Deranleau, D. A. (1969), *J. Am. Chem. Soc. 91,* 4044.
Deranleau, D. A. (1975), *J. Am. Chem. Soc. 97,* 1218.
Deranleau, D. A., Hinman, L. M., and Coan, C. R. (1975), *J. Mol. Biol. 94,* 567.
Deranleau, D. A., and Schwyzer, R. (1969), *Biochemistry 8,* 126.
Dlouha, Y., and Keil, B. (1969), *FEBS Lett. 3,* 137.
Fersht, A. R., and Sperling, J. (1973), *J. Mol. Biol. 74,* 137.
Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H. (1970), *Biochemistry 9,* 1997.
Hess, G. P. (1971), *Enzymes, 3rd Ed. 3,* 232.
Hinman, L. M., Coan, C. R., and Deranleau, D. A. (1974), *J. Am. Chem. Soc. 96,* 7067.
Karrer, P., Schwarzenbach, G., Benz, F., and Solmsson, U. (1936), *Helv. Chim. Acta 19,* 811.
Katrukha, G. S., Zylber, N., and Keil-Dlouha, V. (1973), *FEBS Lett. 29,* 25.
Keil-Dlouha, V., and Keil, B. (1972), *J. Mol. Biol. 67,* 495.
Koshland, D. E., Karkharis, Y. D., and Latham, H. G. (1964), *J. Am. Chem. Soc. 86,* 1448.
Krieger, M., Kay, L. M., and Stroud, R. M. (1974), *J. Mol. Biol. 83,* 209.
Laskowski, M. (1955), *Methods Enzymol. 2,* 8.
Mavridis, A., Tulinsky, A., and Liebman, M. N. (1974), *Biochemistry 13,* 3661.
Morgan, P. N., Robinson, N. C., Walsh, K. A., and Neurath, H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3312.
Oppenheimer, H. L., and Hess, G. P. (1963), *Nature (London) 198,* 689.
Oppenheimer, H. L., Mercouroff, J., and Hess, G. P. (1963), *Biochim. Biophys. Acta 71,* 78.
Porubcan, M. A., Ibanez, I., and Markley, J. L. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 483.
Sigler, P. B., Jeffrey, B. A., Matthews, B. W., and Blow, D. M. (1966), *J. Mol. Biol. 15,* 175.
Spande, T. F., Green, N. M., and Witkop, B. (1966), *Biochemistry 5,* 1926.
Stewart, K. K., and Doherty, F. (1971), *FEBS Lett. 16,* 226.
Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1974), *J. Mol. Biol. 83,* 185.
Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., and Wright, L. H. (1973), *Biochemistry 12,* 4185.
Vandlen, R. L., and Tulinsky, A. (1973), *Biochemistry 12,* 4193.
Villanueva, G. B., and Herskovits, T. T. (1971), *Biochemistry 10,* 3358, 4589.
Walsh, K. A., and P. E. Wilcox (1970), *Methods Enzymol. 19,* 31.
Williams, E. J., Herskovits, T. T., and Laskowski, M. (1965), *J. Biol. Chem. 240,* 3574.
Williams, E. J., and Laskowski, M. (1965), *J. Biol. Chem. 240,* 3580.
Wright, H. T. (1973a), *J. Mol. Biol. 79,* 1.
Wright, H. T. (1973b), *J. Mol. Biol. 79,* 13.

The Interaction of Actin Monomers with Myosin Heads and Other Muscle Proteins†

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ABSTRACT: The simplest interacting unit of actomyosin, viz., single myosin heads (subfragment 1) with actin monomers, has been studied at physiological ionic strength, by isolating the actin molecules from each other on a solid support. The interaction is characterized by a binding constant of 10^5 to 10^6 M^{-1} in the temperature range 4–30 °C. It is endothermic with a standard enthalpy of 24 ± 10 kcal mol^{-1}, and a standard entropy of 110 ± 40 eu. It is thus, like many protein–protein association processes, entropy-driven. Despite the high affinity of the association, which is comparable in its binding constant to that of subfragment 1 with F-actin, there is only very small activation of myosin ATPase. The ionic-strength dependence of the interaction shows unusual features. Binding of the proteins of the relaxing system to the monomeric actin was also examined: troponin binds both in the presence and absence of calcium ions, but neither tropomyosin nor the tropomyosin–troponin complex was found to bind significantly. Monomeric actin has also been examined as a function of ionic strength by spectroscopic methods; it appears that conformational differences between the G and the F state are the consequence of polymerization, and not of the change in ionic strength required to bring the conversion about.

In approaching a complete description of the mechanism by which the interaction of myosin heads with the components of the actin helices, constituting the thin filaments of skeletal muscle, leads to relative motion, it is necessary to define each binding process individually in terms of its intrinsic thermodynamic and kinetic parameters. Compared with the function of a single multi-subunit enzyme, which can be described in terms of a small number of equilibrium constants, the system is obviously extremely complex. It is still a matter of debate whether the two heads on a single myosin molecule are functionally identical, and practically nothing is known about the interaction of each head with its binding site on a single actin molecule. This interaction must be studied in isolation if the nature of the interaction of the two-headed myosin molecule with the constellation of accessible monomers in the actin filament, both in the presence and absence of ATP, is to be understood, as well as the relation of the process to the reported

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changes in the configuration of the thin filaments in the course of contraction (Wakabayashi et al., 1975), and the manner in which it is modulated by the regulatory proteins present in the thin filament.

The difficulty in studying the basic interaction in anything approaching physiological solvent conditions is that the monomeric G-actin polymerizes rapidly to the fibrous form. One therefore has the choice of attempting a specific chemical modification, which will leave the protein in the native conformation, while inhibiting interaction with its neighbors in the filament, or of isolating the monomer by coupling G-actin at low ionic strength to an isoluble matrix. We have shown (Chantler and Gratzer, 1973) that the G-actin coupled to a Sepharose gel matrix remains substantially in the native state, as judged by retention of its nucleotide, and that it is able specifically to bind myosin, and separated myosin heads (subfragment 1). We present here the results of an equilibrium study of its interaction with subfragment 1. Thermodynamic parameters for the binding process have been obtained. The extent of activation of the myosin ATPase by the monomeric bound actin, and its dependence on ionic strength have been determined. The interaction of the relaxing factor complex, and of tropomyosin and troponin separately, with the bound actin have been studied, and the conformational basis of the polymerization of G-actin has been examined by spectroscopic methods.

Materials and Methods

Proteins. Rabbit skeletal muscle actin was prepared from the acetone powder with ATP and calcium chloride under the conditions described by Spudich and Watt (1971), and purified by one polymerization cycle followed by exposure to 0.6 M potassium chloride to dissociate relaxing system proteins. Some experiments were also performed with actin prepared by the somewhat different procedures of Martonosi (1962) and of Drabikowski and Gergely (1964). Actin concentrations were determined spectrophotometrically, using a specific absorptivity, $E_{1cm}^{1\%} = 11.0$ at 280 nm (Houk and Ue, 1974). Rabbit skeletal myosin was prepared according to Perry (1955), and subfragment 1 by digestion with soluble papain by the procedure of Margossian and Lowey (1973), followed by precipitation with 55% ammonium sulfate. Specific absorptivities, $E_{1cm}^{1\%}$, at 280 nm of 5.3 (Perry, 1955) and 7.7 (Young et al., 1965) were used for concentration determinations. The relaxing protein complex was prepared from acetone powder residue (Spudich and Watt, 1971), and troponin was prepared by extraction with lithium chloride (Ebashi et al., 1971). In addition, the troponin–tropomyosin complex was fractionated by ion-exchange chromatography on a hydroxyapatite column, using a linear phosphate gradient, as described by Eisenberg and Kielley (1974). For concentration determinations, specific absorptivities of 3.1 (Holtzer et al., 1965) and 1.1 (van Eerd and Kawasaki, 1973) were used for tropomyosin and troponin, respectively. The preparations were free of contaminating proteins as judged by polyacrylamide gel electrophoreses in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969).

Chemical Modifications. The coupling of G-actin to a Sepharose 4B matrix was effected by the cyanogen bromide activation procedure (Porath et al., 1967) under the conditions previously described (Chantler and Gratzer, 1973). Before use the Sepharose was extracted with 2% sodium dodecyl sulfate for 15 min with stirring. Gels with "spacer arms" were prepared by way of the *N*-hydroxysuccinic imidoester of agarose (Cuatrecasas and Parikh, 1972), and a commercial activated matrix (Enzacryl Polyacetal, Koch-Light Laboratories), used as described by Epton and Thomas (1971), was also tried. Photo-oxidized actin in which one histidine and one methionine residue were destroyed was prepared according to Offer et al. (1972).

Dansyl myosin subfragment 1 was prepared by prior dialysis of the protein against 0.1 M sodium bicarbonate, adjusted to pH 8.0 (Horton and Koshland, 1967); dansyl chloride (1–2% by weight) was then added in 0.5 ml of cold acetone to 10 ml of the protein (1–2 mg/ml) at 4 °C with stirring (Weber, 1952). After 12 h, suspended material was removed in a bench-top centrifuge and the solution was dialysed for 48 h or more with several changes into the working buffer. The concentration of dansyl groups in the protein was determined spectrophotometrically (Hartley and Massey, 1956).

Stability of Product. The matrix-bound actin has, rather surprisingly, a somewhat reduced stability compared with free G-actin under the same conditions (2.5 mM Tris, 0.2 mM calcium chloride, 0.5 mM dithiothreitol, pH 7.2 at 4 °C), and over a period of days its ability to bind myosin heads is progressively lost. The decay follows first-order kinetics and parallels precisely the irreversible loss of nucleotide (experiments with γ-^{32}P-labeled actin). The half-life for binding activity is about 40 h after preparation of the matrix–protein conjugate. The gels were therefore always used within hours of preparation.

Binding Studies. The concentration of G-actin coupled to Sepharose 4B was determined by digestion with Pronase, followed by colorimetric ninhydrin analysis as previously described (Chantler and Gratzer, 1973). Analysis for actin-bound nucleotide both spectrophotometrically, and with ^{32}P-γ-labeled ATP also follows the procedure described in the same paper. The G-actin–agarose gel samples were washed free of dithiothreitol and excess nucleotide, which were normally present in the buffer, immediately before binding assays.

In measuring the amount of noncovalently bound protein in the gel matrix (whether to ensure that all unbound actin had been eliminated after the coupling reaction, or to measure the amount of myosin subfragment 1 retained by the actin) a technical point of general importance arises. We have found that when a gel is washed with 6 M guanidine hydrochloride, which, as an excellent denaturant, can be used to dissociate noncovalent interactions, varying quantities of an ultraviolet-absorbing impurity are invariably leached out. Since this has an absorption maximum at 280 nm, it interferes strongly with spectrophotometric protein determination in such eluates. The spectrum is in fact indistinguishable from that of galactose, and is due to the release of soluble material from the Sepharose matrix. This is evidently of high molecular weight, because there is a sizeable scattering background, and is continually generated with the passage of guanidine hydrochloride through the matrix. Sodium dodecyl sulfate appears to be a much more satisfactory dissociating agent. It does extract a small amount of the ultraviolet absorbing material from the Sepharose but this is sufficiently small that a satisfactory correction can be made.

The total volume available to the binding protein was determined by partition experiments in the blank matrix. The hold-up volume per volume of packed gel was determined by the elution of blue dextran (Pharmacia) from a short column. The equilibrium binding experiments with subfragment 1 were conducted mainly with dansylated protein, which has somewhat diminished ATPase activity, but is unchanged in respect of binding to F-actin (Kasuya and Takashina, 1965). Concentrations of the labeled subfragment 1 in solution were de-

termined fluorimetrically. Equilibration was found to be complete only after about 40 min, and a period of 1 h was routinely used, the sample being maintained in a thermostated water bath with fairly brisk stirring from an overhead paddle stirrer. A molar excess of subfragment 1 over actin of up to 4 was used. The subfragment 1 bound was examined after elution from the gel by polyacrylamide electrophoresis in sodium dodecyl sulfate and was indistinguishable from the sample applied. No actin was eluted. The results were expressed in terms of Scatchard plots for each temperature, from which binding constants were extracted. The binding of tropomyosin and troponin and their complex to the matrix-bound G-actin was observed in the same way, but equilibrium studies were not performed. The solvent in this case was that of Hitchcock (1975), i.e., 0.05 M sodium chloride, 0.01 M imidazole, 2 mM magnesium chloride, 0.5 mM dithiothreitol, pH 7.0, with and without calcium chloride, or EGTA (0.1 mM). The conditions for experiments involving elution of subfragment 1 by ATP-containing buffers were as previously described (Chantler and Gratzer, 1973).

ATPase Determinations. ATPase activity in free solution was measured by colorimetric determination of orthophosphate formation (Allen, 1940). In the mixture of subfragment 1 with Sepharose gels a more sensitive method proved desirable and ^{32}P-γ-labeled ATP was added to the assay solvent (0.04 M potassium chloride, 2 mM magnesium chloride, 2.5 mM Tris, 2 mM ATP, pH 7.0) to an activity of 2×10^4 dps. The labeled ATP was first passed through a column of Dowex 1 in the chloride form to remove radioactive orthophosphate. The following procedure was used, and is based on that of Lymm and Taylor (1970): 0.25- or 0.5-ml aliquots of the reaction mixture, removed at various times, were mixed with 1 ml of 2.5 N hydrochloric acid, followed by 1 ml of 0.2 M potassium phosphate, pH 7.0, as carrier, 3 ml of distilled water was next added and then the phosphomolybdate reagent (10% ammonium molybdate and 0.2 M triethanolamine, 2:1). After 2 min the precipitate was collected on a 0.45-μm Millipore filter and washed with 10 ml of a solution containing 10% ammonium molybdate, 0.2 M triethanolamine, perchloric acid, and water (2:1:1.5:2.5, v/v). The membranes were then deposited on planchettes and counted in a Nuclear-Chicago gas-flow counter.

Instrumental. Sedimentation velocities were measured with a Spinco Model E ultracentrifuge at 60 000 rpm using Schlieren optics. Fluorescence measurements were performed in a Hitachi MPF 3L spectrofluorimeter, equipped with a thermostated cell block, maintained at constant temperature (±0.1 °C). Circular dichroism was measured in a Cary 61 instrument, and proton magnetic resonance spectra were measured with a Bruker 270-MHz pulsed Fourier transform spectrometer. Protein solutions (10 mg/ml or higher) were prepared by dialysis into D_2O. Up to 14 000 pulses were accumulated and the residual water signal was eliminated by water-proton decoupling. The spectra were indexed relative to Me_4Si.

Results

Binding of Subfragment 1 to Immobilized Actin. Binding profiles were obtained by equilibration of subfragment 1 solutions with the actin-containing gels at physiological ionic strength and at a series of temperatures. The data were represented by Scatchard plots, of which a typical example is shown in Figure 1. In all cases linear plots were obtained, reflecting within the limits of experimental error, a single strong binding process. Figure 1 also shows, at high concentrations of subfragment 1, a limb of low slope, corresponding to weak,

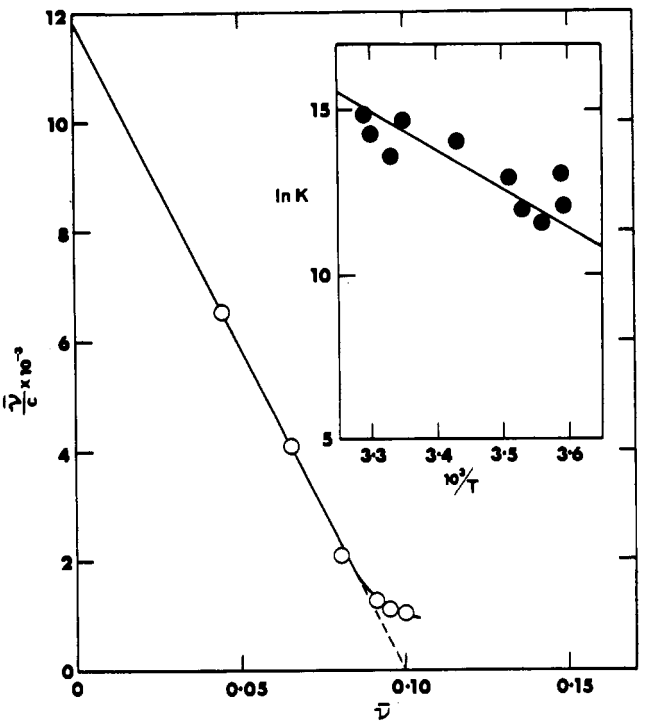


FIGURE 1: Typical Scatchard plot for binding of subfragment 1 to monomeric matrix-bound actin (temperature 16.5 °C). Inset: van't Hoff plot for binding.

possibly nonspecific binding. The strong binding process was associated with a binding constant between 1×10^5 M^{-1} at 4 °C to 2×10^6 M^{-1} at 31 °C, i.e., a standard free energy of binding between −6 and −8.5 kcal mol^{-1}. These experiments which were performed with a series of different agarose–actin preparations yielded the van't Hoff plot shown in Figure 1 (inset). The binding is an endothermic process, and assuming $\Delta C_p = 0$, we obtain a standard enthalpy of association of 24 ± 10 kcal mol^{-1}, and a corresponding entropy of 110 ± 40 eu.

Effect of Immobilized Actin on Subfragment 1 ATPase. The method of assay here used, employing radioactively labeled ATP, has allowed us to define the effect of matrix-bound actin on the ATPase activity of subfragment 1 within closer limits than achieved previously (Chantler and Gratzer, 1973). Figure 2 shows the rate of liberation of radioactive phosphate from ATP by subfragment 1 under saturating conditions, at low ionic strength, in the presence of Sepharose-bound monomeric actin and the same concentration of Sepharose alone as a control. The ATPase rate is retarded in the presence of Sepharose 4B, compared with free solution, probably because of an unstirred layer of substrate around the matrix that limits diffusional access to the enzyme, with a consequent increase in apparent Michaelis constant; this is a familiar effect in immobilized enzyme systems (Axén et al., 1970; Goldman et al., 1971). It is also clear, however, that the bound actin is responsible for a small activation of the ATPase, which never exceeds about 2. The precision of this result is probably limited by the difficulty of sampling uniform quantities of gel.

Effect of Ionic Strength on Binding and Interaction with ATP. In the absence of ATP, diminution of the ionic strength to 0.04 led to some decrease in the extent of binding. The addition of magnesium ions to a concentration of 5 mM produced no detectable effect. The dissociation of the complex of subfragment 1 and the monomeric actin by ATP was by contrast affected by ionic strength in a profound and unexpected manner. In a buffer of ionic strength in excess of 0.1 only a

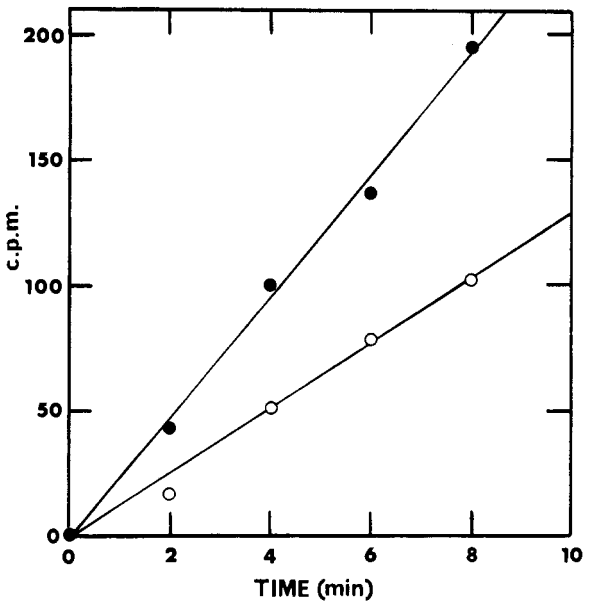


FIGURE 2: Effect of matrix-bound monomeric actin on ATPase activity of subfragment 1: rate of release of [^{32}P]orthophosphate from γ-labeled ATP under saturating conditions by subfragment 1 in the presence of Sepharose 4B containing no actin (open circles) and in the presence of a similar quantity of matrix coupled to monomeric actin (filled circles). Concentration of subfragment 1: 0.09 mg/ml. The buffer contains 2.5 mM Tris, 2 mM magnesium chloride, 2 mM ATP, pH 7.0.

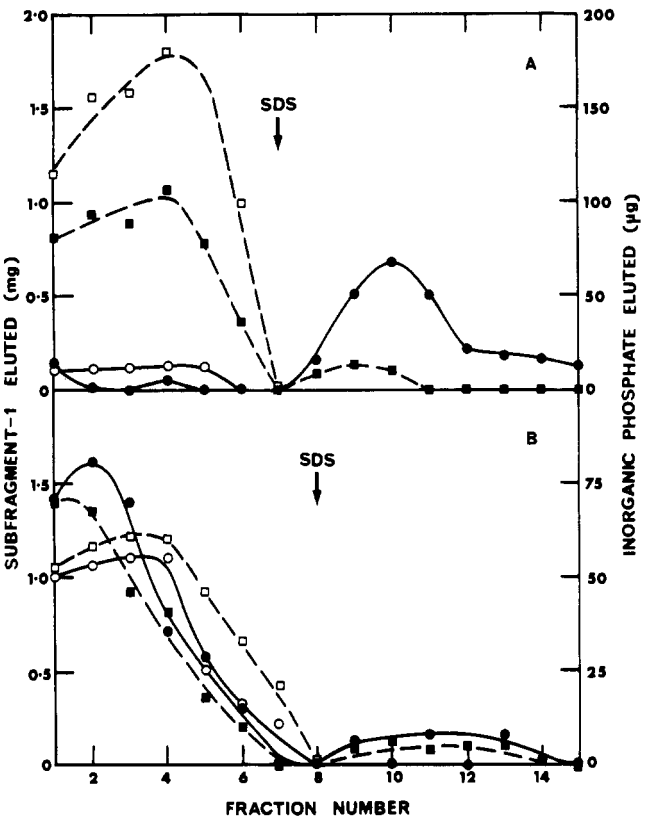


FIGURE 3: Dissociation of bound subfragment 1 from actin-agarose column by ATP and liberation of orthophosphate at low and at high ionic strength. (A) High ionic strength (2.5 mM Tris, 5 mM magnesium chloride, 5 mM ATP, 0.1 M sodium chloride, pH 7.0): elution of subfragment 1 from Sepharose–actin (●); and control of Sepharose with no bound actin (■); (left-hand ordinate), release of orthophosphate from labeled ATP by subfragment 1 in the presence of Sepharose–actin (○); and of Sepharose containing no actin (□) (right-hand ordinate). At the point indicated the eluting buffer is changed to include 1% sodium dodecyl sulfate. (B) Low ionic strength (2.5 mM Tris, 2 mM magnesium chloride, 2 mM ATP, pH 7.0). Symbols as in A.

small fraction of bound subfragment 1 was eluted from the actin column by 5 mM ATP (Figure 3a), the rest being readily dissociated by sodium dodecyl sulfate. If the ionic strength was greatly reduced by omitting the sodium chloride from the buffer, the ATP eluted the subfragment 1 quantitatively from the column (Figure 3b).

There was a parallel and completely reproducible effect on ATPase activity: significant turnover occurred only at low ionic strength. This effect is also shown in Figure 3.

Effect of Ionic Strength on Conformation of Monomeric Actin. In order to determine whether the differences in the functional properties of G- and F-actin arise from the different ionic strengths in which they exist, or from the polymerization brought about by the change in ionic strength, some spectroscopic studies were undertaken. Murphy (1971) has shown that the circular dichroism spectra of G- and F-actin are appreciably different, notably in the region of aromatic absorption, where there are small Cotton effects. We have observed the same change, and we have followed its development when G-actin is allowed to polymerize in the cell. The optical change was found to follow the development of the viscosity and is not, therefore, due to a conformational change induced by salt, which might trigger the polymerization process. The lag phase due to the formation of nuclei for polymerization (Oosawa and Kasai, 1962) could be clearly observed. Photo-oxidized actin (Martonosi and Gouvea, 1961: Offer et al., 1972), in which destruction of some histidine residues was the predominant modification, was also examined. In terms of sedimentation rate and ultraviolet absorption spectrum, this material resembled native G-actin. Preparations of this kind showed no response to increased ionic strength. The circular dichroism in the peptide absorption region gave evidence of some loss of α-helix, and therefore denaturation, but the extent of this effect varied from one preparation to another. In the best preparations by this criterion the aromatic Cotton effects resembled those of G-actin and evinced no change when salt was added, thus confirming that the change in circular dichroism on polymerization results from the association itself. An attempt was made to examine the proton magnetic resonance spectra at 270 MHz of the photo-oxidized actin at low and at high ionic strengths, and to compare these with that of G-actin. No satisfactory results could be obtained for the latter since even at the lowest workable concentrations (ca. 4 mg/ml) severe broadening of the signals occurred, indicative of concentration-dependent aggregation. The spectra of photo-oxidized actin were also not of high quality, but in the low-field region three C-2 histidine and one tyrosine ring proton signal could be observed. These were unchanged in position when salt was added, suggesting again that ionic strength has little direct effect on the conformation of G-actin.

Binding of Tropomyosin and Troponin to Immobilized Actin. The ability of monomeric matrix-bound actin to bind proteins of the relaxing system in physiological solution conditions was examined. The stoichiometric complex of troponin and tropomyosin was not retained by the actin-containing gel. Tropomyosin alone also showed no binding whatever. Troponin, however, consisting of the three species, Tn-I, Tn-N, and Tn-C, was strongly bound, both in the presence of calcium ions (0.1 mM) and in their absence (0.1 mM EGTA), the solvent conditions being those of Hitchcock (1975). The ratio of troponin bound to actin monomers was higher than the binding ratio for subfragment 1, presumably by reason of steric factors.

Effect of Coupling Arm on Binding. The effect of increasing the distance between the protein molecule and the matrix in the hope of improving steric access to the binding species was studied with two derivatives. The polyacetal derivative (Enzacryl) bound a large amount of actin, and indeed it was difficult to control the density of coupling. Moreover ATP was adsorbed by the gel, which evidently has appreciable ion-exchange capacity. No satisfactory results were obtained with this material. The succinimide–agarose derivative (Cuatrecasas and Parikh, 1972) proved more satisfactory, though a high ultraviolet background was encountered in the sodium dodecyl l sulfate eluates; this necessitated the use of Pronase, which was found to be sufficiently active in the presence of sodium dodecyl sulfate to degrade the eluted protein for colorimetric ninhydrin analysis. The fractional binding of subfragment 1 was not consistently higher than that to the derivative made by cyanogen bromide activation, and in fact the trend was toward lower binding. All equilibrium studies were, therefore, performed on the materials prepared in the conditions described, using cyanogen bromide activation of Sepharose.

Discussion

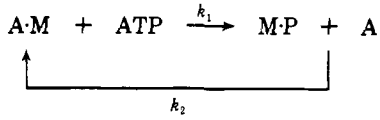
Monomeric Nature of Bound Actin. At the concentration of actin in the gels used in the foregoing experiments (i.e., about 0.6 mg per g of wet weight), calculations (Green and Toms, 1973) indicate that less than 1.5% of the bound molecules should be in a position to interact with another actin molecule. The results of Chan and Mawer (1972), working with aldolase at similar protein subunit concentrations, indeed indicate that this may be an overestimate. The direct evidence for the absence of any significant proportion of polymerized actin in the gels comprises (1) the ability of all the bound nucleotide to exchange rapidly, (2) the identity of the bound nucleotide as ATP, not ADP (Chantler and Gratzer, 1973), and (3) failure of the bound actin to bring about any important enhancement of subfragment-1 ATPase. The thermodynamic data derived for the binding process may, therefore, be taken to refer to the interaction between monomeric proteins. Very recently experiments with column-bound actin have been reported by Bottomley and Trayer (1975), which have led to conclusions differing at several points from ours, notably with regard to ATPase activation, the ionic strength dependence of dissociation of the complexes by ATP, and the ability of the matrix-bound actin to interact with tropomyosin. The primary difference between our experiments and those of Bottomley and Trayer is that in theirs the concentration of actin, both in solution during coupling and on the matrix, and also the degree of activation of the Sepharose were more than an order of magnitude greater. Calculations (Green and Toms, 1973) predict about 25% self-association of bound actin under these conditions. The above criteria for the monomeric nature of the bound actin, other than the ATPase activity, were not examined, and a more precise analysis of the source of the differences is not therefore possible.

Thermodynamic Characteristics of Binding of Subfragment 1. The binding constants for subfragment 1, which may be regarded as the intrinsic association constant for the actomyosin system, are in the range 10^5 to 10^6 M^{-1}, and stand in marked contrast to the low ATPase activation. Measurements of binding of subfragment 1 to F-actin (Young, 1967; Margossian and Lowey, 1973; Marston and Weber, 1975) place the binding constant in this range. The binding process is an entropy-driven reaction, dependent presumably on the displacement of water from hydrophobic contact areas, as observed in many other protein–protein systems (see e.g., Lauffer, 1971). A contact site involving ten or so aliphatic side chains (Nozaki and Tanford, 1971) would be compatible with the 100 eu characterizing the binding reaction. If both heads in myosin are functionally identical in this respect, then the free energy of binding of myosin to F-actin will be given by

$$\Delta F^\circ_M = 2\Delta F^\circ_H - RT \ln 55.5$$

if there are no steric constraints on the binding of the second myosin head when the first has bound to another monomer in the actin strand, and if at the same time one can neglect entropy effects, other than translational. (The subscript M refers to myosin, H to a single head (subfragment 1), and the second term on the right represents the unitary entropy resulting from the immobilization of the second head.) At 25 °C this leads to a binding constant for myosin to F-actin of no less than 1.6×10^{14} M^{-1}.

ATPase Activation. Our findings on ATPase activation are entirely consistent with the results of Offer et al. (1972), whose work on G-actin and photo-oxidized actin appears to leave no room for doubt that the capacity of these monomeric species to activate myosin ATPase is small. Their value for activation by G-actin amounts to a factor of less than five, extrapolated to infinite actin concentration. The results reported by Bottomley and Trayer (1975) are in conflict with these observations. The G-actin–subfragment-1 system responds quite differently from actomyosin to changes in ionic strength. The scheme of ATPase activation put forward by Taylor and his co-workers (see Taylor, 1972, 1973) may be written in a greatly simplified form, thus:



A represents F-actin, M myosin and P the products, ADP and orthophosphate, the intermediate rate constants being subsumed in k_1 and k_2. In the steady state the ratio of [M·P] to [A·M] is then given by $k_1[ATP]/k_2[A]$. The values obtained for k_1 and k_2 (H. White, personal communication) are 10^6 and 10^4 M^{-1} s^{-1}, respectively, at ionic strength 0.04. Thus the system is always predominantly dissociated in the presence of ATP, but the degree of association is higher at the lower ionic strength. Whereas the effect of actin on the ATPase reaction is predominantly to promote the isomerization of the myosin-product complex to a rapidly dissociable state (Bagshaw and Trentham, 1974), it appears (J. Sleep and H. White, personal communication) that other steps in the reaction scheme are also perturbed by F-actin. It therefore seems likely, considering especially the anomalous ionic strength dependence in our system, that the small activation of ATPase may be adventitious, and unrelated to that elicited by F-actin.

The question arises, however, whether a small activation of ATPase by monomeric actin may in fact be sufficient to explain the large activation engendered by F-actin, by reason of the large size of the actomyosin domain; this might be envisaged as accelerating the rate of reaction in much the same way as that between reactants in a micelle or within the double-layer of a polyelectrolyte (Morawetz, 1970). We have calculated from the diffusion coefficient of subfragment 1 and an estimated domain size for F-actin, derived in turn from its sedimentation coefficient (Kikuchi et al., 1974), the rate of diffusional escape of subfragment 1 from such a domain (see e.g., Alberty and Hammes, 1958). Neglecting electrostatic

contributions, the resulting first-order rate constant is of the order of 10^4 s^{-1}. Since the maximum pseudo-first-order rate constant for actomyosin ATPase is about 10 s^{-1}, the small enhancement of activity by monomeric actin cannot account for the extent of activation observed in actomyosin.

Thus the activation mechanism of actin does not operate when the protein is in the monomeric state, even in physiological solution conditions, and despite the fact that the binding affinity for myosin heads is fully developed. Dimers of actin, formed by chemical cross-linking, have been found similarly ineffective in enhancing myosin ATPase activity (Knight, 1976). The result is compatible with evidence (Bárány and Bárány, 1959; Perry and Cotterill, 1964) that separate sites on the myosin heads are responsible for the primary binding to, and ATPase activation by, F-actin, and similarly that myosin-binding and ATPase activation are separable functions on the F-actin molecules (Mühlrad et al., 1968; Chantler and Gratzer, 1975). It seems likely, therefore, that the explanation for the failure of monomeric actin to activate the ATPase lies in the distribution of the primary binding and the ATPase activation functions between separate actin monomers (which may be on opposite strands of the helix).

Interaction of Troponin and Tropomyosin with Monomeric Actin. The strong interaction of troponin with the monomeric actin is of some interest and, presumably, signifies that a 1:1 interaction of troponin I (Ohtsuki et al., 1967; Greaser and Gergely, 1971) with the actin monomers is the determinant of the binding process, and that the site is preserved in the disaggregated actin. Troponin binds whether or not calcium ions are present, as it does to F-actin (Potter and Gergely, 1974; Hitchcock, 1975). The failure of tropomyosin to bind the actin monomer is unsurprising, in view of the evidence that its interaction with F-actin involves a run of seven consecutive monomers (Hitchcock et al., 1973). The failure of the stoichiometric tropomyosin–troponin complex (Spudich and Watt, 1971) to bind to actin monomers is less expected, and must probably be explained in conformational terms. Since there is now strong evidence that the actin filaments undergo geometrical changes in the course of muscular contraction in situ (Vibert et al., 1972; Wakabayashi et al., 1975), and indeed in isolation (Loscalzo et al., 1975), it is reasonable to infer that one or more conformational states, required both for activation of the myosin ATPase and for attachment of the quaternary complex of relaxing proteins, are inaccessible to the monomeric species.

The circular dichroism changes, which we have observed to be a consequence of polymerization, parallel the aromatic difference spectra described by Higashi and Oosawa (1965). The circular dichroism effect is sufficiently extensive to suggest that it corresponds to conformational changes, rather than merely interactions of aromatic residues at the subunit interfaces. This indicates in turn the existence of linked equilibria, whereby polymerization traps a conformational form, not observed in the monomer. It is possible that this conformational adaptability is a component in the action of thin filaments in muscle.

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References

Alberty, R. A., and Hammes, G. G. (1958), *J. Phys. Chem. 62,* 154–159.

Allen, R. J. L. (1940), *Biochem. J. 34,* 858–865.

Axén, R., Myrin, P. A., and Janson, J. C. (1970), *Biopolymers 9,* 401–413.

Bagshaw, C. R., and Trentham, D. R. (1974), *Biochem. J. 141,* 331–348.

Bárány, M., and Bárány, K. (1959), *Biochim. Biophys. Acta 35,* 293–309.

Bottomley, R. C., and Trayer, I. P. (1975), *Biochem. J. 149,* 365–379.

Chantler, P. D., and Gratzer, W. B. (1973), *FEBS Lett. 34,* 10–14.

Chantler, P. D., and Gratzer, W. B. (1975), *Eur. J. Biochem. 60,* 67–72.

Chan, W. W. C., and Mawer, H. (1972), *Arch. Biochem. 149,* 136–145.

Cuatrecasas, P., and Parikh, I. (1972), *Biochemistry 11,* 2291–2299.

Drabikowski, W., and Gergely, J. (1964), in Biochemistry of Muscular Contraction, Gergely, J., Ed., Boston, Mass., Brown Little, p 125.

Ebashi, S., Wakabayashi, T., and Ebashi, F. (1971), *J. Biochem. (Tokyo) 69,* 441–445.

Eisenberg, E., and Kielley, W. W. (1974), *J. Biol. Chem. 249,* 4742–4748.

Epton, R., and Thomas, T. H. (1971), *Aldrichimica Acta 4,* 61–65.

Goldman, R., Kedem, O., and Katchalski, E. (1971), *Biochemistry 10,* 165–172.

Greaser, M. L., and Gergely, J. (1971), *J. Biol. Chem. 246,* 4226–4233.

Green, N. M., and Toms, E. J. (1973), *Biochem. J. 133,* 687–700.

Hartley, B. S., and Massey, V. (1956), *Biochim. Biophys. Acta 21,* 58–70.

Higashi, S., and Oosawa, F. (1965), *J. Mol. Biol. 12,* 843–865.

Hitchcock, S. E. (1975), *Eur. J. Biochem. 52,* 255–263.

Hitchcock, S. E., Huxley, H. E., and Szent-Györgyi (1973), *J. Mol. Biol. 80,* 825–836.

Holtzer, A., Clark, R. J., and Lowey, S. (1965), *Biochemistry 4,* 2401–2411.

Horton, H. R., and Koshland, D. E. (1967), *Methods Enzymol. 11,* 856–880.

Houk, T. S., and Ue, K. (1974), *Anal. Biochem. 62,* 66–74.

Kasuya, M., and Takashina, H. (1965), *Biochim. Biophys. Acta 99,* 452–463.

Kikuchi, M., Maruyama, K., and Noda, H. (1974), *J. Biochem. (Tokyo) 75,* 1103–1111.

Knight, P. J. (1976), Ph.D. Thesis, London University.

Lauffer, M. (1971), in Subunits in Biological Systems, Timasheff, S. N., Fasman, G. D., Ed., New York, N.Y., Marcel Dekker, pp 149–199.

Loscalzo, J., Reed, G. H., and Weber, A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3412–3415.

Lymm, R. W., and Taylor, E. W. (1970), *Biochemistry 9,* 2975–2983.

Margossian, S. S., and Lowey, S. (1973), *J. Mol. Biol. 74,* 313–330.

Marston, S., and Weber, A. (1975), *Biochemistry 14,* 3868–3873.

Martonosi, A. (1962), *J. Biol. Chem. 237,* 2795–2803.

Martonosi, A., and Gouvea, M. N. (1961), *J. Biol. Chem. 236,* 1338–1344.
Morawetz, H. (1970), *Acc. Chem. Res. 3,* 354–360.
Mühlrad, A., Corsi, A., and Granata, A. L. (1968), *Biochim. Biophys. Acta 162,* 435–443.
Murphy, Y. (1971), *Biochemistry 10,* 3723–3728.
Nozaki, Y., and Tanford, C. (1971), *J. Biol. Chem. 246,* 2211–2217.
Offer, G., Baker, H., and Baker, L. (1972), *J. Mol. Biol. 66,* 435–444.
Ohtsuki, I., Masaki, T., Nonomura, Y., and Ebashi, S. (1967), *J. Biochem. (Tokyo) 61,* 817–819.
Oosawa, F., and Kasai, M. (1962), *J. Mol. Biol. 4,* 10–21.
Perry, S. V. (1955), *Methods Enzymol. 2,* 582–588.
Perry, S. V., and Cotterill, J. (1964), *Biochem. J. 92,* 603–608.
Porath, J., Axén, R., and Ernback, R. (1967), *Nature (London) 215,* 1491–1492.
Potter, J. D., and Gergely, J. (1974), *Biochemistry 13,* 2697–2703.
Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246,* 4866–4871.
Stone, D. B., Prevost, S. C., and Botts, J. (1970), *Biochemistry 9,* 3937–3947.
Taylor, E. W. (1972), *Ann. Rev. Biochem. 41,* 577–616.
Taylor, E. W. (1973), *Curr. Top. Bioenerg. 5,* 201–231.
van Eerd, J. P., and Kawasaki, Y. (1973), *Biochemistry 12,* 4972–4980.
Vibert, P. J., Haselgrove, J. C., Lowy, J., and Poulsen, F. R. (1972), *J. Mol. Biol. 71,* 757–767.
Wakabayashi, T., Huxley, H. E., Amos, L. A., and Klug, A. (1975), *J. Mol. Biol. 93,* 477–497.
Weber, G. (1952), *Biochem. J. 51,* 155–167.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Young, M. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 2393–2400.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem. 240,* 2428–2436.

Infrared Spectroscopic Studies of Carbonyl Horseradish Peroxidases†

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ABSTRACT: Infrared difference spectra, $Fe^{II}CO$ vs. Fe^{III}, of horseradish peroxidase isoenzymes A2 and C were recorded from 2000 to 1800 cm^{-1}. Under alkaline conditions, pH 9, both isoenzymes exhibit two CO stretching bands, at 1938 and 1925 cm^{-1} for A2 and at 1933 and 1929 cm^{-1} for C. As the pH is lowered the low-frequency band for each isoenzyme decreases in intensity with a concommitant appearance and increase in intensity of a band at 1906 and 1905 cm^{-1} for the A2 and C isoenzymes, respectively. These changes conform to pK values of 6.7 for the A2 and 8.8 for the C isoenzymes of horseradish peroxidase. The interpretation of the infrared results was simplified by the observation that a linear relationship exists between the redox potential, E_{m7}, for the Fe^{III}/Fe^{II} system vs. the infrared CO stretching frequency, ν_{CO}, for cytchrome a_3, hemoglobin, myoglobin, and cytochrome P-450 cam with substrate. This relationship suggests that the primary force altering ν_{CO} in these heme proteins is a variation in electron density at the heme iron and not direct protein interactions with the CO ligand. The horseradish peroxidase infrared bands in the 1930-cm^{-1} region correlate well with this relationship. The large deviation of the 1905-cm^{-1} band from the linear relationship and its dependence upon hydrogen ion concentration are consistent with horseradish peroxidase having a single CO binding site which can hold in two geometries, one of which contains an amino acid moiety capable of forming a hydrogen bond to the carbonyl oxygen.

The infrared spectra of most carbonyl heme proteins exhibit a single band due to the C–O stretching mode of CO bound to the heme iron, and only in a few instances have more than one band been detected. Blood from some animal species shows more than one band because of the presence of hemoglobin variants with deviant binding sites (Barlow et al., 1973). Homogeneous preparations of myoglobin from various species yielded spectra with a main band at 1944 cm^{-1} and a shoulder at 1935 cm^{-1}, which led to the assumption of a site allowing two geometries for CO binding (McCoy and Caughey, 1971). Recent reports of multiple CO stretching bands for the CO compounds of horseradish peroxidase (1933 and 1905 cm^{-1}) (Alben and Bare, 1973) suggest a site capable of binding CO in more than one manner. To explore this possibility we have compared the infrared spectra of the CO compounds of two horseradish isoperoxidases and studied in detail the effects of variations in pH.

Materials and Methods

The acid type HRP[1] A2, pH 3.9, was isolated as described by Paul and Stigbrand (1970) and Marklund et al. (1974). It gave A_{403}/A_{280} = 4.08 and was homogeneous in polyacryl-

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[1] Abbreviations used are: HRP, horseradish peroxidase; Hb, hemoglobin; Mb, myoglobin; ir, infrared; E_{m7}, midpoint potential at pH 7; ν_{CO}, wavenumber in cm^{-1} for the CO stretching absorption band; $\Delta\nu_{CO}$, the half-bandwidth of this band.