composition or for the occurrence of a rate-limiting conformational change on myosin without a bound nucleotide.

In conclusion it can be stated that blocking the SH_1 site of myosin does not affect the ability of HMM to bind to actin in the absence of ATP nor does it affect the cyclic interaction of HMM with actin and ATP. On the other hand, the blocking of the SH_1 site of myosin does decrease the actin activation of the HMM ATPase and evidence was presented in this paper that this may be explained by a decreased rate of conversion from the refractory to the nonrefractory state in SH_1-blocked HMM.

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The Dissociation Constant of the Actin–Heavy Meromyosin Subfragment-1 Complex†

Steven Marston and Annemarie Weber*

ABSTRACT: We measured the binding of [^{14}C]iodoacetamide labeled heavy meromyosin subfragment-1 (S-1) to F-actin by sedimenting the actin–S-1 complex and assaying the radioactivity remaining in the supernatant. The apparent dissociation constants (K_d) at 25°, pH 7.0, were 0.01 to 0.04 μM at 0.027 and 0.08 ionic strengths and 0.07 to 0.14 μM at 0.14 ionic strength. K_d was not altered when the troponin–tropomyosin complex was bound on the actin, nor was it affected by free calcium concentration in the range 10^{-4} to 10^{-9} M. Measurements of the displacement of labeled S-1 from actin by native S-1 showed labeling had not altered K_d. In control experiments we found that at the low actin concentrations used (0.01–0.5 μM) not all of the actin sedimented and, furthermore, the data suggested that some of the S-1 in the supernatant was bound to supernatant actin. Our estimation of K_d, based on the assumption that all the supernatant S-1 was free, therefore resulted in an apparent K_d greater than the true K_d. We minimized the effect of the supernatant actin artefact by using only the data for high ratios of S-1 to actin, where no less than 75% of the actin sedimented; we estimate that the true K_d values could not be less than half the apparent K_d values.

Actin and myosin, the major proteins involved in muscle contraction, bind very tightly to each other in the absence of ATP. It has long been believed that the cycling of the myosin bridges in muscle involves the formation and dissociation of these so-called rigor complexes (Huxley, 1969). They most likely represent an intermediate state in actin-activated ATP hydrolysis by myosin. The dissociation constant for the rigor complex is one of the determinants for the overall reaction rate of ATP hydrolysis by actomyosin whose value adds to the kinetic analysis which has made considerable progress in recent years (Kanazawa and Tonomura, 1965; Lymn and Taylor, 1971, Trentham et al., 1972; Bagshaw and Trentham, 1974; Marston, 1973).

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Previous workers have reported a dissociation constant of about 1 μM for the binding of actin to isolated active sites of myosin (heavy meromyosin subfragment-1, S-1) (Young, 1967; Margossian and Lowey, 1973). However, in our hands both direct and indirect experiments indicated that the dissociation constant is probably much lower. We therefore reinvestigated actin–S-1 binding using radioactively labeled S-1. We found the dissociation constant is at least an order of magnitude less than previously published values.

Materials and Methods

Protein Preparations. An acetone powder was prepared from muscle residue after the extraction of myosin (Feuer et al., 1948; Szent-Györgyi, 1951). Monomeric actin (G-actin) was extracted from the powder with 20 vol of "depolymerizing solution" (10 mM Tris (pH 8.5), 0.1 mM ATP, 0.2 mM $CaCl_2$, and 0.2 mM dithiothreitol) and isolated by reversible polymerization–depolymerization (Rees and Young, 1967). Troponin and tropomyosin were removed from the extract by sedimenting polymerized actin in the presence of 0.8 M KCl at pH 8.5 (Spudich and Watt, 1971). The troponin–tropomyosin complex was isolated by the method of Ebashi and Ebashi (1964).

Pure polymeric actin (F-actin) was prepared by polymerizing G-actin with 0.1 M KCl and 1 mM $MgCl_2$. Regulated actin was prepared by polymerizing G-actin in the presence of three times its weight of troponin–tropomyosin complex. The regulated and pure F-actins were sedimented at 140,000g for 1 hr and the pellets were redispersed in "low ionic strength" buffer (10 mM imidazole (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM dithiothreitol, and 10 mM KCl) (Bremel and Weber, 1972).

Heavy meromyosin subfragment-1 (S-1) was prepared by a technique based on that of Margossian and Lowey (1973). A myosin gel (10 mg/ml in 10 mM imidazole (pH 7.0)–0.2 M KCl) was incubated with papain (3 μg/mg of myosin) for 12 min at 25°. Then the pH was lowered to 6.4 with 0.2 M imidazole (pH 5.6) and iodoacetic acid was added to a final concentration of 1 mM to stop proteolysis. The reaction mixture was immediately diluted with 8 vol of cold 10 mM imidazole (pH 7.4)–0.5 mM dithiothreitol. S-1 was isolated by ammonium sulfate precipitation; the fraction precipitating between 50 and 65% saturating ammonium sulfate was collected, dissolved, and dialyzed against 10 mM imidazole (pH 7.0) for 10 hr. Finally, insoluble material was removed by centrifuging for 10 min at 50,000g. Purified S-1 retained 80–90% of the EDTA ATPase activity of the parent myosin (15–17 sec^{-1}).

S-1 (50 nmol) was radioactively labeled by incubating with an equimolar amount of [^{14}C]iodoacetamide (54 mCi/mmol) in 10 mM imidazole (pH 7.5)–0.1 M KCl at 0° for 24 hr. The excess label was then removed by dialysis against low ionic strength buffer. Stoichiometry of labeling was usually 0.9–1.1 mol of iodoacetamide/mol of S-1. After labeling the EDTA ATPase activity was completely inhibited, while MgATPase activity was increased threefold compared with native S-1.

Sedimentation Experiments. Iodoacetamide-labeled S-1 at a final concentration of about 0.025 μM and F-actin in the range 0–0.4 μM were mixed in a final volume of 2 ml of low ionic strength buffer (10 mM imidazole (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM, dithiothreitol and 10 mM KCl, I = 0.027). Higher ionic strengths were obtained by adding the appropriate quantity of KCl. In some experiments, free calcium concentration was reduced to 10^{-9} M by substituting 4 mM EGTA[1] for 10 mM KCl.

Thirty minutes was allowed for equilibrium and then the mixtures were centrifuged for 2 hr at 49,000g (Sorvall RC2B centrifuge, SM24 rotor), 25°, to sediment F-actin and the F-actin–S-1 complex. The height of the fluid column was 1 cm. Portions (1.0 ml) of the supernatant were carefully removed and their radioactivity was assayed by liquid scintillation counting. Control experiments showed that in the absence of actin about 10% of S-1 sedimented as would be expected from its sedimentation coefficient of 3 S (Young 1967), whereas at high concentrations (above 10 μM) over 90% of actin was spun down. Assuming the actin still sediments at the low concentrations employed, it is possible to measure the amount of labeled S-1 bound to actin by comparing the amount of radioactivity remaining in the supernatant when S-1 is centrifuged in the presence and absence of F-actin:

$$[\text{actin–S-1}] = [\text{S-1 added}] \times \frac{(\text{cpm in absence of actin} - \text{cpm in presence of actin})}{(\text{cpm in absence of actin})}$$

Appropriate corrections were made for the small fraction of radioactivity (about 5% free label plus 4% inactive labeled S-1) which did not sediment even under optimum conditions (50 μM F-actin, 20 μM S-1, 0.1 ionic strength). Assuming one S-1 binds per actin (Margossian and Lowey, 1973), the concentration of free actin can then be estimated:

$$[\text{free actin}] = [\text{added actin}] - [\text{actin–S-1}]$$

Since added S-1 concentration was not the same in every experiment, the results are plotted as the fraction of S-1 "bound" as a function of [free actin].

Protein Fluorescence Measurements. To determine how much of the actin sedimented during our experiments, Figure 3, the concentration of protein remaining in the supernatant after sedimentation was measured by fluorimetry (Perkin Elmer MPF-2A, excitation at 276 nm and emission at 330 nm). Actin concentrations as low as 40 nM may be measured provided that the imidazole buffer, which fluoresces slightly, is replaced by phosphate. In experiments where iodoacetamide labeled S-1 was sedimented with actin, the concentration of S-1 in the supernatant was estimated from the radioactivity and the fluorescence contributed by S-1 subtracted (a control experiment showed that the fluorescence of S-1 was not altered by binding the actin). Under our conditions the fluorescences of G-actin and F-actin were the same (Lehrer and Kerwar, 1972); thus we may determine the concentration of actin in the supernatant without knowledge of its state of polymerization.

ATPase Activity. Myosin and S-1 "EDTA ATPase" activities were measured in 10 mM Tris (pH 8.0), 2 mM ethylenediamine-N,N'-tetraacetic acid, 0.5 M KCl, and 5 mM ATP at 25°. After 0.5 or 1 min, the reaction was stopped with trichloroacetic acid and the phosphate released was estimated by the method of Taussky and Schorr (1953).

S-1 "Mg^{2+} ATPase" and actin-activated ATPase activities were measured by an NADH-linked enzyme assay (Bergmeyer, 1965). The actin and myosin were incubated with 0.1 mg/ml of pyruvate kinase, 0.1 mg/ml of lactic dehydrogenase, 0.5 mM phosphoenol pyruvate, 0.04 mM NADH, and 1 mM MgATP in low ionic strength buffer.

[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

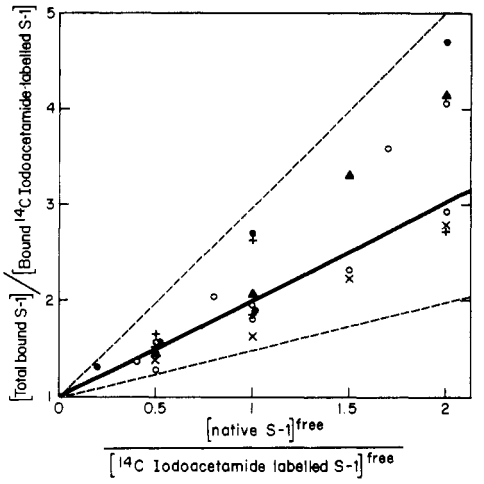

FIGURE 1: Competition of [^{14}C]iodoacetamide labeled S-1 and native S-1 for F-actin. The full line is calculated for $K_d = K_d^*$ and the dotted lines for $K_d = 2K_d^*$ and $K_d = \frac{1}{2} K_d^*$. Incubation conditions: actin, 0.8 μM; labeled S-1, 1.0 μM; native S-1, 0–3 μM; 10 mM imidazole (pH 7.0); KCl see below; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$; 0.2 mM dithiothreitol. Sedimentation for 2 hr at 49,000g, 25°; symbols: pure actin at ionic strengths 0.027 (○), 0.08 (x), and 0.14 (△); regulated actin at ionic strengths 0.027 (●), 0.08 (+), and 0.14 (▲).

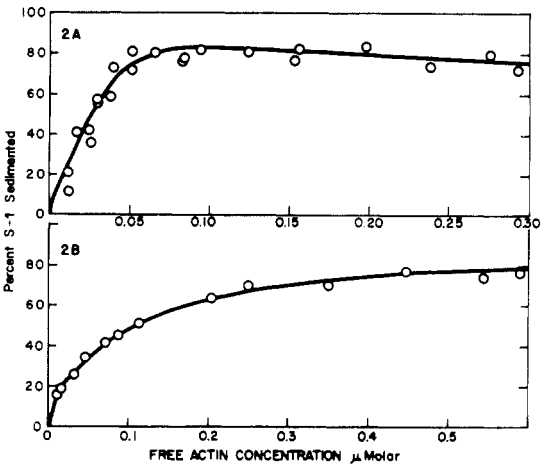

FIGURE 2: Fraction of [^{14}C]iodoacetamide labeled S-1 sedimented with actin after 2 hr centrifugation at 49,000g, 25°. Incubation medium: 10 mM imidazole (pH 7.0), KCl see below, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM dithiothreitol: (A) 0.025 μM S-1 and regulated actin at 0.027 ionic strength; (B) 0.06 μM S-1 and pure actin at 0.14 ionic strength.

Table I: Actin Binding to 0.7 μM [^{14}C] Iodoacetamide Labeled S-1.[a]

Concn Actin Added (μM)	Concn Free Actin (μM)	Concn Free S-1 (μM)	Concn Acto–S-1	% of S-1 Bound
2.5	1.81	0.03	0.69	96
2.0	1.32	0.04	0.68	94
1.5	0.82	0.04	0.68	94

[a] Conditions: low ionic strength buffer, pH 7.0; $I = 0.027$; 25°; centrifugation, 2 hr at 49,000g.

For each ATP molecule hydrolyzed, one NADH is oxidized. The rate of NADH oxidations was determined by recording the change in optical density at 340 nm (Gilford 300-N spectrometer with Thermocuvette and Radiometer REC 51 recorder).

We assumed molecular weights of 110,000 for S-1 and 45,000 for pure actin. For regulated actin we assumed one troponin-tropomyosin complex, mol wt 150,000, for every seven actin monomers (Bremel and Weber, 1972).

Results

In preliminary experiments we observed that actin and the isolated active sites of myosin (heavy meromyosin subfragment-1, S-1) combined with unexpectedly high affinity in the absence of ATP (cf. Young, 1967; Margossian and Lowey, 1973) since complex formation was nearly complete at micromolar protein concentrations (Table I). Therefore, it was necessary to conduct our measurements at nanomolar protein concentrations. Labeling the S-1 with [^{14}C]iodoacetamide of high specific activity allowed us to determine the amount of S-1 that sedimented with actin filaments in concentrations as low as 5×10^{-9} M.

Labeling of the "SH_1" sulfydryl group of S-1 (Burke et al., 1974; Stone, 1973) did not significantly affect its binding to actin (Seidel, 1973). This was demonstrated by measuring the competition of native and labeled S-1 for actin. Increasing concentrations of native S-1 were added to preequilibrated (30 min) mixtures of 0.8 μM actin and 1.0 μM labeled S-1 (S-1*), thereby displacing some S-1* from the actin. After 0.5 hr equilibration actin–S-1 complexes were sedimented and the amount of S-1* in the supernatant was assayed. Since binding is stoichiometric at 1 μM the amount of S-1* displaced from actin depends on the relative concentrations and dissociation constants of the native and labeled S-1 according to the simple equation:

$$\frac{[\text{total bound S-1}]}{[\text{bound S-1*}]} = \frac{K_d^*[\text{S-1}]^{free}}{K_d[\text{S-1*}]^{free}} + 1$$

In Figure 1 [total bound S-1]/[bound S-1*] is plotted as a function of the ratio of free native to free labeled S-1 at several ionic strengths. The mean slope is close to unity indicating that $K_d^* = K_d$.

The low protein concentrations used for these measurements presented some difficulties that are illustrated by the typical "binding curve" at low ionic strength; Figure 2A. When actin was added in increasing amounts to a constant amount of S-1, the fraction of S-1 sedimented first increased, as expected, until about 80% sedimented, but surprisingly, on increasing actin further the fraction of S-1 sedimenting decreased. This was not due to inactivation of S-1 by a contaminant of actin since at high actin concentrations (about 50 μM) sedimentation of S-1 was restored to more than 95% of the added S-1.

The explanation of this behavior is probably found in the experiments of Figure 3. It occurred to us that actin may depolymerize when diluted to these low concentrations and that the depolymerized actin may compete with the actin filaments for S-1, thereby retaining S-1 in the supernatant. Figure 3 presents evidence in favor of depolymerization. Measurements of protein tryptophan fluorescence indicated that, in the absence of S-1, the fraction of actin which sedimented decreased from over 90% to about 40% when actin was diluted from 50 μM to less than 0.5 μM and maintained at these concentrations for 2–3 hr (incubation and centrifugation period). The fraction of actin sedimenting is substantially increased by the presence of S-1; at 100 nM actin the fraction sedimenting goes from 40% in the absence of S-1 to 75% with 0.03 μM S-1 and 86% with 0.08 μM S-1. It is interesting that S-1 caused an amount of actin to sediment that was larger than its equivalent concentration. Nevertheless, as actin concentration increased the effect of

Table II: Apparent Dissociation Constants of Acto-S-1 Complex at 25°, pH 7.0.

	No. Expt	Ionic Strength	Dissoc[a] Constant (μM)	Range of Data (%)[b] S-1 Sedimenting	S-1 Concn (μM)	S-1 Preparation[c] No.
Pure actin	1	0.027	0.014 ± 4	31–74	0.02	21
	2		0.024 ± 5	27–95	0.04	22
	3		0.008 ± 2	46–83	0.03	28
	4		0.027 ± 5	32–80	0.02	23
(145,000g)	5	0.027	0.016 ± 10	13–84	0.04	28
(145,000g)	6		0.004 ± 1	23–77	0.04	28
	7	0.08	0.010 ± 2	13–86	0.04	27
	8		0.008 ± 2	9–82	0.04	28
	9	0.14	0.071 ± 6	16–82	0.08	28
Regulated actin	10	0.027	0.012 ± 5	37–82	0.02	23
	11		0.019 ± 3	17–80	0.025	23
	12	0.08	0.041 ± 10	11–83	0.03	24
	13		0.035 ± 6	21–77	0.025	24
(10^{-9} M Ca^{2+})	14	0.08	0.026 ± 5	23–88	0.03	24
(10^{-9} M Ca^{2+})	15		0.043 ± 20	10–84	0.03	24
	16	0.14	0.149 ± 18	12–72	0.06	24

[a] Mean dissociation constant and standard error were calculated by the statistical procedure of Wilkinson (1961) using data from 0 to 0.1 μM free actin concentration (i.e., up to 75–85% sedimentation of S-1) except for the experiments at 0.14 ionic strength where all the data were used. [b] Total range of data collected; the upper limit is a maximum in most cases, except for the 0.14 ionic strength experiments where the percentage sedimenting was still increasing at the highest actin concentrations used. [c] Each experiment used a fresh actin preparation.

S-1 was diluted out and with a tenfold excess of actin over S-1 the fraction of actin not sedimenting was quite substantial (Figure 3).

If the actin which did not sediment was capable of binding S-1 it would retain S-1 in the supernatant. At 0.027 and 0.08 ionic strengths this effect would result in the biphasic curve observed in Figure 2A since the actin-S-1 binding approaches saturation at less than 0.1 μM actin where a high proportion of actin is sedimenting. Above 0.1 μM not much more S-1 can be bound, but increasing amounts of S-1 would be retained by actin in the supernatant resulting in a decrease in the S-1 sedimenting. In contrast, at high ionic strength (Figure 2B) S-1 binding is far from saturation over the actin concentration range where the fraction of actin sedimenting decreases; consequently the curve is roughly hyperbolic.

Apparent dissociation constants calculated from our measurements are presented in Table II. At 0.027 and 0.08 ionic strengths we used only the data in the range of actin concentrations 0–0.1 μM where the effect of nonsedimenting actin is at a minimum; at 0.14 ionic strength, we used all the data. We have estimated that even in the limiting case where all the supernatant actin bound S-1 with the same affinity as did the sedimenting actin the apparent dissociation constant would not have been reduced by more than 50%. For example, the value derived from experiment 11 (Table II) only decreases from 0.019 to 0.014 μM. Furthermore, this correction is too large since, as the total actin increased, the amount of S-1 in the supernatant did not increase as much as the actin in the supernatant did (Figures 2 and 3).

The dissociation constants were not significantly altered by raising the ionic strength from 0.027 to 0.08 (Table II). Increasing it further, to 0.14, increased the constant by five- to tenfold. It should be stressed that the scatter is too large to exclude the possibility of a twofold difference in the dissociation constant at 0.027 and 0.08 ionic strength.

The presence of regulatory proteins on the actin filament did not significantly alter the binding of S-1, nor did the calcium concentration in the range from 10^{-4} to 10^{-9} M. In

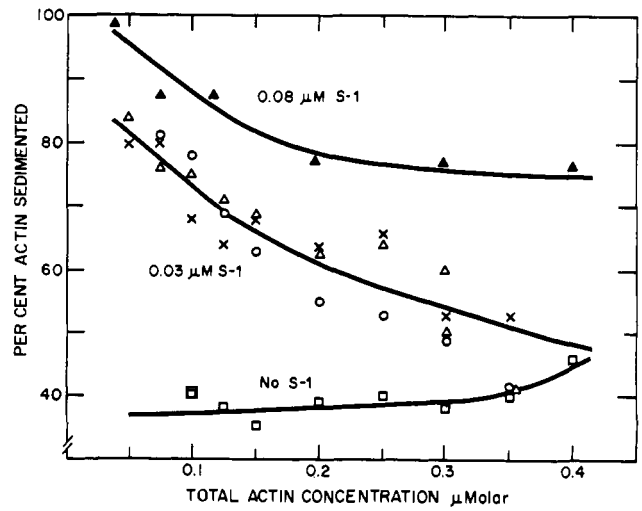

FIGURE 3: The fraction of actin sedimenting at low actin concentrations. Incubation medium: 5 mM phosphate (pH 7.0), KCl see below, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$; centrifugation for 2 hr at 49,000g, 25°. Symbols: pure actin, I = 0.027 (□); pure actin + 0.03 μM S-1, I = 0.027 (○); pure actin + 0.03 μM S-1, I = 0.08 (x); pure actin + 0.03 μM S-1, I = 0.14 (△); pure actin + 0.08 μM S-1, I = 0.14 (▲).

view of the similarities of the dissociation constants, we thought it necessary to determine whether, at these low actin concentrations, tropomyosin and troponin remain bound on the actin filaments. We therefore labeled the troponin-tropomyosin complex with [^{14}C]iodoacetamide. When this complex was bound to 0.5 μM actin, the acto-S-1 ATPase activity was at least 80% calcium sensitive and when we diluted actin from 0.5 to 0.02 μM, the quantity of complex bound on actin did not decrease.

Discussion

The use of the ultracentrifuge to measure the binding of a slowly sedimenting molecule like S-1 to a rapidly sedimenting one, such as F-actin, is a standard technique (Steinberg and Schachman, 1966). However, using this technique to measure dissociation constants (Young, 1967;

Margossian and Lowey, 1973) involves certain assumptions. (1) The disturbance of equilibrium during sedimentation due to changes of actin concentration at the boundary is negligible. (2) The equilibrium is not disturbed by the change in hydrostatic pressure as the actin and acto-S-1 move down the centrifuge tube. Equilibrium will be affected by pressure if there is a significant change in molecular volume, V (cubic centimeters per mole), when actin and S-1 bind thus:

$$\Delta(\ln K) = -(\Delta P \Delta V)/RT$$

where R is the gas constant (ergs per kelvin per mole), T is the absolute temperature (kelvin), and ΔP is the change in pressure (dynes per square centimeter) (Kegeles, et al., 1967). We believe pressure effects, if they occur, are small since the apparent dissociation constant measured at 145,000g, where the pressure at the bottom of the 1-cm fluid column was 142×10^6 dyn cm^{-2} above that at the miniscus, was only slightly less than the apparent dissociation constant measured at 50,000g ($\Delta P = 50 \times 10^6$ dyn cm^{-2}) (Table II). (3) The S-1 which sediments represents all the bound S-1 and that which does not sediment is free. Some uncertainty exists about supernatant S-1 since we do not know whether some of it is bound to supernatant actin (Figure 3; see Results section). It is likely that much of the supernatant actin was in the monomeric form since the protein concentrations were so low and, since ATP was absent, the monomeric actin may have been inactivated. We minimized the error at 0.027 and 0.08 ionic strengths by using only the data in the range of actin concentrations where at least 75% of the actin sedimented. Oosawa and his colleagues (Oosawa and Kasai, 1962) demonstrated that actin polymerization is comparable to a condensation process in which the actin is all polymeric above a critical concentration and all monomeric below it. According to Oosawa's data, concentrations below 1 μM are well below the critical level; our observations (Figure 3) that the extent of depolymerization reached only about 60% in the 2–3 hr of incubation and centrifugation is in accordance with the low rate constants for depolymerization. A shift in the critical concentration to lower values by myosin fragments has been suggested by the experiments of Yagi et al. (1965) and those of Cooke and Morales (1971) for salt-free solutions. Our data suggest that S-1 also has this effect at 1 mM magnesium and with ionic strengths of 0.03 to 0.14. The observation that S-1 affects more than an equivalent amount of actin is presumably related to the mechanism of depolymerization and therefore not directly related to the topic under discussion.

The apparent dissociation constants of the actin-S-1 complex based on the three assumptions discussed above are presented in Table II. We estimate (see Results) that even in the limiting case where supernatant actin binds S-1 as tightly as the sedimented actin, the apparent dissociation constant is not greater than twice the true dissociation constant.

It is not clear why our dissociation constants are so much lower than previously published (Young, 1967; Margossian and Lowey, 1973). We have obtained the same dissociation constants with S-1 prepared from myosin by our method or by the method of Margossian and Lowey and from myofibrils by the method of Cooke (1972), so we do not believe the difference is due to a difference in S-1 preparations. When we measured actin-S-1 binding by light scattering at 300 nm we found, as with the sedimentation method (Table I), that binding was stoichiometric at 0.5 μM S-1 and actin. This result supports our assertion that sedimentation produced a valid measurement of actin-S-1 binding.

The dissociation constant of 0.02 μM at 0.08 ionic strength agrees reasonably with the value of 0.025 μM at 0.1 ionic strength, calculated from rate constants of association and dissociation (White and Taylor, personal communication). Our values of the dissociation constant are relatively insensitive to ionic strength; since White and Taylor observe a tenfold reduction of the association rate with a change of ionic strength from 0.01 to 0.1, it seems likely that the rate of dissociation also decreases with increasing ionic strength.

S-1 binding to regulated actin has been shown to increase the affinity of troponin for calcium (Bremel and Weber, 1972), yet, surprisingly, we did not observe the reciprocal effect—an increase in S-1 binding affinity at 10^{-4} M compared with 10^{-9} M free calcium (Table II). Nevertheless, our results are not at odds with Bremel's since changes in the dissociation constant of the actin-S-1 complex would only have been observed if calcium binding caused a large free-energy change or had a highly cooperative effect on S-1 binding. For example, we calculated that a contribution of binding energy up to 3000 cal due to calcium binding on troponin would be hidden in the scatter of data provided the actin-S-1 binding energy change was distributed evenly over four or more of the seven actin controlled by each troponin-tropomyosin complex.

In summary at 25° and low ionic strength with 1.0 mM $MgCl_2$ at pH 7.0 (conditions used for most kinetic studies), the dissociation constant of the F-actin-S-1 complex is about 0.02 μM. On increasing ionic strength to 0.14 binding becomes weaker and the dissociation constant increases five- to tenfold. The affinity between actin and the active site of myosin is so high that dissociation of the complex—a crucial step for the shortening of muscle—requires considerable expenditure of energy. The energy is provided by binding of ATP to myosin, which explains why it is necessary for myosin to have such a high affinity for ATP (Wolcott et al., 1974; Mannherz et al., 1974).

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DNA Binding by Cyclic Adenosine 3′,5′-Monophosphate Dependent Protein Kinase from Calf Thymus Nuclei†

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ABSTRACT: Cyclic adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase and proteins specifically binding cAMP have been extracted from calf thymus nuclei and analyzed for their abilities to bind to DNA. Approximately 70% of the cAMP-binding activity in the nucleus can be ascribed to a nuclear acidic protein with physical and biochemical characteristics of the regulatory (R) subunit of cAMP-dependent protein kinase. Several peaks of protein kinase activity and of cAMP-binding activity are resolved by affinity chromatography of nuclear acidic proteins on calf thymus DNA covalently linked to aminoethyl Sepharose 4B. When an extensively purified protein kinase is subjected to chromatography on the DNA column in the presence of 10^{-7} *M* cAMP, the R subunit of the kinase is eluted from the column at 0.05 *M* NaCl while the catalytic (C) subunit of the enzyme is eluted at 0.1–0.2 *M* NaCl. When chromatographed in the presence of histones, the R subunit is retained on the column and is eluted at 0.6–0.9 *M* NaCl. In the presence of cAMP, association of the C subunit with DNA is enhanced, as determined by sucrose density gradient centrifugation of DNA–protein kinase complexes. cAMP increases the capacity of the calf thymus cAMP-dependent protein kinase preparation to bind labeled calf thymus DNA, as determined by a technique employing filter retention of DNA–protein complexes. This protein kinase preparation binds calf thymus DNA in preference to salmon DNA, *Escherichia coli* DNA, or yeast RNA. Binding of protein kinases to DNA may be part of a mechanism for localizing cyclic nucleotide stimulated protein phosphorylation at specific sites in the chromatin.

It appears likely, on the basis of several recent studies, that certain of the effects of cyclic adenosine 3′,5′-monophosphate (cAMP)[1] upon growth and differentiation of eukaryotic cells are due to the ability of this cyclic nucleotide to influence transcription. cAMP or its butyryl derivatives have been observed to stimulate incorporation of radioactive precursors into RNA of cells from uterus (Sharma and Talwar, 1970), adrenocortex (Nussdorfer and Mazzocchi, 1972), thyroid (Wilson and Wright, 1970), into RNA of human lymphocytes (Averner et al., 1972; Rosenfeld et al., 1972), and into RNA of nuclei from rat liver cells (Jost and Sahib, 1971; Dokas et al., 1973). cAMP has been implicated in the activation of specific genes in *Drosophila* salivary glands (Leenders et al., 1970; Rensing and Hardeland, 1972). cAMP-induced morphological changes in rat sarcoma (Korinek et al., 1973) and neuroblastoma (Bondy et al., 1974) cells have been linked to effects of the cyclic nucleotide upon mRNA synthesis. cAMP-induced phosphodiesterase synthesis in chicken embryo fibroblasts has been observed to be dependent upon increased transcription (Russel and Pastan, 1974).

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetate; PK II, a protein kinase fraction from calf thymus.