ ratio tends to plateau at a value close to 1. This suggests that in the myosin monomer in pyrophosphate a permanent dipole moment makes a major contribution to the orientation, as also proposed by Hvidt et al. (1984).

Discussion

Orientational relaxation times for myosin in PPi media were found to be dependent on PPi concentration and on pH, indicating that in these media myosin has a strong tendency to form aggregates. Myosin was shown to form minifilaments at pH 7.0 in 5 m*M* sodium pyrophosphate. These high order structure have been reported to dissociate into dimers (pH 8.0, 5 m*M* PPi) and then into monomers when pH and PPi concentration are increased (Reisler et al. 1986). This accounts for the observed variations of apparent K_{sp} in PPi media.

Table 2. Apparent $\tau_{E=0}$ values (in μs) as a function of concentration in various media. Myosin concentration: C in mg/ml. *R*: reference medium: 0.3 *M* NaCl, 0.02 *M* PO4, pH 7.3. PPi: pyrophosphate at indicated concentrations and pH's

Conc. \ Solvent:		*R*	PPi (11 °C)				(24.4 °C)
	pH	7.3	10 m*M* 8.0	5 m*M* 8.5	10 m*M* 8.5	10 m*M* 9.0	10 m*M* 9.0
0.10	$\tau_{E=0}$		53±5	54±3	52±4		30±5
0.25	$\tau_{E=0}$		65±5	70±5	58±4	57±4	34±5
0.50	$\tau_{E=0}$	22±2	98±2	78±8	65±5	63±4	35±5
0.75	$\tau_{E=0}$	22±2			70±5	67±3	
1.00	$\tau_{E=0}$	22±2		102±10	76±5	75±5	39±5
1.50	$\tau_{E=0}$	22±2		117±10	96±8	90±8	41±5
2.00	$\tau_{E=0}$	22±2		159±5	103±10	103±10	46+5
3.00	$\tau_{E=0}$	22±2					48±5
4.00	$\tau_{E=0}$	22±2					
6.00	$\tau_{E=0}$	22±2					

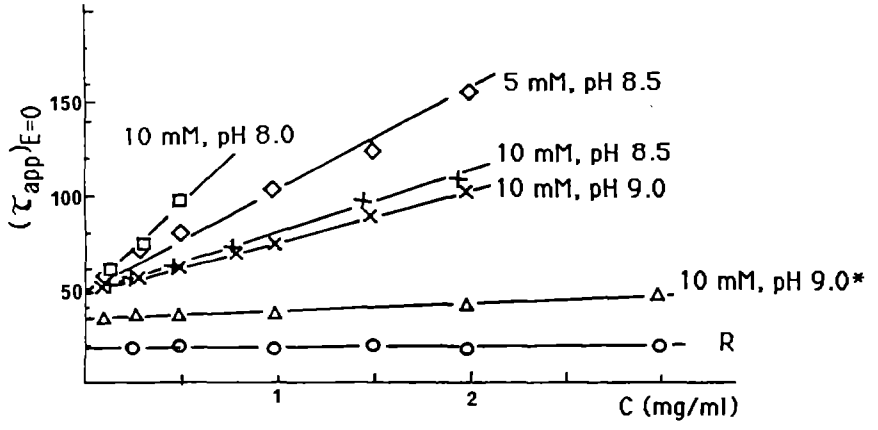

Fig. 5. Dependence of extrapolated relaxation times $(\tau_{app})_{E=0}$ on myosin concentration in different PPi media and in solvent *R*. Temperature 11 °C except condition *:24.4 °C

Relaxation time values and monomer-dimer equilibrium

Assuming an equilibrium between monomers and parallel dimers with a 43 nm stagger (Burke and Harrington 1972; Herbert and Carlson 1971; Davis et al. 1982) (with a negligible amount of higher n-mers), an apparent fit by a pseudo-monoexponential function was expected to yield values between 37 μs and 120 μs (at 11 °). Intermediate values reported in Table 2 indicate that significant amounts of dimeric material are present at pH and PPi concentrations used here and that these amounts may be higher than generally thought.

In summary, apparent K_{sp} and $\tau_{E=0}$ values as recorded here are strongly dependent on pH, PPi and myosin concentration; a result in full agreement with that of Hvidt et al. (1984). Moreover, we found the standard relaxation time of myosin to be independent of temperature (in the 11–24° range), as was the relaxation time of the rod itself (Hvidt et al. 1984).

These results contrast with the observation that in 0.3 *M* NaCl, 0.02 *M* PO_4 at pH 7.3, K_{sp} and τ values are independent both of field strength and of myosin concentration and $\tau_{20,w}$ is also independent of temperature. A monoexponential function fits the decay curve and yields a standardized relaxation time of 17 μs. Thus, under these conditions, monomeric myosin is the only species apparently present up to protein concentrations where intermolecular interactions, rather than polymer function, can account for the observed deviation from the Kerr law.

Standardized rotational relaxation times

If we suppose that monomeric myosin in solution assumes the same shape in the absence of field regardless of the composition of the medium (*R* or PPi) and that this shape is a quasi-linear arrangement, as derived from a τ value of 38 μs, then the low value recorded in buffer *R* could be explained by a transient bending of the molecules induced by the applied field. However, the fact that such transient bending is not observed in PPi in itself constitutes evidence that the media *R* and PPi have different effects on the myosin molecule. The hypothesis that in 0.3 *M* KCl, a conductive medium, higher currents which result in local heating could induce a spontaneous or a field induced deformation of myosin finds no basis either in current interpretation of the electric birefringence or in current observations. The only effect that may be present could be related to an ionic screen effect occurring at high ionic strength, the consequence of which should be observed mainly in an induced ionic polarization. In this case, because the molecule is more flexible, relaxation times would be shorter owing to internal molecular mo-

tions. If the observed 17 μs value were an average of a 37 μs relaxation time associated with global motion and a much shorter relaxation time associated with flexibility, a noticeable variation would have been observed in our electric field range, contrary to our result. Lastly, at 11 °C, heat evolved in the solution by a single pulse raises the temperature in the Kerr celly by less than 0.2 °C (as verified experimentally) and the time between pulses is such that the temperature returns to the set value before the next pulse is applied.

With regard to whether the difference observed in the two kinds of solvents signifies a change in myosin conformation, the conformation of smooth muscle myosin was shown to vary with ionic strength, changing from a nearly straight conformation in 0.6 *M* KCl to a bent form (in which the LMM region of the rod was bent back on the S2) in 0.15 *M* KCl, with a corresponding change in intrinsic viscosity. (Trybus et al. 1982). It was also observed that while increasing salt concentration stabilizes the major portion of the coiled coil rod in skeletal myosin, chloride ion specifically destabilizes a domain near the S2/LMM junction (Stafford 1985) as observed by a proteolytic susceptibility study in the concentration range of 0.6 to 2 *M* NaCl. This suggests that chloride imparts some flexibility to the myosin rod. Thus chloride exerts opposite effects in these two different muscles although it must be remarked that these experiments were performed in very different concentration ranges. However, our salt concentration was well below those of this proteolytic susceptibility study of rod and we observed that while the rod assumes the same straight configuration in both 0.3 *M* KCl and 10 m*M* PPi, the behaviour of whole myosin is different in these two media.

NaCl and KCl solutions in the 0.5–0.6 *M* concentration range have been used in most studies of the hinge in rod or myosin. Our electric birefringence study in the same type of solvent at high ionic strength permits a direct comparison of results obtained by different approaches under similar conditions. However, as mentioned above, chloride ion has been shown to influence the conformation of a limited zone at the S2/LMM junction. This effect could be suspected of inducing a flexibility not present in other media closer to physiological conditions.

Cleavage of Acanthamoeba myosin II by chymotrypsin removes a COOH-terminal segment of 66 amino acids. The new species can no longer form filaments but can still form parallel dimers (Kuznicki et al. 1985). The observed relaxation time, 8.2 μs (Wijmenga et al. 1987), of this new species in 10 m*M* imidazole-HCl, pH 7.0, as well as in 0.1 *M* KCl, was found to be compatible with a myosin molecule bent at 36 nm from the carboxyl terminus in the native molecule with an average bend angle of 110–120°, assuming the heads are oriented at 180° (Garcia de la Torre and Bloomfield 1981; Wijmenga et al. 1987). Thus, under their conditions of low salt other than PPi and KCl, electric birefringence indicated that a hinge is present in the myosin tail, a result in agreement with what we have found using 0.3 *M* KCl.

Rodgers and Harrington (1987) observed a marked decrease of the radius of gyration of skeletal myosin rod with temperature centered at 40 °C. Since the fraction helix curve was shifted by about 5 °C with respect to the radius of gyration vs. temperature profile, the change in radius of gyration was taken to indicate the presence of a bend in the rod appearing at higher temperature. This agrees well with the observations that, at low temperature, bending is independent of temperature and, in particular, the rod assumes a nearly straight structure in KCl (Cardinaud and Bernengo 1984) as well as in PPi (Hvidt et al. 1984). This again draws attention to the fact that the heads in the myosin molecule impart some flexibility to the tail, which is not observed on isolated rods in the low temperature range.

Thus, the considerable difference in electric birefringence behaviour of myosin solutions in PPi and solvent *R* strongly suggests that PPi has a direct effect on myosin rod conformation and stiffens the tail. Our attempt to determine K_{sp} values showed that myosin solutions in pyrophosphate buffers are monodisperse only at high pH and within a limited myosin concentration range, a characteristic which restricts the usefulness of pyrophosphate containing media for electric birefringence studies. That PPi binding induces a particular conformation in myosin may be related to its role in the formation of minifilaments. Assuming that myosin molecules must possess a certain degree of flexibility and pliancy to be stably inserted in a normal length filament, one could speculate that PPi binding to specific sites stiffens the myosin tail and the resulting strain is such that the packing of the molecule is limited.

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