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In Eq. 44, an editorial error was made. The correct equation is:

\[ p(0) = p(α) - 2c^2μ_0Q^2K^2 ≥ p(α) - (1/2)c^2μ_0 \]  \[ \text{[44]} \]

In Eqs. 45 and A6, printer’s errors were made. The correct equations are:

\[ μ = c^{-2}p(α) + μ_0[1 + ηF - (1/2)ν^2(1 + 2νF)] \]  \[ \text{[45]} \]

\[ F = -(3/2)c^2V \sin^2 θ(r/a^2) - \left[ j_i(βr/j_i(λ)) / (1) - (λ^2/j_i(λ)) \right], \]  \[ \text{[A6]} \]

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\[ F = -(3/2)\alpha^4V \sin^2 \theta[(r/a)^2 - [\lambda_j(\lambda)/j_j(\lambda)]^2 = [\lambda_j(\lambda)/j_j(\lambda)]. \]  \[\text{[A6]}\]

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Affinity of myosin S-1 for F-actin, measured by time-resolved fluorescence anisotropy

(Actin-myosin S-1 affinity/fluorescence depolarization)

STEFAN HIGHSMITH, ROBERT A. MENDELSON, AND MANUEL F. MORALES

Cardiovascular Research Institute, University of California, San Francisco, Calif. 94143

Contributed by Manuel F. Morales, October 28, 1975

ABSTRACT The association constant for myosin subfragment-1 (S-1) and actin was measured, using a new application of fluorescence depolarization which capitalizes on the fact that S-1 has high rotational mobility while F-actin does not. Uncoupling of the time dependences of the anisotropy decay and the association/dissociation phenomena allowed the experimentally determined anisotropy decay curve to be fitted by a sum of two terms weighted by the mole fractions of the free and bound S-1. At 4°C, ionic strength 0.16 M, and pH 7.0, the association constant K_a is (1.73 ± 0.35) x 10^10 M^-1 at infinite dilution. This makes the −ΔG° of binding of F-actin to S-1 similar to the −ΔG° of binding of ATP to S-1, and the possible physiological relevance of the similarity to muscle contraction is discussed.

Our object is to obtain the affinity constant for the simplest interaction between myosin and actin (specifically between the "S-1" moiety of myosin and F-actin), namely, the formation of the so-called "rigor complex". Present speculations (27) are that in a portion of the cycle of interactions underlying muscle contraction the system passes successively through two or more (S-1)-actin complexes, the rigor complex being the last and most stable; in such schemes the binding of an intact ATP to S-1 then dissociates the rigor complex, allowing the cycle to begin anew. It is thus of manifest importance to know such quantities as the ΔG of S-1 to actin binding, the ΔG of S-1 to ATP binding, and so on. We are making a beginning on the ΔG of S-1 to actin binding by measuring ΔG°, i.e., −RTlnK_a, where K_a is the association constant. Estimations of K_a based on light-scattering, viscosity, etc. have been made over the years, but, as the quantities measured were always high-order averages, the estimates were always questionable. Not until recently are there preliminary results with less ambiguous methods. We are aware of two such efforts besides our own, namely, those of Lowey and Margossian (1, 2) and Marston and Weber (3), both based on sedimentation techniques.

We elected to measure K_a by a versatile, but somewhat novel technique, which therefore has to be described and established in this paper. Since the molecular weight of F-actin is several million, and that of S-1 only 10^5, we based our method on the difference in rotational mobility between free and bound S-1. This mobility is measured by the time decay of r, the anisotropy of the fluorescence from a dye attached to S-1, after pulse excitation with polarized light. Since decay is very rapid, it does not "couple" with the binding transitions, and r of a multicomponent system turns out to be the number average of the rs of the individual components. This simplicity of underlying theory is not always shared with transport methods. Steady-state fluorescence polarization methods have occasionally been used to characterize equilibria among muscle proteins (4), but, besides being tedious, such methods have the disadvantageous requirement that either temperature or bulk viscosity be widely varied.

The fluorometer used in this work is substantially that previously used to study the segmental flexibility of myosin (5), and recently modified to study the species in an actin-S-1 ATPase system (6). In each experiment 2.5 x 10^7 photons were collected in 25 min.

THEORY OF THE METHOD

The natural function that expresses the directional properties of the emission from a set of n fluorophores is the anisotropy, r = (I|| - I⊥)/(I∥ + 2I⊥), where I∥ and I⊥ are the measured fluorescence emission intensities parallel and perpendicular, respectively, to the axis of polarization of the exciting light. The denominator is proportional to the total intensity, I_total. If the emissions from the fluorophores are non-interacting but heterogeneous (i.e., there are N_i of the ith kind, r is in general not separable according to i. However, as first pointed out by Wahl (7), if the total intensities are equal for all species, then r is a sum of terms, each term being a product of the mole fraction x_i and molar anisotropy r_i. The total intensities of labeled S-1 with various ligands (including actin), have been measured in our laboratory as a function of time (6). Although differences in the excited state lifetimes (and therefore in the total intensities) have been detected, they are invariably of the order of 1%; so, for our purposes, to assume them equal is a reasonable approximation. Thus, for a solution of labeled S-1 in different states, all of which have equal total intensities, the measured system anisotropy (7) is,

\[ r_{ssys}(t) = \sum_i x_i r_i(t) \]  

[1]

In the case of binding equilibria, one must consider the velocity constants of association and dissociation, k_a and k_d, and examine what effects they have on Eq. [1]. Stryer (8) has commented qualitatively on this question. Also, an analogous case in nuclear magnetic resonance spin relaxation was treated thoroughly and elaborately by Zimmerman and Brittin (9). We can show by elementary methods that, in agreement with the conclusions of both aforementioned papers, if the rate of exchange, k_a[A] + k_d, is much smaller than the rates of decay, \( \Phi_F^{-1} + \Phi_B^{-1} \Phi \) (the rotational correlation time, and F and B refer to free and bound states of S-1), then Eq. [1] becomes,

\[ r_{ssys}(t) = x_F r_F(0)e^{-t/\Phi_F} + x_B r_B(0)e^{-t/\Phi_B} \]  

[2]

If, on the other hand, the rates of exchange are much larger

Abbreviations: S-1, myosin subfragment 1; 1,5-IAEDANS, (N-iodoacetyl-N'-1-sulfo-5-naphthyl)ethylenediamine; Tris, N-(tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
than the rates of decay, then

$$r_{yy}(t) = r(0) \exp \left[ \frac{1}{2} \left( \frac{1}{t} - \frac{1}{t_0} \right) \right]$$  \[[3]\]

In principle, either equation could be fitted to data so as to obtain the $x$s. Furthermore, for the decay of the fluorescent intensities, $I_{yy}(t) = \sum x_i I_i(t)$, which corresponds to Eq. [1]. If the $I_i$s are all approximately equal, then this equation is useless, but if they are not, then this equation can be fitted to data to obtain the $x$s and therefore $k_a$.

**EXPERIMENTAL**

S-1 was prepared by the method of Cooke (10). Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis and Sephadex G-200 column chromatography showed a single major component corresponding to the heavy chain fragment of S-1. S-1 was approximately 70% labeled by incubation with (N-iodoacetyl-N'-sulfo-5-naphthyl)ethyleneediamine (1,5-IAEDANS) (26) in a 1 to 0.7 ratio (S-1 to dye at $\mu M$ concentrations) at 4°C in the dark for 24 hr. Actin was prepared by the method of Spudich and Watt (11) and generously given to us by Dr. R. Cooke. All chemicals were of the highest commercial grade.

All solutions were made up from concentrated stock solutions and contained 0.15 M KCl, 0.010 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), pH 7.0. Measurements were made at 4°C with S-1 and actin concentrations between 0.2 and 1.2 $\mu M$.

**RESULTS CONCERNED WITH ESTABLISHING THE METHOD**

It will develop later that for the binary mixture of interest (free and bound S-1), In $r$ versus $t$ is curvilinear, so of course it is desirable to measure $\ln r(t)$ over as large a range of $t$ as possible. This range, however, is experimentally limited at both ends. At early times it is limited because the excitation flash is not a delta function. *A priori* one cannot be sure that a flash of finite duration and complex form will not (when convoluted with the emission) produce *late* distortions in $r(t)$, or (when scattered in the solution) will not manage to pass the filters and produce *early* distortions in $r(t)$. At late times the range is always limited because the total emission ($e^{-t}$-th time for 1,5-IAEDANS is about 20 nsec) progressively dims, and the error in measuring and computing $r(t)$ becomes unacceptable. As a result of the experiments to be described, we concluded that data collected between 25 and 145 nsec were acceptable for analysis.

Earlier (5), we convoluted our lamp flash with model equivalent spheres of rotational correlation time, $\Phi = 220$ nsec, and found that in the range 0–175 nsec $\ln r(t)$ is not distorted by the flash. In the present work we examined for possible scattering artifacts by studying matched solutions of S-1 and F-actin, one in which the S-1 was fluorescent-labeled and one in which it was not. In Fig. 1 are shown the total intensity ($I + 2I_\perp$) and intensity difference ($I_\perp - I_\parallel$) for the labeled case, and the total (scattered and filter fluorescence) intensity for the unlabeled case. When the anisotropy for the labeled S-1 and actin was calculated from intensities that were “corrected” by subtracting out the intensities for the unlabeled case, it was found to differ from the uncorrected anisotropy by less than 1% beyond a point that is 25 nsec after the lamp peak; so we settled on 25 nsec as the lower limit of the usable range. The upper limit of 145 nsec was simply set by conservatively inspecting data dispersion as a function of time.

The next methodological question that we considered was whether coupling between fluorescence decay and chemical transformations occurs, i.e., whether either Eq. [2] or Eq. [3] is an acceptable approximation. If,

$$A + S \underset{k_a}{\overset{k_d}{\rightleftharpoons}} A \cdot S$$

then how does, $k_d[A] + k_{-a}$, compare with $\Phi_F^{-1} + \Phi_B^{-1}$? (Here “A” stands for actin.) Finlayson et al. (12) report for myosin that $k_d = 1.4 \times 10^8 M^{-1} sec^{-1}$. An upper limit for $k_{-a}$ is therefore, $k_{-a} = k_d/K_a$, where $K_a$ is the smallest value in the modern literature (1, 2). Finally, we substitute the largest concentration used in this work for $[A]$, and thus obtain an upper limit for $k_d[A] + k_{-a}$, but find that it is still orders of magnitude smaller than $\Phi_F^{-1} + \Phi_B^{-1}$, which is $5 \times 10^8 sec^{-1}$. Therefore, it is Eq. [2] that is applicable to our experiments.

**RESULTS OBTAINED USING THE METHOD**

Fig. 2 shows typical measurements of $\ln r_{yy}(t)$ versus $t$ obtained with mixtures of F-actin and S-1. In this figure the actin concentration decreases from top to bottom, the bottom curve being for S-1 alone. If $10^4$-fold magnesium pyrophosphate is added to any of the systems described by the top curves, the systems generate the bottom curve, within experimental error. For reasons explained above, data of this sort were fitted to Eq. [2] in the range 25 nsec < $t$ < 145 nsec. However, a further simplification of Eq. [2] is possible because $\Phi_B$, the rotational correlation time of S-1 bound to F-actin, is very large. Since $\Phi_B$ is large and the excited state lifetime is short, measurement by the present method difficult, but using a saturating concentration of actin in a very
long-run experiment, we estimate \( \Phi_B \) to exceed \( 6 \times 10^9 \) nsec; similarly Mihashi and Wahl (13) estimate \( 10 \times 10^9 \) nsec. Using a method better adapted to this issue ("saturation transfer" electron spin resonance), Thomas et al. (14) found \( \Phi_B \approx 180 \times 10^9 \) nsec. Thus, in our time-range we are justified in assuming that in Eq. [2], \( \exp(-t/\Phi_B) \approx 1 \). In earlier work (5), we have found that \( r_F(t) \) is satisfactorily given by a single exponential. Here we have re-evaluated the parameters and find \( \Phi_F = 231 \pm 20 \) nsec and \( r_F(0) = 0.233 \) (10 trials). Thus, knowing that \( \Phi_B \rightarrow \infty \), and measuring \( r_F(0) \) and \( \Phi_F \) in experiments with S-1 alone, our problem is reduced to fitting (by weighted least squares techniques) the experimental \( r_{sys}(t) \) to,

\[
r_{sys}(t) = r_F \cdot 0.233 \exp \left[ -t/(231 \times 10^{-9} \text{sec}) \right] + (1 - x_F) r_B(0) \tag{5}
\]

so as to find the best values of the parameters \( x_F \) and \( r_B(0) \). When this was done, the mean \( r_B(0) \) in 21 experiments was 0.251. This value is significantly different from that of \( r_B(0) \).

From the theory of depolarization (15), Mendelson et al. (5) showed that \( r_B(0) \) should depend only on the square of the cosine of the angle between the absorption and emission dipoles of the (S-1)-attached dye—an angle not likely to depend on actin ligation; so we have to consider that some effect is lowering \( r_B(0) \), i.e., that back extrapolation of ln \( r(t) \) from later times is giving an "apparent" \( r_B(0) \). At the suggestion of Prof. C. Weber, we measured \( r_B(0) \) in highly viscous 90% glycerol solution, and found it to be 0.257, very nearly the value of \( r_B(0) \). This still leaves several explanations of the lowering effect open. For example, since the lamp flash is not truly a delta function of intensity, the time at which excited fluorophores really begin to rotate is not definite, and it is possible that the back extrapolation of ln \( r(t) \) intersects \( t = 0 \) at a time when in fact some rotation has already occurred. With S-1 immobilized through actin ligation on glycerol viscosity, however, no appreciable rotation would have occurred, and the "true" \( = r_B(0) \) value of \( r(0) \) would be recorded. Another possibility is that the fluorophore, while covalently attached, is firmly bound most of the time, but is loose and in very rapid relative motion for a part of the time. In this case, \( r_B(0) \) is larger than \( r_F(0) \) because upon binding to actin, the looseness of the dye is reduced*. In either case, \( r_F(0) \) is an apparent value but still the one to be used in analyzing the data, and \( \Phi_F \) is unaffected.

Examination of the behavior of macromolecules by a method based on observing labeled macromolecules is always open to the question of whether labeling per se can affect the behavior. We sought to reassure ourselves on this point by a set of experiments in which a constant concentration of actin was presented with a constant total concentration, but variable proportions, of labeled and unlabeled S-1. If the affinity constant of labeled S-1 is \( K \), and that of the unlabeled S-1 is \( \alpha K \), it can be shown rigorously from Mass Law arguments that in such an experiment the fraction of labeled S-1 that is free \( (x_F) \) changes with proportion if \( \alpha \neq 1 \), but remains constant if \( \alpha = 1 \). Table 1 shows that when the percentage of labeled S-1 was varied from 70% down to

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*Exact treatment of attached dye motion superimposed on S-1 motion is well nigh impossible, but at least conceptually it is easy to see what such superimposition would do to \( r_B(0) \). For simplicity we can imagine that the attached dye exists either "firmly bound" (for a fraction of the time, \( f \)) or "loose" (for a fraction of the time, \( 1-f \)), and furthermore that when the dye is "loose" its angular velocity relative to its attachment adds to the angular velocity of S-1. Because of the proportionality of angular velocity to rotational diffusion coefficient the latter would be \( D(B) \) [or \( 1/\Phi_B \)] for the firmly bound dye and \( D(B) + D(S) \) [or \( 1/\Phi_B \) + \( 1/\Phi_{geo} \)] for loose dye, ignoring numerical factors.

**Table 1. Effect of labeling on \( x_F \)**

<table>
<thead>
<tr>
<th>% Labeled</th>
<th>( x_F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>0.323</td>
</tr>
<tr>
<td>35</td>
<td>0.312</td>
</tr>
<tr>
<td>52</td>
<td>0.344</td>
</tr>
<tr>
<td>70</td>
<td>0.309</td>
</tr>
</tbody>
</table>

(See text). Values obtained for the mole fraction of free S-1, \( x_F \), for solutions of 0.418 \( \mu \)M S-1 and 0.424 \( \mu \)M F-actin at \( 4^\circ \)C in 0.15 M KCl, 0.010 M Tes, pH 7.1, when the degree of labeling of S-1 is varied between 18 and 70%.

---

Fig. 2. Anisotropy decay. The points are experimental data for the fluorescence depolarization of 1,5-IAEDANS-labeled S-1; the lowest curve is for \( 0.428 \times 10^{-9} \) M S-1; the upper data are for solutions of S-1 with, in ascending order, 0.623, 1.92, and 25.98 \( \mu \)M F-actin. The decay of S-1 alone is well approximated by a single exponential (see text and ref. 5) represented by the solid line in the lowest curve. The solid lines for the upper curves result from varying the parameters \( x_F \) and \( r_B(0) \) to give a weighted best least squares fit of Eq. [5] to the data in the range of 25-145 nsec. Association constants for 21 solutions of S-1 and actin were determined from the values obtained for \( x_F \) in each solution. The upper curve does not extrapolate to the mean value of \( r_B(0) \) because of variation in preparation.
18% no detectable trend in $\sigma_F$ was observed, indicating that within the accuracy of our experiments the affinity constant of "native" S-1 is unaffected by labeling.

**DISCUSSION**

While the treatment that we have given to our data is at some points semi-empirical [namely, we have ascribed $r_F(0)$ > $r_F(0)$ to the existence of rapid processes (of whose identity we are unsure) occurring prior to the onset of depolarization by thermal rotation of S-1, and we have assumed that two states (bound and free) account for all subsequent phenomena], we feel that the theoretical framework of the method is as well established as for any current alternatives, and that we have realized some of the anticipated benefits, such as sensitivity extending to the sub-micromolar range and relative ease of experimentation. Furthermore, although some interaction of the S-1 surface exists between binding of actin and labeling of the thiol known as "SH1" (16), we have shown here that this effect does not translate into a detectable difference between affinities of labeled and unlabeled S-1, i.e., labeling per se does not affect the quantity that we measure. Fig. 3 shows that $K_a$ is larger the larger the ratio of total S-1 to total actin. This could conceivably be because S-1 copolymerizes, so to speak, with actin, but that is an unlikely explanation because from the data of Marston and Weber (3) our actin would be at least 90% polymerized anyway. So we have to imagine that either free S-1's repel one another, driving themselves into ligation with actin, or conversely, bound S-1's attract one another either directly or through actin. Either of these effects could have interesting functional consequences; so we are presently attempting to distinguish between them.

Because of differences in the manner of preparation of S-1, solvent composition, and temperature (muscle protein associates are typically very endothermic), differences between our estimate of $K_a$ ($1.7 \times 10^6$ M$^{-1}$) and those of others (Table 2) do not necessarily reflect differences in quality of measurement, and quite possibly the general similarity of results is more significant than the differences. Our estimate of $K_a$ corresponds to a $\Delta G^\circ$ of S-1 to actin binding of about $-7.88$ kcal/mole ($-32.9$ kJ/mole). The $\Delta G^\circ$ of S-1 to ATP binding is not easily measured because of the hydrolysis that S-1 catalyzes, so it has been estimated indirectly from a Michaelis constant (17, 18), by analogy with ADP (19), by analogy with thio-ATP (20), by a column method (21), and as a ratio of separately measured velocity constants (22). The $\Delta G^\circ$ values generally lie between $-6$ and $-7$ kcal/mole. To estimate the $\Delta G^\circ$'s from $\Delta G^\circ$'s is tenuous. The ATP concentration in muscle is thought to be much higher than the concentrations of cross bridges and actin monomers, but in real muscle the latter are assembled into filaments and held close to one another. It is thus possible that the $\Delta G^\circ$'s as well as the $\Delta G^\circ$'s will turn out to be comparable.

It was pointed out many years ago (23) that the free energy decrement on myosin-to-ATP binding had to be much greater than the decrement accompanying the subsequent hydrolytic step, and therefore that binding was probably the step associated with "energy transfer". These expectations have been confirmed by modern measurements (24). However, recent general considerations of energy transfer have dealt with the interaction between ATP and myosin. To do so is mistaken; myosin alone cannot be the transducer, and in fact the free energy decrement on binding would be largely nullified by the increment required to desorb ADP. Elsewhere, one of us (25) has proposed a better way to think of the process. The essential feature is that at the beginning of the work-performing branch of the ATP-myosin-actin cycle the $-\Delta G$ of binding ATP to S-1 should only slightly exceed the $\Delta G$ of "unbinding" actin from S-1. The present measurement of the latter quantity suggests that this requirement is in fact met.

S.H. is a Postdoctorate Fellow of the USPHS, and M.F.M. is a Career Investigator of the A.H.A. This research was supported by USPHS Grant HL-16683, and N.S.F. Grant GB-24992-X. We are grateful to Dr. S. Marston for early information about his related work, to Prof. G. Weber for helpful suggestions about experimental strategy, to Prof. Jean Botts for suggestions about one of the proofs used in this paper, to Prof. L. Peller for several helpful discussions, and to Mr. Tom Ferrin for computer assistance. Our 1,5-IADENS was generously supplied by Dr. Richard Haugland.

**Table 2. Association constants for myosin subfragment-1 (S-1) and F-actin obtained in this and other laboratories**

<table>
<thead>
<tr>
<th>$K_a (M^{-1})$</th>
<th>Conditions</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(1.73 \pm 0.35) \times 10^4$</td>
<td>0.15 M KCl, 0.01 M Tes, pH 7.1, 4°C</td>
<td>Fluorescence depolarization (this work)</td>
</tr>
<tr>
<td>$3.1 \times 10^2$</td>
<td>0.10 M KCl, 0.10 M Tris-HCl, 0.001 M MgCl$_2$, pH 7.6, 6–8°C</td>
<td>Sedimentation (UV analysis)</td>
</tr>
<tr>
<td>$(1.4 \pm 0.6) \times 10^7$</td>
<td>0.12 M KCl, 10 mM imidazole, 1 mM MgCl$_2$, 4 mM EGTA, 0.2 mM dithiothreitol, pH 7.0, 25°C</td>
<td>Sedimentation (1°C analysis)</td>
</tr>
</tbody>
</table>

M KCl, 0.010 M Tes, pH 7.1, when the degree of labeling of S-1 is varied between 18 and 70%.