Nagel, R. L., Ranney, H. M., and Kucinskis, L. L. (1966), *Biochemistry 5*, 1934.
Nozaki, K., and Tanford, C. (1967), *J. Biol. Chem. 242*, 4731.
Orttung, W. H. (1965), *J. Amer. Chem. Soc. 87*, 924.
Orttung, W. H. (1968a), *J. Phys. Chem. 72*, 4058.
Orttung, W. H. (1968b), *J. Phys. Chem. 72*, 4066.
Orttung, W. H. (1968c), Document No. 10070, ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington, D. C. See Orttung (1968b), for ordering information.
Orttung, W. H. (1969a), *J. Amer. Chem. Soc. 91*, 162.
Orttung, W. H. (1969b), *J. Phys. Chem. 73*, 418.
Orttung, W. H. (1969c), *J. Phys. Chem. 73*, 2908
Perutz, M. F. (1965), *J. Mol. Biol. 13*, 646.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature 219 (London)*, 131.
Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., and Kilmartin, J. V. (1969), *Nature (London) 222*, 1240.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem. 19*, 73.
Scatchard, G., and Pigliacampi, J. (1962), *J. Amer. Chem. Soc. 84*, 127.
Takashima, S. (1965), *J. Phys. Chem. 69*, 2281.
Tanford, C. (1957), *J. Amer. Chem. Soc. 79*, 5340.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Tanford, C., and Kirkwood, J. G. (1957), *J. Amer. Chem. Soc. 79*, 5333.
Tanford, C., and Nozaki, Y. (1966), *J. Biol. Chem. 241*, 2832.
Wyman, J., Jr. (1964), *Advan. Protein Chem. 19*, 223.
Wyman, J., and Ingalls, E. N. (1941), *J. Biol. Chem. 139*, 877.
Yip, Y. K., and Bucci, E. (1968), *J. Biol. Chem. 243*, 5948.

Actin–Heavy Meromyosin Binding. Determination of Binding Stoichiometry from Adenosine Triphosphatase Kinetic Measurements*

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ABSTRACT: Enzyme kinetic measurements of the effects of actin on the heavy meromyosin adenosine triphosphatase (ATPase) are used for a study of the binding of heavy meromyosin to actin. In the absence of free magnesium ions, the potassium-activated ATPase of heavy meromyosin is inhibited by actin, and this inhibition is used as a quantitative measure of binding. The activation of the heavy meromyosin ATPase by actin in the presence of magnesium ions is also used as a measure of binding. Both kinds of data, when analyzed according to the theory of multiple equilibria, give simple binding curves with a binding stoichiometry of 1 mole of heavy meromyosin/mole of monomers in F-actin.

It is generally accepted that the molecular basis for the contraction of muscle is an interaction between the two major contractile proteins, actin and myosin (Szent-Györgyi, 1951; Huxley, 1960, 1969); however, there is still considerable question about one of the most fundamental features of the actin–myosin interaction, namely, the stoichiometric binding ratio of the actin to the myosin. The number of monomers in F-actin which interact with each myosin molecule is of particular interest because myosin probably contains two active subunits (Stracher and Dreizen, 1966; Slayter and Lowey, 1967; Lowey *et al.*, 1969; Nauss *et al.*, 1969) which, when separated from the molecule as subfragment 1, are both capable of binding actin (Mueller and Perry, 1962; Young, 1967). The actin monomers, on the other hand, are single polypeptide chains (Rees and Young, 1967). On this basis, one might expect that each myosin molecule would have two binding sites for actin and would combine with two monomers in the F-actin filament.

Unfortunately, it is difficult to investigate the actin–myosin binding directly under physiological conditions because myosin aggregates at low ionic strength, but experiments can be carried out at high ionic strength where myosin is disaggregated, or using heavy meromyosin, an early proteolytic digestion product of myosin which retains the actin binding and ATPase-active region of the myosin intact but does not aggregate at low ionic strength (Szent-Györgyi, 1953). On the basis of electron microscope observations of the actin–heavy meromyosin complex, Huxley (1963) concluded that each actin monomer could bind one molecule of heavy meromyosin. However, while some studies of actin–

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heavy meromyosin binding in the ultracentrifuge have supported this conclusion (Young, 1967; Tawada, 1969), there have been several light-scattering studies with myosin at high salt and with heavy meromyosin (Gergely and Kohler, 1958; Ikkai and Ooi, 1969; Tonomura *et al.*, 1962; Finlayson *et al.*, 1969; Sekiya *et al.*, 1967) which have suggested a binding ratio of one myosin or heavy meromyosin molecule per two actin monomers. The latter measurements involve the assumption that the light-scattering intensity is a valid linear measure of the amount of complex coexisting with the dissociated proteins in an equilibrium mixture, but on the other hand, the ultracentrifuge methods may be subject to various technical difficulties and also to uncertainties due to the effects of pressure (Ikkai and Ooi, 1966, 1969).

We report here a binding study based on a rather different approach. In connection with our studies on the ATPase kinetics of the acto–heavy meromyosin system, it appeared to us that the extent of actin–heavy meromyosin binding might be inferred from the effect of actin on the heavy meromyosin ATPase. Although this approach also has its share of assumptions and uncertainties, these are, in the main, different from those involved in the physicochemical work, and furthermore, these measurements have the advantage of being made in the presence of ATP. The actin–heavy meromyosin binding is calculated from ATPase measurements over a range of free heavy meromyosin concentrations, and a limiting binding stoichiometry is obtained by extrapolation according to the theory of multiple equilibria (Scatchard, 1949). Our first experiments are based on the finding that actin inhibits the potassium-activated ATPase of myosin in the absence of free Mg ions (Barron *et al.*, 1966); for the binding study, we use the inhibition of the heavy meromyosin ATPase by actin as a measure of the amount of heavy meromyosin bound to actin. In the second group of experiments, on the other hand, the more familiar actin-activation of the heavy meromyosin ATPase in the presence of Mg serves as the measure of binding. In both cases, a stoichiometry near 1 mole of heavy meromyosin/mole of actin monomers is obtained.

Methods

Actin and heavy meromyosin were prepared from rabbit skeletal muscle and their concentrations measured according to the customary procedures of this laboratory (Eisenberg and Moos, 1967, 1968), except that the salt solution used for washing the F-actin was altered as specified below for the two groups of experiments. In both cases, the final F-actin solution was prepared by dispersing the washed pellets, with the aid of brief sonication, in the specified solvent with about 0.1 mM added ATP, and then removing the free nucleotides by two treatments with Dowex 1-X4 anion-exchange resin. The actin and heavy meromyosin were always used within a week after preparation. The ATPase rates were measured with an automatic pH-Stat at pH 7, at an initial ATP concentration of 2 mM, and the relation between the pH-Stat record and the amount of ATP hydrolyzed was determined from the amount of titrant used in the complete hydrolysis of the known total amount of ATP under the conditions of the experiment.

The binding calculations depend crucially on the molecular weights of the proteins, and the values we have used are 46,000 for actin and 350,000 for heavy meromyosin. Although higher molecular weights for actin have been used in the past, values near 46,000 have been obtained recently by both physical (Rees and Young, 1967) and chemical (Tsuboi, 1968; Johnson *et al.*, 1967) methods, and consistent with this value, our actin preparations always contain 20–22 μmoles of bound nucleotide/g of protein. The molecular weight for heavy meromyosin is less firmly established; we have selected 350,000 (Mueller, 1964) because this value was obtained with heavy meromyosin prepared by approximately the same digestion procedure we have used. Our heavy meromyosin preparation showed no flow birefringence at low ionic strength when viewed in a hand polariscope, indicating no contamination with myosin or light meromyosin, and on Sephadex G-200 chromatography, it moved as a single peak with no sign of smaller fragments such as might be produced by overdigestion with trypsin.

Results

Actin Inhibition of the Heavy Meromyosin ATPase in the Absence of Free Mg^{2+}. In the first group of experiments, estimates of heavy meromyosin–actin binding are derived from measurements of the actin inhibition of the heavy meromyosin ATPase in the absence of free Mg. In order to reduce the free Mg concentration in the final reaction mixtures to a sufficiently low level for these experiments, the solvent used in the actin preparation was 0.1 M KCl–10 mM imidazole buffer (pH 7) with no added Mg salts, and the Mg-chelating agent 1,2-diaminocyclohexanetetraacetic acid was added to the experimental mixtures.

In Figure 1, we show that the heavy meromyosin ATPase is indeed inhibited by actin in the absence of Mg^{2+}, just as was reported previously for myosin (Barron *et al.*, 1966). At high actin concentrations, nearly 90% inhibition of the heavy meromyosin ATPase was achieved. However, there does appear to be a residual ATPase activity which is not abolished even at the highest actin concentration we have used. This residual activity does not appear to be due to free magnesium ions remaining in the system because, as shown in Figure 1b, it is observed even at an ionic strength above 0.3, and is abolished by substitution of sodium for potassium ions in the mixture (cross in Figure 1b), neither of which would be expected for a magnesium-activated acto–heavy meromyosin ATPase. Apparently the acto–heavy meromyosin complex has a small but finite potassium-activated ATPase activity in the absence of free magnesium ions.

In order to use the inhibition of the heavy meromyosin ATPase by actin for a quantitative study of actin–heavy meromyosin binding, we make the assumption that the extent of inhibition is a linear measure of the fraction of the heavy meromyosin which is bound to actin. A similar approach was used by Straus and Goldstein (1943) in determining the number of binding sites for a reversible inhibitor on an enzyme, and only a slight modification is required in our case to take account of the residual ATPase activity which apparently remains when all the heavy meromyosin is bound to actin. We assume that the actin-bound heavy meromyosin exhibits an ATPase activity per mole equal to V_i, the minimum activity measured at high actin concentration in Figure 1a, and that the free heavy meromyosin exhibits an ATPase activity equal to V_0, the measured ATPase

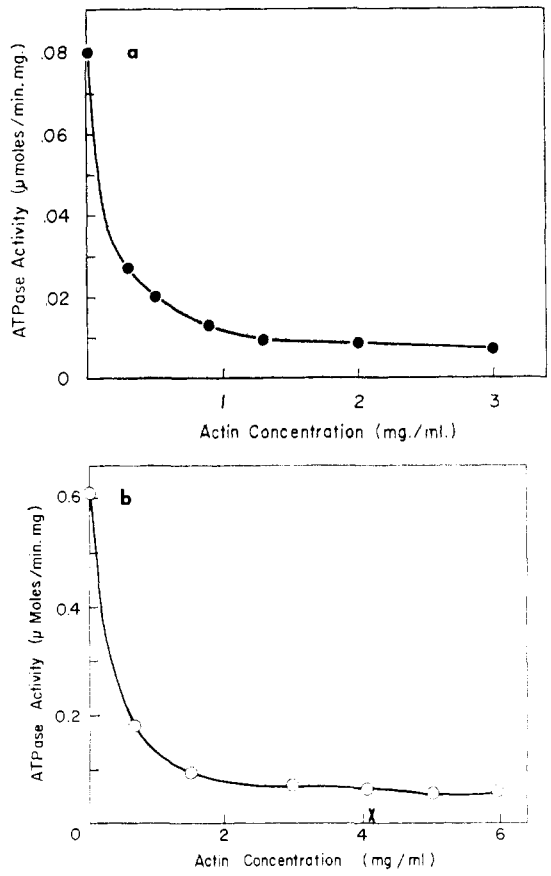

FIGURE 1: Inhibition of heavy meromyosin ATPase by actin. All samples contained 2 mM ATP, 5 mM 1,2-diaminocyclohexanetetraacetate, 10 mM imidazole buffer (pH 7), and 1 mM ethylene glycol bis(aminoethyl ether)tetraacetate. (The latter was not required for this work, but was added for reasons related to another project for which these data were to be used.) Temperature: 25°. (a) 0.12 M KCl, 1.5 mg of heavy meromyosin/ml. (b) ○, 0.3 M KCl; ×, 0.05 M KCl plus 0.25 M NaCl; heavy meromyosin concentration varied between 0.4 and 1.2 mg per ml.

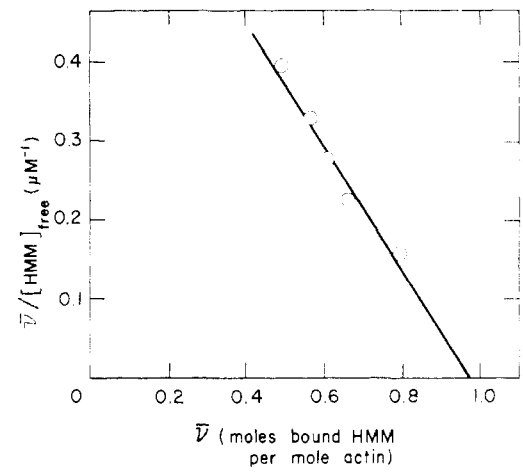

FIGURE 2: Scatchard plot of actin–heavy meromyosin binding, from actin inhibition of heavy meromyosin ATPase under conditions of Figure 1a. Actin concentration constant at 0.3 mg/ml; heavy meromyosin concentration varied between 1.5 and 3.5 mg per ml.

activity per mole of heavy meromyosin in the absence of actin. On this basis, if the observed ATPase rate per mole of total heavy meromyosin (HMM) is called v, we can write

$$v[\text{HMM}]_{\text{total}} = V_0[\text{HMM}]_{\text{free}} + V_i[\text{HMM}]_{\text{bound}} \quad (1)$$

Combining this with the statement of conservation of heavy meromyosin

$$[\text{HMM}]_{\text{total}} = [\text{HMM}]_{\text{free}} + [\text{HMM}]_{\text{bound}} \quad (2)$$

we can obtain the relation:

$$[\text{HMM}]_{\text{bound}} = [\text{HMM}]_{\text{total}} \frac{V_0 - v}{V_0 - V_i} \quad (3)$$

By means of eq 2 and 3, we can use the measured ATPase activities, v, V_0, and V_i, to derive values for both the free and actin-bound heavy meromyosin concentrations in the presence of a given total concentration of F-actin. These results are then treated according to the theory of multiple equilibria (Scatchard, 1949) to obtain a binding stoichiometry. This theory predicts that, if all of the heavy meromyosin binding sites on the F-actin are identical and independent

$$\frac{\bar{\nu}}{[\text{HMM}]_{\text{free}}} = \frac{1}{k}(n - \bar{\nu}) \quad (4)$$

where $\bar{\nu}$ is the number of moles of actin-bound heavy meromyosin per mole of monomer in the total F-actin added; k is the intrinsic dissociation constant of the actin–heavy meromyosin complex; and n is the maximum value of $\bar{\nu}$ at infinite heavy meromyosin concentration, *i.e.*, the stoichiometric binding ratio of heavy meromyosin to actin. According to eq 4, a Scatchard plot of $\bar{\nu}/[\text{HMM}]_{\text{free}}$ *vs.* $\bar{\nu}$ should be linear if the above assumptions are valid, with a slope equal in magnitude to $1/k$ and an intercept on the abscissa equal to n.

For our binding experiments, various amounts of heavy meromyosin were added to a fixed concentration of F-actin, and from the ATPase activity of each mixture, the concentrations of free and bound heavy meromyosin were calculated as described and plotted according to eq 4. Figure 2 shows a typical example of such a plot, and it does indeed appear to be linear. The values of k and n derived from the slope and abscissa-intercept of this plot are shown in Table I, together with those obtained in two other such experiments using different preparations of both actin and heavy meromyosin. The values obtained by Young (1967) from ultracentrifuge measurements in the absence of ATP are also given for comparison. In all three of our experiments, the abscissa-intercept, n, was near unity, indicating that one molecule of heavy meromyosin can bind to each monomer in F-actin. If each heavy meromyosin molecule bound to two actin monomers, the intercept on the abscissa of our plots would be 0.5, which does not appear to be compatible with the data; in fact, for most of the experimental points (see Figure 2, for example) the total amount of actin present would not be sufficient to account for the observed inhibition if the binding ratio were 1 mole of heavy meromyosin/2 moles of actin monomer.

TABLE I: Stoichiometry, n, and Intrinsic Dissociation Constant, k, of the Acto–Heavy Meromyosin Complex.[a]

Expt	n (Moles of Heavy Meromyosin/Mole of Actin)	k (μM)
1	1.0	0.8
2	1.1	2.1
3	0.9	1.4
Young (1967)	1.1	2.3

[a] Conditions for our experiments were as described in Figure 2. Young's experiments were carried out in the absence of ATP but in the presence of 0.4 mM $MgCl_2$, at a temperature of 6–8°, with 0.04 M KCl.

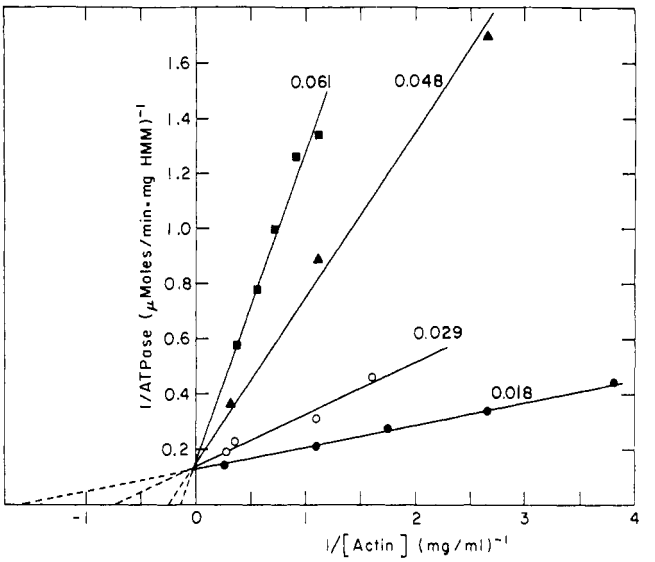

FIGURE 3: Effect of ionic strength on acto–heavy meromyosin ATPase system. The total ionic strength is indicated on each line. Samples contained 2 mM ATP, 2.4 mM (○, ●, ▲) or 1.0 mM (■) $MgCl_2$, 10 mM imidazole-HCl buffer (pH 7), and KCl to make up the balance of the ionic strength. Heavy meromyosin concentration was 0.04 mg/ml for ○ and ●, and 0.08 mg/ml for ▲ and ■. Temperature 25°.

A further point to note is that the one-to-one binding of heavy meromyosin to actin in our experiments leads to nearly complete inhibition of the heavy meromyosin ATPase (Figure 1), which implies that, if heavy meromyosin has two ATPase sites (Schliselfeld and Bárány, 1968; Lowey and Luck, 1969; Nauss *et al.*, 1969; Eisenberg *et al.*, 1969), both these sites are somehow affected by the binding of only one actin monomer.

Actin Activation of the Heavy Meromyosin ATPase in the Presence of Mg^{2+}. In relation to the problem of muscle contraction, the foregoing study has the drawback that it is carried out in the absence of Mg^{2+}, so we felt it would be of interest to use our ATPase kinetic approach to estimate the actin–heavy meromyosin binding ratio in the presence of Mg and ATP where actin activates the heavy meromyosin ATPase as it does *in vivo*. For this purpose, it was necessary to find conditions where conveniently measurable ATPase rates are obtained at concentrations of heavy meromyosin and actin which are suitable for a binding study. The conditions of our earlier study of acto–heavy meromyosin kinetics (Eisenberg and Moos, 1968) were not satisfactory because the effective affinity of actin for the heavy meromyosin–ATP complex was somewhat too weak and the maximum ATPase activity of the acto–heavy meromyosin complex was much too high. However, we have found that these difficulties can be overcome by working at very low ionic strength to increase the affinity of actin for the heavy meromyosin–ATP complex and at low temperature to reduce the ATPase activity. In order to achieve a sufficiently low ionic strength in the final reaction mixtures in these experiments, the solvent used for the actin preparation was 3 mM $MgCl_2$ containing 10 mM imidazole (pH 7) with no added KCl.

The effect of ionic strength on the acto–heavy meromyosin ATPase system is illustrated by Figure 3, which shows double-reciprocal plots of ATPase rate against actin concentration at a series of different ionic strengths. The linearity of the plots indicates that the simple kinetic analysis used previously is applicable down to ionic strengths below 0.02. Furthermore, it is clear that, in agreement with our earlier results (Eisenberg and Moos, 1968) and those of Tawada and Oosawa (1969), and in contrast to those of Szentkiralyi and Oplatka (1969), the apparent affinity of actin for the heavy meromyosin–ATP complex increases steadily with decreasing ionic strength while there is no change in the maximum ATPase of the acto–heavy meromyosin complex. Qualitatively, the observed effect of ionic strength on the apparent affinity is consistent with the view that electrostatic interactions are predominantly involved in the actin–heavy meromyosin binding.

The effect of temperature is shown in Figure 4. It can be seen that at 6° the measured ATPase rates, as well as the extrapolated rate at infinite actin concentration, are roughly 40-fold lower than those at 25° (note the difference in the ordinate scales of Figure 4a,b), while the apparent affinity of actin for the heavy meromyosin–ATP complex seems to be somewhat strengthened at the lower temperature. This large temperature coefficient of the acto–heavy meromyosin ATPase rate is reminiscent of the behavior of actomyosin (Levy *et al.*, 1959; Bárány, 1967) and of myosin modified by *p*-hydroxymercuribenzoate or dinitrophenol (Levy *et al.*, 1962). Since we are dealing here with the extrapolated acto–heavy meromyosin ATPase rate at infinite actin concentration, our temperature coefficient cannot be affected by dissociation of the acto–heavy meromyosin. Hence, by analogy, the large temperature coefficient of actomyosin ATPase as compared with the temperature coefficient of the myofibrillar ATPase or the speed of muscle contraction is probably not simply due to actomyosin dissociation as was proposed by Bárány (1967). Actually, as noted above, lower temperature does not seem to promote dissociation of acto–heavy meromyosin. Of course detailed conclusions cannot be drawn from data at only two temperatures, and further work on the effects of temperature on the acto–heavy meromyosin ATPase system is currently in progress.

The reduced ATPase activity at low temperature, together with the increased actin–heavy meromyosin affinity at low

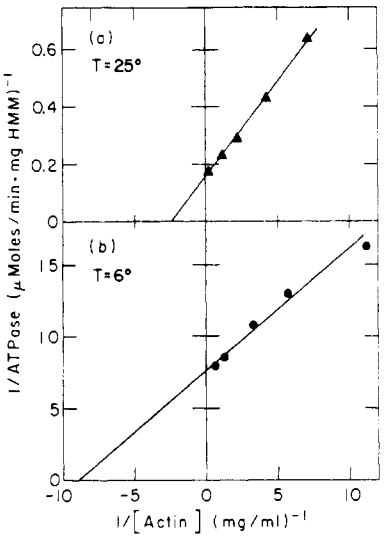

FIGURE 4: Effect of temperature on acto-heavy meromyosin ATPase system. Samples contained 2.0 mM ATP, 2.4 mM $MgCl_2$, 7 mM imidazole-HCl buffer (pH 7), and 2.3 mM KCl, for a total ionic strength of 0.017. (a) Temperature, 25°; heavy meromyosin concentration, 0.04 mg/ml. (b) Temperature, 6°; heavy meromyosin concentration, 0.96 mg/ml. The actin concentration in part b was corrected by subtracting the amount of bound actin, calculated from the measured ATPase rates assuming a one-to-one molar binding ratio of actin to heavy meromyosin. This correction is negligible in part a.

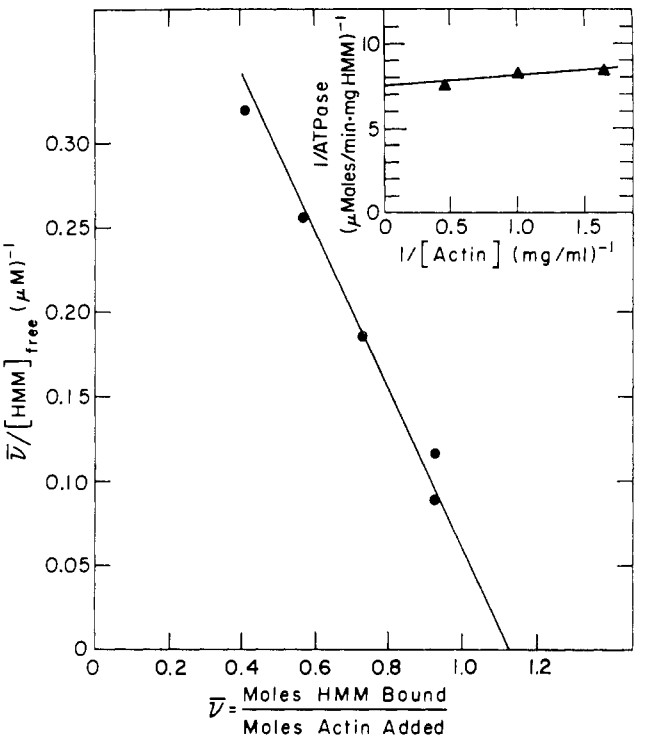

FIGURE 5: Scatchard plot of actin–heavy meromyosin binding, from actin activation of heavy meromyosin ATPase in the presence of Mg. Samples contained 2 mM ATP, 2.4 mM $MgCl_2$, and 4 mM imidazole-HCl buffer (pH 7), for a total ionic strength of 0.013. Binding plot (main graph): Actin concentration fixed at 0.24 mg/ml; heavy meromyosin concentration varied between 1.26 and 5.82 mg per ml. Reciprocal plot (inset), for determination of V_{max}: heavy meromyosin concentration fixed at 0.97 mg/ml; actin concentration varied. Both plots were run the same day with identical conditions.

ionic strength, provide suitable conditions for the study of actin–heavy meromyosin binding in the presence of ATP and Mg. To carry out this study, the ATPase rate was measured with different amounts of heavy meromyosin added to a fixed amount of actin, and the bound heavy meromyosin concentration was calculated from the observed ATPase rate according to the relation

$$[HMM]_{bound} = [HMM]_{total} \frac{v - V_0}{V_{max} - V_0} \quad (5)$$

This relation is derived by the same logic as eq 3. Here, V_{max} is the maximum ATPase activity of the acto-heavy meromyosin complex, determined by extrapolation of a double-reciprocal plot under the same conditions, and as before, v is the measured ATPase rate and V_0 is the ATPase activity of heavy meromyosin in the absence of actin. The free heavy meromyosin concentration is again obtained from eq 2, and the results are plotted according to eq 4.

Figure 5 shows a typical example of a Scatchard plot obtained in this way, and it is evident that the experimental points obey the linear relationship required by the theory if all heavy meromyosin binding sites on actin are identical and independent. There is no evidence of interference between heavy meromyosin molecules binding at neighboring sites even when the actin is well over half-saturated with heavy meromyosin (*cf.* Szentkiralyi and Oplatka, 1969). The apparent intrinsic dissociation constant obtained from the slope of this line is 2.6×10^{-6} M, which agrees rather well with the value of 2.0×10^{-6} M obtained from the abscissa-intercept of Figure 4b. Of primary interest, however, is the abscissa-intercept in Figure 5, which indicates a limiting binding stoichiometry near 1 mole of heavy meromyosin/mole of actin monomers. Repeat experiments also gave essentially the same result. Thus, not only is a one-to-one combining ratio obtained in the absence of Mg ions as shown above, but the binding is also one-to-one in the presence of MgATP where actin activates the heavy meromyosin ATPase, as it does *in vivo*.

Discussion

In this paper we have derived a stoichiometry of actin–heavy meromyosin binding from measurements of the effects of actin on the heavy meromyosin ATPase kinetics under two different sets of conditions. We have arrived at the conclusion that, both in the absence of free Mg where actin inhibits the heavy meromyosin ATPase and in the presence of Mg where actin activates the ATPase, heavy meromyosin binds to F-actin in a ratio of 1 mole of heavy meromyosin/mole of actin monomers.

It must be remembered, however, that this conclusion depends on certain assumptions. First of all, as in most protein–protein binding studies, we must assume that our protein preparations are homogeneous and not significantly denatured, and that we are using correct values for the molecular weights. Our procedures for preparing the proteins and the basis for our choice of molecular weights have been

described in previous papers and in the Methods section. To reconcile our data with a binding ratio of one heavy meromyosin per two actin monomers would require that our molecular weight for actin is too high or that for heavy meromyosin too low by a factor of two, which does not seem likely, or that our heavy meromyosin preparation is half-denatured. The latter also seems rather unlikely because our maximum MgATPase activities for heavy meromyosin at infinite actin have been consistent over several years and are no lower than those obtained in other laboratories (Tawada and Oosawa, 1969; Lowey *et al.*, 1969).

The major assumption upon which our study rests, of course, is that the effect of actin on the heavy meromyosin ATPase can indeed be used as a linear measure of actin–heavy meromyosin binding. It must be emphasized that this assumption of a direct relationship between enzymic activity and actual moles of actin bound could be in error. In principle, one can only determine stoichiometry when the free concentration of one component can be determined in an equilibrium situation, and this condition may not be fulfilled in this study. Therefore we cannot be at all certain that we are really determining the true stoichiometry of binding by our method. On the other hand, a direct measurement of actin–heavy meromyosin binding, particularly in the presence of ATP, is an extremely difficult technical problem at the present time, and consequently we felt justified in approaching the binding problem in this indirect way.

While it is possible that some complexity of the ATPase kinetics of our system gives rise to a misleading result for the binding stoichiometry, our confidence in our conclusion is strengthened by the fact that the binding plots appear linear with reasonable slopes, and that the same one-to-one binding ratio was obtained both in the presence and absence of Mg in spite of the very different characteristics of the ATPase kinetics in these two cases. Our result also agrees with that obtained by Young (1967) in the only previously published study in which the theory of multiple equilibria was applied. Furthermore, for the experiments in the presence of Mg, the simple kinetic scheme which we have previously used to describe the acto–heavy meromyosin ATPase kinetics leads to a proportionality between the actin activation of the heavy meromyosin ATPase at saturating substrate concentration and the amount of heavy meromyosin bound to actin. While the actual kinetics may of course be more complex, there is no evidence as yet from our experiments at varied actin and ATP concentrations (Eisenberg and Moos, 1970) which would require modification of this kinetic scheme.

If we accept the conclusion that the actin–heavy meromyosin binding stoichiometry is indeed one-to-one, *i.e.*, that every monomer in F-actin can bind a molecule of heavy meromyosin, an interesting problem arises in relation to the fact that heavy meromyosin seems to have two ATPase active sites per molecule, corresponding presumably to the two globular heads seen in the electron microscope. First of all, it must be noted that the result of the actin-activation studies in the presence of Mg cannot be explained by postulating that the two heads of the heavy meromyosin molecule bind to two neighboring actin monomers at high actin concentration whereas only one of the two heads binds to the actin when heavy meromyosin is present in excess and crowding occurs. On the contrary, in terms of the actual data, the value of unity for the abscissa intercept in Figure 5 means that the acto–heavy meromyosin ATPase activity per mole of actin monomer at infinite heavy meromyosin concentration is the same as that per mole of heavy meromyosin at infinite actin concentration (V_{max} in the double-reciprocal plot). Therefore, if both heads bind at infinite actin concentration and their ATPase is activated, then likewise at infinite heavy meromyosin concentration there must be two heads bound and activated per actin monomer. On the other hand, it may be that only one of the two heads of a heavy meromyosin molecule can ever bind at a time, even with excess actin, as was suggested by Young (1967) to explain his finding that subfragment 1 also binds to actin in a one-to-one ratio. However, the data on actin inhibition of the ATPase in the absence of Mg indicate that, if the latter is the case, the binding of actin to one head of the heavy meromyosin apparently influences both since the heavy meromyosin ATPase can be almost completely inhibited by actin under these conditions.

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References

Bárány, M. (1967), *J. Gen. Physiol. 50, No. 6, Part 2*, 197.
Barron, S., Eisenberg, E., and Moos, C. (1966), *Science 151*, 1541.
Eisenberg, E., Barouch, W. W., and Moos, C. (1969), *Federation Proc. 28*, 536.
Eisenberg, E., and Moos, C. (1967), *J. Biol. Chem. 242*, 2945.
Eisenberg, E., and Moos, C. (1968), *Biochemistry 7*, 1486.
Eisenberg, E., and Moos, C. (1970), *J. Biol. Chem. 245*, 2451.
Finlayson, B., Lymn, R. W., and Taylor, E. W. (1969), *Biochemistry 8*, 811.
Gergely, J., and Kohler, H. (1958), *in* Conference on the Chemistry of Muscular Contraction, Tokyo, Igaku-Shoin, Ltd., p 14.
Huxley, H. E. (1960), *in* The Cell, Brachet, J., and Mirsky, A. E., Ed., Vol. 5, New York, N. Y., Academic, p 365.
Huxley, H. E. (1963), *J. Mol. Biol. 7*, 281.
Huxley, H. E. (1969), *Science 164*, 1356.
Ikkai, T., and Ooi, T. (1966), *Biochemistry 5*, 1551.
Ikkai, T., and Ooi, T. (1969), *Biochemistry 8*, 2615.
Johnson, P., Harris, C. I., and Perry, S. V. (1967), *Biochem. J. 105*, 361.
Levy, H. M., Sharon, N., and Koshland, D. E., Jr. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 785.
Levy, H. M., Sharon, N., Ryan, E. M., and Koshland, D. E., Jr. (1962), *Biochim. Biophys. Acta 56*, 118.
Lowey, S., and Luck, S. M. (1969), *Biochemistry 8*, 3195.
Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42*, 1.
Mueller, H. (1964), *J. Biol. Chem. 239*, 797.
Mueller, H., and Perry, S. V. (1962), *Biochem. J. 85*, 431.
Nauss, K. M., Kitagawa, S., and Gergely, J. (1969), *J. Biol. Chem. 244*, 755.
Rees, M. K., and Young, M. (1967), *J. Biol. Chem. 242*, 4449.
Rizzino, A. A., Eisenberg, E., Wong, K. K., and Moos, C. (1968), *Federation Proc. 27*, 519.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Schliselfeld, L. H., and Bárány, M. (1968), *Biochemistry 7*, 3206.
Sekiya, K., Takeuchi, K., and Tonomura, Y. (1967), *J. Biochem. (Tokyo) 61*, 567.
Slayter, H. S., and Lowey, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1611.
Stracher, A., and Dreizen, P. (1966), *Curr. Topics Bioenergetics 1*, 153.
Straus, O. H., and Goldstein, A. (1943), *J. Gen. Physiol. 26*, 559.
Szent-Györgyi, A. (1951), Chemistry of Muscular Contraction, 2nd ed, New York, N. Y., Academic.
Szent-Györgyi, A. G. (1953), *Arch. Biochem. Biophys. 42*, 305.
Szentkiralyi, E. M., and Oplatka, A. (1969), *J. Mol. Biol. 43*, 551.
Tawada, K. (1969), *Biochim. Biophys. Acta 172*, 311.
Tawada, K., and Oosawa, F. (1969), *J. Mol. Biol. 44*, 309.
Tonomura, Y., Takura, S., and Sekiya, K. (1962), *J. Biol. Chem. 237*, 1074.
Tsuboi, K. K. (1968), *Biochim. Biophys. Acta 160*, 420.
Young, M. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2393.

Structure of α1-CB8, a Large Cyanogen Bromide Produced Fragment from the α1 Chain of Rat Collagen. The Nature of a Hydroxylamine-Sensitive Bond and Composition of Tryptic Peptides*

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ABSTRACT: As a first step in the determination of the primary structure of an extended sequence in collagen, the 22 peptides resulting from tryptic digestion of rat collagen α1-CB8 were separated and their compositions determined. The peptides accounted for the entire cyanogen bromide produced fragment (mol wt 24,000) and no variant peptides were found. These observations provide additional support for the identity of the two α1 chains in the rat collagen molecule. The task of peptide separation was facilitated by the finding that treatment of α1-CB8 with alkaline hydroxylamine yielded two fragments which could be separated and analyzed individually. Although hydroxylamine cleavage resulted in the formation of a new NH_2-terminal glycine, several lines of evidence indicated that hydroxylaminolysis of a peptide bond was not involved. The experimental data suggest that the hydroxylamine-sensitive bond exists as the cyclic imide, anhydroaspartylglycine, which forms by cyclization of an asparaginyl (or aspartyl) side chain with the subsequent (glycyl) amide nitrogen in the polypeptide chain. Molecular models demonstrate that cyclization of an asparaginyl side chain is sterically hindered by the presence of a side chain on the subsequent amino acid. Collagen may therefore be particularly susceptible to hydroxylamine cleavage because of its high glycine content and the increased probability of asparaginyl-glycyl sequences. The elucidation of the nature of the hydroxylamine-sensitive bond in α1-CB8 provides an alternative explanation for many of the chemical findings previously believed to result from the existence of ester or other nonpeptide bonds in collagen. It is therefore probable that collagen chains are synthesized as single unbroken polypeptides, and that intrachain subunits linked by nonpeptide bonds do not exist in the protein.

In collagen, a fibrous protein, important intramolecular interactions appear to be limited to the hydrogen bonds which form between the polypeptide backbones of the three intertwined left-handed helical chains (Ramachandran, 1967). As a consequence of this structure, amino acid side chains extend radially from the central core of the molecule and are primarily involved in intermolecular interactions, as might be expected in a protein which forms structural aggregates. However, because of the highly specific packing of molecules in the collagen fibril and the necessity for interactions with other macromolecules, the requirements for specificity in the amino acid sequence of collagen chains are at least as stringent as those in globular proteins. This specificity is reflected in the relative constancy of the structure of the helical regions of the collagen molecule in vertebrate species (Bornstein, 1968; Bornstein and Kang, 1970).

The determination of the amino acid sequence of a linear polypeptide such as collagen, combined with knowledge of the conformation of the peptide backbone, may therefore be expected to provide much of the information required to elucidate the relation of structure to function. As a step in this direction, and in order to ensure that any existing sequen-

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