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Segmental Flexibility of the S-1 Moiety of Myosin†

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ABSTRACT: The flexibilities of rabbit myosin and heavy meromyosin—labeled on the S-1 moiety with the fluorophore *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)ethylenediamine—have been examined by the nanosecond fluorescence depolarization technique. It was found that there is considerable flexibility within heavy meromyosin which is localized near the (S-1)–(S-2) connecting joint. The S-1 moiety of myosin depolarized more slowly than free S-1 but with a relaxation time in agreement with a model in which S-1 is free to pivot at the (S-1)–(S-2) connection. S-1 was found to be highly elongate with an axial ratio of greater than 3.5 if considered a hydrated prolate ellipsoid. These results are shown to harmonize with recent suggestions that S-1 rolls on actin to produce muscle contraction.

Current speculations concerning the manner in which a myosin "cross bridge" attaches to the thin filament and imparts a mechanical thrust invariably assume that in the myosin molecule there is at least one point of flexibility between two segments. The existence of such a point has been inferred from enzyme susceptibilities and from morphological features of the filament assemblies. It occurred to us that the more direct approach which Stryer (1968), Tao (1969), and Yguerabide *et al.* (1970) used to examine for "segmental flexibility" in immunoglobulin might also be applicable to myosin.[1] Myosin too is Y-shaped, but its "stem" (light meromyosin (LMM) and S-2 portions) is very long. Here we have used the technique of fluorescence polarization decay to characterize the motion of the two "arms" (S-1 moieties), both when they are integral parts of the Y (as in myosin or heavy meromyosin (HMM)) and when they have been cut free of the stem (as in S-1), and then have argued that the observed relationship between these motions could only be obtained if the arms are *flexibly* attached to the stem. The fluorescence polarization decay technique consists of attaching a fluorophore to the single special (fast-reacting) SH group on each S-1 moiety; then we excite these fluorophores by a brief flash of plane-polarized (‖) light and measure the intensity of the two components ($I_{\parallel}$ and $I_{\perp}$) of the ensuing fluorescence as a function of time. From these intensities is constructed the "polarization anisotropy," $r(t) \equiv (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$; this function decays with time because the directional preference imposed by the polarized flash is progressively randomized by the rotational Brownian movement of the molecule bearing

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[1] In a pioneering investigation using steady-state fluorescence, Young *et al.* (1965) concluded what we shall conclude here, *viz.*, that there is segmental flexibility in myosin; however, the agreement between studies is only apparent. We find the rotational correlation time of the moving segments to be much greater than do they. Also, we find that the moving segments move more slowly when incorporated into the molecule than when free, but that the difference is just what mechanics predicts for attachment by a "swivel joint"; Young *et al.* find the motion to be the same whether the segments are incorporated or free.

the absorption and emission dipoles. This movement is characterized by rotational diffusion constants. It should be possible to express $r(t)$ in terms of diffusion constants and the orientation of the dipoles referred to the geometry of the molecule. Recently this important problem was definitively solved by Belford *et al.* (1972) for an arbitrary ellipsoid.

Experimental Procedure

Flourescence Labeling and Protein Preparation. To label myosin we employed *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)-ethylenediamine (IAEDans), developed, characterized, and donated to us by Hudson (1970). IAEDans resembles 1-anilino-8-naphthalenesulfonate in its fluorescence properties and iodoacetamide in its reactivity toward protein SH groups. As with iodoacetamide the Ca^{2+}-ATPase activity saturates at about five times control value as the degree of labeling with IAEDans is increased. There are 2 mol of dye/mol of myosin at the lowest concentration of dye which saturates the activity. This behavior has been taken to indicate that these two dye molecules have reacted with the two fast-reacting or "S_1" thiols of myosin, *i.e.*, that there is one dye molecule per S-1 moiety, equivalently situated. Actually our degree of labeling was less than saturation, as we incubated mixtures in the proportion 1.8:1 of dye and rabbit myosin (prepared according to a method developed by Botts and described in Stone (1970) or according to Tonomura *et al.* (1966)) for 24 hr at 4° with pH 7; this practice further assured specificity of labeling. Labeled myosin was purified either by DEAE-Sephadex or by Sepharose 2B chromatography for fluorescence measurements, and such myosin also became the parent material for preparation of labeled subfragments. Labeled heavy meromyosin (HMM) was prepared from labeled myosin according to Lowey *et al.* (1969), except that the latter was exposed to trypsin at pH 6.2 for only 30 sec at 25°. Under these circumstances the yield of HMM is very poor; on the other hand, after chromatography on Sepharose 4B such HMM was practically homogeneous on sodium dodecyl sulfate gel electrophoresis. Labeled S-1 was prepared from labeled myosin by the method of Lowey *et al.* (1969) and by Stone and Perry's modification (lower papain concentration) thereof (1972), and it was purified by chromatography on Sephadex G-200. Stone and Perry's method reduced the heterogeneity on sodium dodecyl sulfate electrophoresis to two (heavy chain) bands of roughly equal density. The time constant for $r(t)$ decay did not change, however, indicating that the heterogeneity removed probably arose from breaks in the polypeptide backbone that did not appreciably alter the tertiary structure of S-1. A few experiments were made with S-1 prepared directly from *psoas* fibers (Cooke, 1972), then labeled, and chromatographed. Such material gave 20% longer time constants, but these are not reported here because we wished to compare myosin, HMM, and S-1 labeled in precisely the same way. F-actin and G-actin were prepared from acetone-dried crude actin powder, according to Stone *et al.* (1970).

Apparatus for Measuring Flourescence Polarization Decay. A block diagram of our apparatus is Figure 1. An RC charging circuit employing a 4.7 megohm resistor and the capacitance of 3 cm of (General Radio 874L) air dielectric coaxial cable steadily generated light flashes at 30–125 kHz. Air flow across the gap cooled the electrodes. These flashes were plane polarized by a 1 cm × 1 cm Glan-Thompson prism, passed through a Corning 7-60 input filter transmitting between 330 and 380 nm (half-maximum points), and entered a thermojacketed and defogged sample cell held at 4°. The fluorescence at 90°

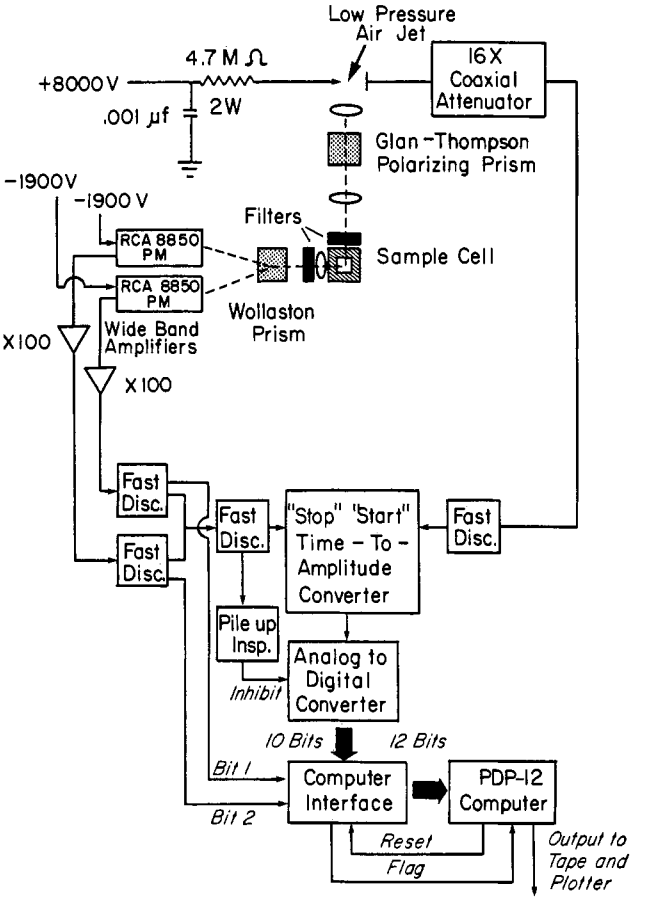

FIGURE 1: Block diagram of apparatus for measuring fluorescence anisotropy decay in the nanosecond time range.

from incidence passed through two output filters (Corning 0-51 and 3-72) transmitting above 420 nm. A Wollaston prism split this radiation into two orthogonally polarized beams diverging by 20°. Each beam then struck an RCA 8850 photomultiplier tube operating at photocathode-to-ground potentials below 1900 V in order to minimize dark current noise. Wide-band amplifiers (Hewlett-Packard 462A) increased the photon pulses to above the discrimination level of the timing triggers (E. G. & G., Inc., T140/N). The "start" pulse for the TAC, the time-to-amplitude converter (Ortec 437 A), was supplied directly by the light flash signal. The "stop" pulse was obtained from either discriminator. Time difference information from the TAC was digitized (with 512 channel precision) by a Northern Scientific 622 analog-to-digital converter. This information was transmitted *via* a program-interrupting interface to a Digital Equipment Corporation PDP-12 computer. Two bits of the 12-bit computer word were set by the fast discriminators to identify the data according to originating photomultiplier. Subsequently, a Cal-Comp plotter read out the desired functions. To ensure that the probability of data collection be time independent after starting the TAC the total data rate was kept below 10% of the start rate. Additionally, a pile-up rejection unit (Ortec 404A) excluded data from photons arriving within 10^{-5} sec of one another. Typical flash and data acquisition rates were 75 and 6 kHz, respectively.

The time axes were calibrated by using measured lengths of RG 213/U coaxial cable. The time delay introduced in these

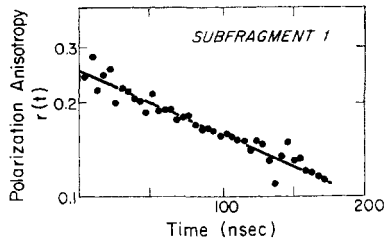

FIGURE 2: Anisotropy of fluorescence *vs.* time. Conditions: 0.05 mg/ml of subfragment-1 (Stone–Perry method), 0.15 M KCl, and 0.05 M Tris, pH 7.9. From solid line, $r(0) = 0.255$, $\Phi = 220 \pm 5$ nsec.

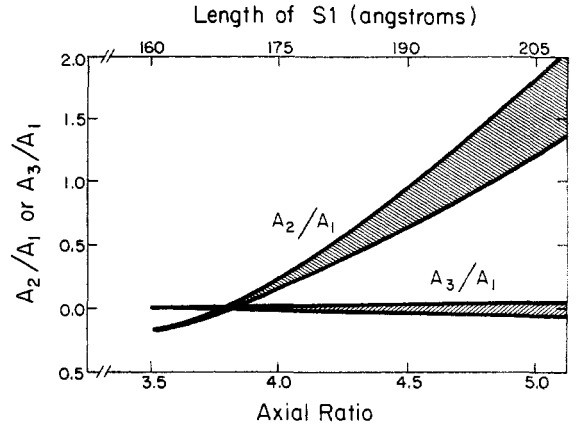

FIGURE 3: Acceptable (*i.e.*, generate adequate representations of S-1 anisotropy decay data) values of certain ratios of the three BBW coefficients as functions of compatible values of axial ratio (S-1 particle treated as a prolate ellipsoid of revolution).

cables was checked by (General Radio 874-L30) rigid air dielectric lines.

Typically, experiments lasted 8 hr during which approximately 2.0×10^8 photons were detected. No photodenaturation occurred in this time, as shown by repeating the measurements, and by monitoring the total photon detection rate during the experiments.

Analysis and Results

Application of the Belford–Belford–Weber (BBW) Equation to S-1. In the BBW treatment one envisions a rectangular system of coordinates imbedded in an arbitrary ellipsoid in such a way that the z axis (axis 3) coincides with the major axis of the ellipsoid, and that the equator of the ellipsoid lies in the normal plane containing the x axis (axis 1) and the y axis (axis 2). BBW define the transition moments of absorption ($\vec{\alpha}$) and of emission ($\vec{\epsilon}$) by direction cosines, α_1, α_2, and α_3, and ϵ_1, ϵ_2, and ϵ_3. Their "master equation" relates $r(t)$ to the sum of five exponential terms in which the coefficients are of the direction cosines and the exponentials are functions of D_1, D_2, and D_3—the rotational diffusion coefficients around axes 1, 2, and 3. It can be shown that $r(0) = (3/5)\cos^2\mu - (1/5)$, where μ is the angle between $\vec{\alpha}$ and $\vec{\epsilon}$. Recently the BBW equation has been verified in computer experiments (Harvey and Cheung, 1972). Phenomenologically, our $r(t)$ data are adequately represented by a single exponential, *i.e.*, $r(t) = r(0)\exp(-t/\phi)$ (Figure 2). Empirical constants, $r(0)$ and Φ, were chosen by applying the principle of least squares in the range 0–τ, where τ was the lesser of 175 nsec or the time at which the photon counting error produced fluctuations (assuming a Poissonian count distribution) greater than variations in $r(t)$ from other sources. Data after 175 nsec (about nine fluorescence $t_{e^{-1}}$ times of the dye) were ignored for the following reason. In simulations in which a light pulse was *scattered* from a Ludox suspension to generate $L(t)$, and $L(t)$ was convoluted with $J(t)$—the transformation which equivalent spherical molecules would produce—the convolution predicted the expected $I(t)$ (exponential decay) in the range 0–175 nsec, but predicted bizarre behavior thereafter.[2]

Since our S-1 data were describable by a single exponential we assumed for simplicity that S-1 was an ellipsoid of revolution. In this case the choice of one direction cosine is arbitrary, so we set $\alpha_2 = 0$. Also, we let $D_3 \equiv D_\parallel$ and $D_1 = D_2 \equiv D_\perp$. Then $(5/2)r(t) = A_1\exp(-6D_\perp t) + A_2\exp[-(5D_\perp + D_\parallel)t] + A_3\exp[-(2D_\perp + 4D_\parallel)t]$, where $A_1 \equiv (1/4)(3\alpha_3^2 - 1)(3\epsilon_3^2 - 1)$, $A_2 \equiv 3\alpha_3\epsilon_3\alpha_1\epsilon_1$, $A_3 \equiv (1/4)(2\alpha_1^2 - \epsilon_1^2 - \alpha_3^2\epsilon_3^2 + \alpha_3^2 + \epsilon_3^2 - 1)$, and $r(0) = (3/5(\alpha_1\epsilon_1 + \alpha_3 - \epsilon_3)^2 - (1/5)$.

It can be reasonably estimated that the molecular weight of S-1 is about 1.15×10^5, the specific volume about 0.73 ml g^{-1}, and the hydration about 0.2 ml g^{-1}; therefore, the rotational diffusion coefficient of a sphere equivalent to S-1 can be considered known. On the other hand, the axial ratio, ρ, of the particle (considered as a prolate ellipsoid of revolution) is uncertain; for example some electron micrographs (Lowey *et al.*, 1969) suggest that $\rho = 1.5$, while their viscosity data, interpreted by Simha's equation, suggest that $\rho = 5.8$. Therefore, ρ was made one of the two main parameters in our fitting procedure. For reasons evident later we also wanted the polar angles ($\cos^{-1}\alpha_3$ and $\cos^{-1}\epsilon_3$) that $\vec{\alpha}$ and $\vec{\epsilon}$ make with the imbedded axis 3. Our data were treated so as to isolate these quantities.

If we set $\delta \equiv D_\parallel - D_\perp$, then the BBW equation is $(5/2)\cdot r(t) = A_1\exp(-6D_\perp t)\{1 + (A_2/A_1)\exp(-\delta t) + (A_3/A_1)\exp(-4\delta t)\}$, so obviously if either δ is large enough or the ratios of A's are small enough, $A_1\exp(-6D_\perp t)$ will be a good approximation to $(5/2)r(t)$. Of course the larger ρ is the larger is δ, as Perrin's (1934) equations show. The behavior of the equation with varying orientations of $\vec{\alpha}$ and $\vec{\epsilon}$ is harder to express. The direction cosines are expressible in terms of the polar angles, θ_α and θ_ϵ, and one[3] azimuthal angle, ϕ_ϵ. However, $\cos\phi_\epsilon$ can itself be expressed in terms of $\kappa^2 = \cos^2\mu$ and of the polar angles, and κ^2 is obtainable from $r(0)$. Because of these relations the relevant direction cosines—therefore the A's—are functions of the polar angles.

The exploration of parameter values that fit our data was made by computer, as follows. A value of ρ was assumed; this value and information about the equivalent sphere were entered into Perrin's equations to calculate the three exponents, $6D_\perp$, $5D_\perp + D_\parallel$, and $2D_\perp + 4D_\parallel$. Also, values of θ_α and θ_ϵ were assumed; these values determined a set of A values. Entering the D and A values into the BBW equation yielded an $r(t)$, and therefore a "theoretical" $t_{e^{-1}}$—the time that it took for $r(t)$ to fall (exponentially or nearly so) from $r(0)$ to $r(0)e^{-1}$. Separately, our actual data, plotted and processed, yielded an

[2] In limiting our data to this interval we may be adopting an overly conservative view. The bizarre behavior was in fact never observed, either in $I(t)$ or $r(t)$, and perhaps was an artifact of the simulation only, as the simulation employs some optical elements not used in the measurements.

[3] Actually two are necessary, but $\phi_\alpha = 0$ in accordance with the earlier assignment, $\alpha_2 = 0$.

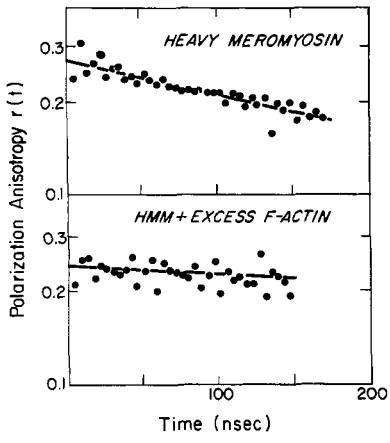

FIGURE 4: Anisotropy of fluorescence *vs.* time. Conditions for HMM: 0.05 mg/ml of HMM, 0.15 M KCl, 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, pH 7.0. Conditions for HMM + a 25 molar excess of F-actin: 0.1 mg/ml of HMM, 9.9 mg/ml of F-actin, 0.05 M KCl, and 0.05 M Tris, pH 7.9.

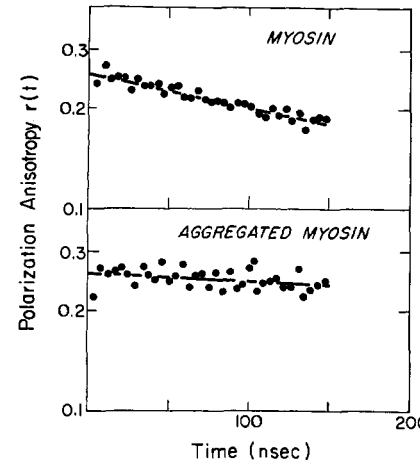

FIGURE 5: Anisotropy of fluorescence *vs.* time. Conditions for myosin: 0.04 mg/ml of myosin (filtered through Sepharose 2B), 0.1 M KCl, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.0.

"experimental" $t_{e^{-1}}$, *viz.*, Φ(S-1) (=220 nsec). If $t_{e^{-1}}$(theor) $\neq$ Φ(exptl), the triple choice of ρ, θ_α, and θ_ϵ was rejected, and we went on to test the next triple choice. By these means we isolated a region in $(\rho,\theta_\alpha,\theta_\epsilon)$-space, any point of which represented an acceptable description of our data.

A single point of this region also defines a point in $(A_2/A_1, A_3/A_1, \rho)$-space. In Figure 3 we have plotted the projections of the acceptable region on the $(A_2/A_1, \rho)$-plane and on the $(A_3/A_1, \rho)$-plane. As may be seen in this figure the minimum ρ—achievable only with the smallest allowable polar angles and corresponding to the presence of the first term and a small negative second term in the BBW ellipsoid of revolution expression—is 3.5, *i.e.*, the least asymmetric S-1 particle compatible with our measurements would have a length of 160 Å and a diameter of 45 Å. These minimal dimensions agree rather well with the most recent morphological modeling (Moore *et al.*, 1970; Miller and Tregear, 1972), so we are encouraged to think that they are "right." If so, we infer that our dye fastens to S-1 in such a way that both its $\vec{\alpha}$ and $\vec{\epsilon}$ make smallish angles with the major axis of S-1. Even if the axial ratio were as large as 5, both $\vec{\alpha}$ and $\vec{\epsilon}$ would still be less than 40°. A paraphrase is to say that because the dye attaches with its optic axes rather aligned with the major axis of S-1 the most effective depolarization motion is end-over-end tumbling, and that it is for this reason that our experiment mainly gives information about $D_\perp$.

Segmental Flexibility in HMM and Myosin. The central conclusion of this work comes from comparing Φ(HMM) (Figure 4) and Φ(myosin) (Figure 5) with Φ(S-1). The mass of HMM (3.4×10^5 daltons) and this difference alone would tend to make Φ(HMM) greater than Φ(S-1). However, this difference aside, if HMM were a perfectly rigid structure its motions would be equivalent to moving two S-1 moieties (each with solvent flow around it), and Φ(HMM) should be considerably greater than twice Φ(S-1), which is is not. Furthermore, in view of the orientations of $\vec{\alpha}$ and $\vec{\epsilon}$ in S-1, appreciable depolarization rates by a rigid HMM would be achieved by having the two S-1 moieties projecting at near 90° from the "stem," but this would give the particle an impossibly long relaxation time. Still, HMM and myosin might be quite rigid, but (in contrast to the situation in S-1) the dye might be able to move relative to the macromolecule, and thus depolarize artifactually nearly as well as labeled S-1. However, when myosin is aggregated into filaments (Table I), or when HMM combines with F-actin, Φ significantly increases, with little or no change (<2%) in fluorescence lifetime—suggesting that Φ increases because segments of the macromolecule become immobilized, not because the environment of the dye is changed.[4] We are thus left with the most likely explanation for the relatively high rate at which HMM or myosin depolarizes—in these macromolecules *there is some sort of motion of the S-1 segments relative to the rest of the structure*, *i.e.*, there is "segmental flexibility" in the sense of Yguerabide *et al.* (1970).

Whatever the motion of the S-1 segments, it is a motion that is somewhat hindered compared to the motion of free S-1, for Φ(HMM) and Φ(myosin) are certainly longer than Φ(S-1); for one thing, it is unlikely that the S-1 segments are attached to the stem by completely "soft" polypeptide chains. One of the two natural segmental motions is torsion. Torsion can be almost ruled out *a priori*, however, because of the $\vec{\alpha}$ and $\vec{\epsilon}$ orientations, and in fact this expectation has been verified by the simulated diffusion technique of Harvey and Cheung (1972). The other, and most likely, segmental motion of the S-1 moieties is rotation—not about the center of mass, as in free S-1, but about the point (junction of S-1 and S-2) at which a segment attaches to the stem. Such a model can be examined if we approximate S-1 by an equivalent rod, and if we assume that the two S-1's attach to a single "universal joint" on the stem. Together the two segments then resemble a "once-broken" rod of length $2l$ and diameter d, where l is the length of either segment. Yu and Stockmayer (1967) have calculated that the $D_\perp$ for either half of a (long) broken rod is twice the $D_\perp$ for the unbroken rod. If this relation applies to our case ($\rho \sim l/d \geq 3.5$) we expect that

$$\frac{\Phi(\text{S-1})}{\Phi(\text{myosin}} = \frac{D_\perp(\text{myosin})}{D_\perp(\text{S-1})} \cong \frac{2D_\perp(2l/d)}{D_\perp(l/d)} \cong 0.4$$

[4] That aggregation into reconstituted thick filaments immobilizes the S-1 appendages suggesting that the point of flexibility is near where S-1 joins the thick filament, *i.e.*, at the junction of S-1 and S-2; if, for example, the point of flexibility were on the equator of S-1 aggregation would be unlikely to cause immobilization. In connection with the immobilization of myosin by binding to actin it is interesting to note that the fluorescence lifetime *is* changed in an S-1–G-actin–ATP system. Thus, conformational changes that do change the dye environment are not always foreclosed.

TABLE I: Characteristics of the Decay of Fluorescence Anisotropy for Different Systems in Each of Which the S-1 Moiety Is Labeled.[a]

System	pH	[KCl] (M)	State	Φ (nsec)[a,b]	r(0)	Fluorescence Lifetime (nsec)[a,c]
S-1[d]	7.0	0.15	Solution	220 ± 5	0.255	20.9 ± 0.5
S-1[e]	6.5	0.10	Solution	235 ± 10	0.26	20.5 ± 0.5
S-1[e]	6.5	0.60	Solution	250 ± 10	0.25	20.4 ± 0.5
HMM[f]	7.0	0.15	Solution	400 ± 15	0.27	21.2 ± 0.5
Myosin[g]	7.0	0.60	Solution	450 ± 20	0.26	20.8 ± 0.5
Myosin[h]	7.0	0.60	Solution	440 ± 15	0.25	20.7 ± 0.5
Myosin[h]	7.0	0.10	Aggregate	1800 ± 400	0.26	20.6 ± 0.5
HMM + excess F-actin	7.9	0.05	Solution	1500 ± 400	0.24	21.1 ± 0.5
S-1[d,i] + excess G-actin and ATP	6.5		Solution	260 ± 10	0.24	22.9 ± 0.5

[a] Estimated by least-squares procedures and reported together with standard deviation. Results of one experiment with each system are shown, but variations from one preparation to another do not exceed 10%. All data were obtained at protein concentrations of less than 0.1 mg/ml. [b] Errors do not include error in time calibration (*ca.* ±3%). [c] Includes estimated error as a result of small electronic variations, during the several month experimental period. [d] Prepared according to Stone and Perry (1972). [e] Prepared according to Lowey *et al.* (1969). [f] 30-sec trypsin digest. [g] Chromatographed on DEAE-cellulose. [h] Filtered through Sepharose 2B. [i] Fraction of S-1 bound to G-actin was not ascertained, so cited Φ and lifetime are lower limits.

The experimentally measured ratio is (Table I) 0.5. Effectively the calculation of Yu and Stockmayer assumes that the break point on the rod is fixed in space. For myosin (stem, 1500 Å) this is undoubtedly a better approximation than for HMM (stem, 500 Å), and possibly it is this difference in the fixation of the center of rotation that accounts for the small difference between Φ(HMM) and Φ(myosin).

Discussion

In summary, the foregoing work has shown that (i) the S-1 moiety is longer than hitherto thought and (ii) the attached S-1 moieties appear to have considerable freedom of motion relative to the "stem" of the myosin molecule. These findings have two implications worth considering.

Firstly, it is easy to accommodate the pronounced asymmetry of S-1 in the thrust mechanism proposed by Huxley and Simmons (1971) (Figure 6). If chemical affinities at the contact between an S-1 and actin cause the former to "roll," thereby generating a torque, $\mathfrak{F}l_1$, around O, or a force, $\mathfrak{F}$, at P, the component of this force along S-2 generates a pull of $\mathfrak{F}\sin(\alpha + \beta)$ along S-2; if S-2 does not sustain a bending moment we need not consider any other component of $\mathfrak{F}$.

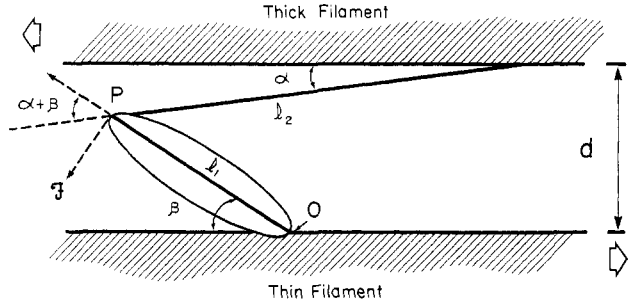

FIGURE 6: Mechanics of force generation of an ellipsoidal S-1 particle rolling on the thin filament and free to pivot at point P. For discussion see text.

Part of the pull along S-2 is used to translate point P along the fiber axis, and it is therefore "useful," while part of it just generates a "useless" compression. These two parts are in the proportion $[l_2^2 - (d - l_1 \sin\beta)^2]^{1/2}/[d - l_1 \sin\beta]$. If d, l_2, and β are thought of as constants of the filament assembly, then it is clear that the "useful"/"useless" proportion increases with l_1. Perhaps that is why Nature chose an elongate S-1.

Secondly, the finding that the S-1 moieties can move as segments independent of the stem lends plausibility to our suggestion (Tokiwa and Morales, 1971; Viniegra-Gonzalez and Morales, 1972; Botts *et al.*, 1972) that a duplex myosin molecule progresses on the thin filament by an "arm-over-arm" movement (arm = S-1 moiety). Had the experimental conclusions been otherwise our concept would have been rendered untenable.

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Binding of Succinate to Aspartate Trancarbamylase Catalytic Subunit. pH and Temperature Dependence of Nuclear Magnetic Resonance Relaxation Times†

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ABSTRACT: The interaction of the inhibitor succinate with the catalytic subunit of *Escherichia coli* aspartate transcarbamylase has been studied by a transient nuclear magnetic resonance method. In the presence of carbamyl phosphate both the succinate proton relaxation rate, $1/T_2$, and the temperature dependence of relaxation change markedly over the pH range 7–10. The data are accounted for by a mechanism featuring two protonated groups on the enzyme affecting succinate binding and titratable over the pH range studied. Four distinct enzyme species are included, one of which does not interact with succinate and does not contribute to the relaxation, two others, one singly protonated and the other doubly protonated, both of which form fast-exchange complexes with succinate, and the fourth, a conformational isomer of the doubly protonated species in which succinate is bound more tightly. The concentration of these species is pH dependent, and pK_a values of 6.9 and 8.2 are calculated for the two titratable groups at the active site. A rotational correlation time of 7×10^{-8} sec is calculated for the fast exchange enzyme–inhibitor complexes suggesting that succinate is bound through both carboxyl groups. Hence the lower pK_a group on the enzyme is probably distinct from the succinate carboxyl group binding sites, its protonation instead catalyzing the conformational change which leads to tighter binding of succinate. The equilibrium constant for the conformational change of the doubly protonated species is 8×10^{-2} with rate constants of 3.2×10^3 sec^{-1} for the forward step and 2.5×10^2 sec^{-1} for the reverse. The higher pK_a group appears to interact directly with succinate, and its deprotonation at higher pH leads to only very weak, if any, binding of the inhibitor.

Allosteric control of the reaction catalyzed by aspartate transcarbamylase from *Escherichia coli* can be fully understood only when the catalytic mechanism itself is known in detail. Toward this end Stark and coworkers have employed a variety of physical techniques in studying the isolated catalytic subunit and have proposed a mechanism for the enzyme-catalyzed condensation of L-aspartate and carbamyl phosphate to form carbamyl aspartate (Collins and Stark, 1969, 1971; Porter *et al.*, 1969; Schmidt *et al.*, 1969; Davies and Stark, 1970; Davies *et al.*, 1970). In this mechanism carbamyl phosphate binds first and a conformational change following aspartate binding serves to force the substrates together and promote the reaction (Collins and Stark, 1969).

Since the time of this proposal several studies have elaborated on aspects of the mechanism. In particular, transient nuclear magnetic resonance (nmr) (Sykes *et al.*, 1970) and temperature-jump methods (Hammes *et al.*, 1971) have been used to study the kinetics of binding of the inhibitor succinate to the catalytic subunit. At pH 7.0 in the presence of saturating carbamyl phosphate the nmr results led to the conclusion that a conformational change takes place when succinate binds and that this change is largely concerted with the binding. The temperature-jump experiments of the binding of succinate to the catalytic subunit were done at pH 7.4 and from these

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