Proton-Dependent Dissociation Equilibrium of Hemoglobin. 2. Surface Pressure Measurements in Monolayers of Horse Hemoglobin(III)†

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ABSTRACT: The molecular weight of hemoglobin(III) in monolayers on aqueous subsolutions has been determined by measuring the surface pressure as a function of the protein surface concentration. The dissociation equilibrium between tetrameric and dimeric hemoglobin(III) was determined for spread as well as adsorbed monolayers. The results were compared with analogous measurements in solution. It was found that the numerical value and the pH dependence of the dissociation constant were similar both in the bulk and in the surface phase of the solution. From these findings it was concluded that the native conformation of hemoglobin(III) is retained after adsorption at aqueous surface.

Protein monolayers adsorbed at the surface of aqueous solutions can be empirically described by an equation of state which is valid in the region of small surface pressures (Bull, 1945a):

$$\pi(A - A_0) = RT/M \qquad (1)$$

where π is surface pressure (dyn/cm), M is molecular weight, A is area per quantity of spread protein (cm^2/g), A_0 is a constant proportional to the area occupied by the protein molecules in the surface (cm^2/g).

According to this equation, it is possible to calculate the molecular weight by measuring the surface pressure π as a function of the surface concentration c^s ($c^s = 1/A$). Such investigations on protein monolayers result in molecular weights which agree fairly well with the molecular weight in solution found by other methods (Guastalla, 1939; Bull, 1945a,b, 1951; Imahori, 1952a,b; Fredericq, 1952; Allan and Alexander, 1954; Harrap, 1955; Benhamou, 1956; Muramatsu and Sobotka, 1962). For the parameter A_0, which has been termed "coarea" in analogy to the corresponding term in the van der Waals equation, a numerical value of roughly 10^7 cm^2/g has been determined for different proteins. Assuming this to be the surface area actually occupied by the adsorbed molecules, it was concluded that adsorption of protein molecules at aqueous surfaces leads to complete unfolding (Bull, 1947; Cheesman and Davies, 1954). The same reasoning came from analogous values of the so-called "limiting area" determined in π/A isotherm measurements on protein monolayers. This conclusion has been disputed by some authors, who had investigated protein monolayers by spectroscopic (Malcolm, 1968; Loeb, 1969, 1971), rheological (Boyd et al., 1973), and other methods (Hamaguchi, 1955, 1956; Baier and Zobel, 1966; Mitchell et al., 1970; Phillips et al., 1975).

In order to contribute to this discussion, we have studied the association equilibrium between the subunits of tetrameric hemoglobin molecules adsorbed at aqueous surfaces. According to our measurements dissociation constants in monolayers closely resemble those in solution. In our opinion this result contradicts the conception that protein molecules are generally unfolded upon adsorption.

The measurements of surface pressure reported here were performed by varying the amount of protein at constant surface area (π/c^s isotherm). The formation of the monolayer was either due to spreading ("spread monolayer") or due to adsorption from the interior of a diluted protein solution to the surface ("adsorbed monolayer").

Materials and Methods

Materials. The preparation of horse hemoglobin(III) is described in the preceding paper (Schroeder et al., 1976). The protein concentration was determined photometrically at 546 nm by the method of Betke and Savelsberg (1950). The extinction coefficient was taken as $\epsilon = 11075$ (l. (mol of heme)$^{-1}$ cm^{-1}) for CN-hemoglobin(III). All chemicals were of reagent grade. Water was doubly distilled in an all-quartz apparatus.

Surface Pressure and Surface Potential Measurements. The surface pressure was determined by the Wilhelmy type hanging plate method (Gaines, 1966). Three kinds of plates were used: platinum plates (30.0 × 20.0 × 0.1 mm), glass plates (24.0 × 24.0 × 0.15 mm), and paper cuts (30.0 × 15.0 × 0.16 mm). Platinum plates were roughened, flamed, thoroughly rinsed with distilled water, and dried before use. Glass plates were treated with hot chromic acid. Paper cuts were equilibrated for several hours in the subsolution buffer. The different kinds of plates gave the same results within the experimental error.

A Model 4102 Sartorius electrobalance was used for surface pressure determinations in the range between 10^{-2} and 1 dyn/cm. Readings were readily reproducible to 2×10^{-3} dyn/cm.

The surface potential was measured by the ionizing electrode method (Gaines, 1966). The air above the aqueous surface was ionized by a 10-μCi ^{238}Pu source and the potential difference between a platinum air electrode and a calomel electrode in the solution was compensated for with a calibrated potentiometer. The balance point was detected with a Model 601 Keithly electrometer. By this procedure potential differences of 1 mV could be read.

The subsolution was contained in a circular glass dish (10-cm diameter, 2-cm depth), which was embedded in an

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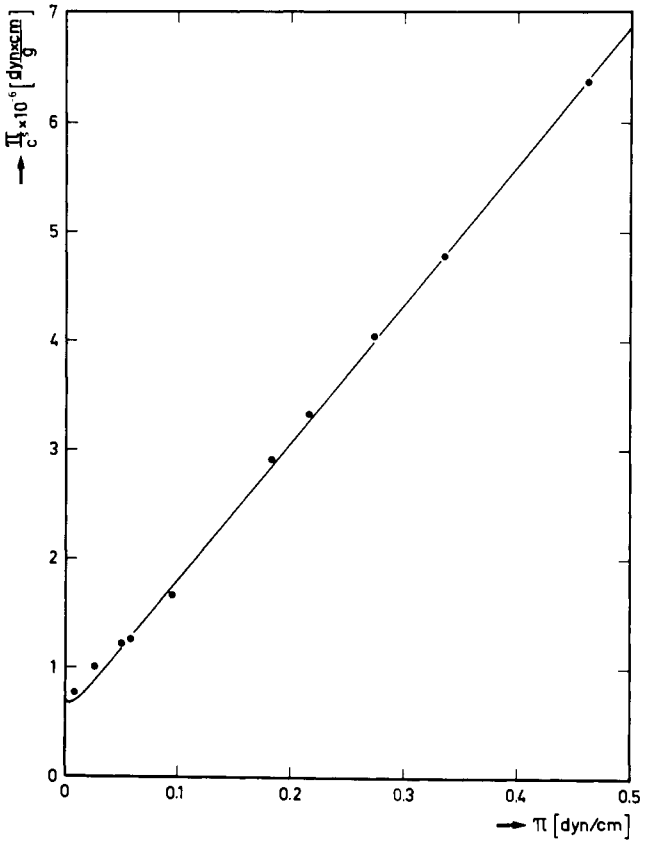

FIGURE 1: Hemoglobin (III) monolayer at pH 5.5. Reduced surface pressure π/c^s over surface pressure π. Subsolution: 0.1 M KCl–0.0035 M citrate buffer. Experimental values (●); the line has been calculated with eq 4 taking $K_d^s = 4.47 \times 10^{-13}$ (mol of heme/cm^2) and $\eta = 0.26$.

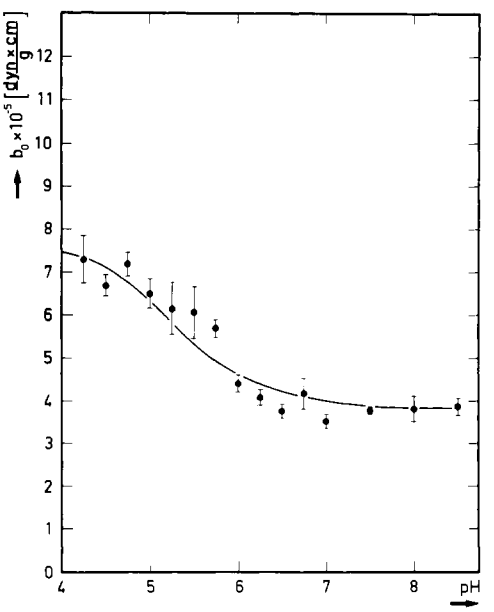

FIGURE 2: Hemoglobin (III) monolayer; pH dependence of the parameter b_0 (eq 2). Symbols represent the mean and the standard error of the mean of eight experiments on the average. The drawn curve was calculated from eq 4 with the corresponding value of K_d^s given by line A in Figure 5. The calculation procedure is described in the text. Subsolution: 0.1 M KCl–0.0035 M citrate or phosphate buffer.

aluminum trough kept at constant temperature (20 °C) by circulating water from a thermostat.

The balance and the electrode system were enclosed in a grounded aluminum box providing both electrostatic shielding and protection of the subsolution surface from contamination and drafts.

Protein Monolayers. Spread Monolayers. Spreading from a protein solution of neutral pH causes incomplete adsorption to the surface and loss into the subsolution. In preliminary experiments optimal conditions for spreading were determined. These investigations resulted in the following procedure: Protein was dissolved to a concentration of 0.01% (w/v) in aqueous solution of 5% (v/v) 1-propanol, and the pH was adjusted to 4.2 by 0.1 M hydrochloric acid. Aliquots of 2×10^{-3} ml of the spreading solution were applied to the surface of the subsolution by means of an Agla micrometer syringe. After 10 min the surface pressure was read. In order to remove surface active impurities before each experiment, the surface layer of the subsolution was carefully sucked off by a glass capillary.

Adsorbed Monolayers. At the surface of protein solutions, adsorbed monolayers form spontaneously. If the initial adsorption layer is removed by suction, dissolved protein diffuses from the interior of the solution into the surface and a new monolayer is formed.

Protein Concentration in the Monolayer. In the case of optimal spreading conditions, the amount of protein transferred per area served as a direct measure for the surface concentration. In the case of incomplete spreading and in adsorbed monolayers, the surface concentration was determined by surface potential measurements. In the pH region covered in these measurements, surface potential and surface concentration are proportional up to a value of $c^s = 0.8 \times 10^{-7}$ g/cm^2 (J. Kubicki, unpublished data).

Buffer Solutions. The subsolution in the monolayer experiments was 0.0035 M buffer in 0.1 M KCl. Potassium citrate was chosen in the range from pH 4 to 6 and potassium phosphate from pH 5.5 to 8.5. In the overlapping region indentical results were obtained with both buffers.

Results and Discussion

Hemoglobin was spread in the region between pH 4.25 and 8.5. The data were examined by plotting the reduced surface pressure π/c^s as a function of the surface pressure π. According to eq 1 such plots will yield linear relations in the case of monodisperse monolayers:

$$\pi/c^s = b_0 + b_1\pi \text{ (dyn cm/g)} \quad (2)$$

$$b_0 = RT/M = 2.44 \times 10^{10} \text{ (dyn cm/g)} \quad (2a)$$

$$b_1 = A_0 \text{ (cm}^2\text{/g)} \quad (2b)$$

Actually we find straight lines for plots of π/c^s over π in the whole pH range covered (Figure 1). In Figure 2 and 3 the parameters b_0 and b_1 of eq 2 obtained by linear regression analysis are presented as a function of pH. b_0 increases with decreasing pH. A plateau is visible at pH >6.5. In this range the average value of b_0 corresponds to a molecular weight of 64 900 ± 1500 (n = 36).[1] At pH <5.0, b_0 approaches a value corresponding to the molecular weight of the dimers. In the region between pH 5 and 6.5 dimeric and tetrameric hemoglobin molecules are coexisting in the monolayer. As far as the dissociation behavior of hemoglobin(III) in surfaces is concerned, the same qualitative statements hold as in the case of hemoglobin(II)O_2 (Benhamou, 1956).

[1] The mean ± standard error of the mean (n number of experiments).

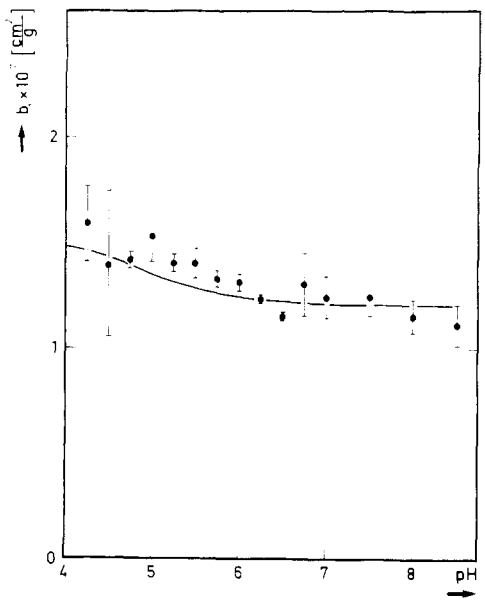

FIGURE 3: Hemoglobin (III) monolayer; pH dependence of the parameter b_1 (eq 2). Symbols, procedure of curve calculation, and subsolution as in Figure 2.

For the quantitative description of a protein monolayer with coexisting dimeric (index 2) and tetrameric (index 4) molecules, eq 1 must be replaced by:

$$\frac{\pi}{c^s}[1 - c^s(1 - \alpha)A_{O4} - c^s\alpha A_{O2}] = (1 + \alpha)RT/M_4 \quad (3)$$

$$c^s = c_2{}^s + c_4{}^s \quad (3a)$$

$$\alpha = c_2{}^s/c^s \quad (3b)$$

Defining:

$$\eta = (A_{O2} - A_{O4})/A_{O4} \quad (3c)$$

and substituting eq 3c in eq 3 gives:

$$\frac{\pi}{c^s} = (1 + \alpha)\frac{RT}{M_4} + (1 + \alpha\eta)A_{O4}\pi \quad (4)$$

If dimeric and tetrameric hemoglobin molecules interact reversibly and equilibrium is attained, this can be described by:

$$K_d{}^s = \frac{(c_2{}^s)^2}{M_4 c_4{}^s/4}\ (\text{mol/cm}^2) \quad (5)$$

Here we consider activity and concentration to be identical. Comparison of the parameters b_0 and b_1 in eq 2 with the analogous terms in eq 4 shows that the latter now are functions of α and according to eq 5 of c^s. Equation 4 no longer represents an exact linear relation between π/c^s and π. However, no significant deviation from linearity was found in the whole c^s and pH range covered by our measurements. This observation raises the question whether in the monolayer a reversible equilibrium between tetramer and dimer hemoglobin molecules really exists. Applying eq 4 and calculating $\pi/c^s = f(\pi)$ with $K_d{}^s$ values varied in a wide range, we found no substantial deviation from linearity down to the lowest surface pressures in our measurements. Consequently eq 2 could be further applied to our experimental data, except that b_0 and b_1 no longer have the same meaning as in eq 2a and 2b. The fact that linearity observed in the $(\pi/c^s)/\pi$ diagrams does not contradict reversible interaction in the monolayer is not positive proof for the existence of an equilibrium. A more convincing check is to demonstrate that the dissociation state of hemoglobin is exclusively determined by the pH and concentration conditions in the surface layer independently of the state existing prior to absorption (Table I). Hemoglobin spread from solutions of pH 4.5 in which it is dimeric onto subsolutions of pH 6.5 is found tetrameric as it is in solution under corresponding conditions. Conversely, spreading from solution of pH 6.5 in which it is tetrameric onto subsolutions of pH 4.5 yields dimers in the surface. Furthermore, if hemoglobin is dissolved in subsolution of pH 6.5, but at concentrations so low that it is completely dissociated into dimers, it is found to be tetrameric after adsorption into the surface. Here association is due to a much higher surface concentration as compared with that in the bulk solution. It is obvious from these findings that hemoglobin in the monolayer can react in both directions: Dissociation of tetramers into dimers occurs as well as association of dimers into tetramers. Therefore we feel justified in assuming a true equilibrium between the two association states of hemoglobin found in the monolayer.

TABLE I: Comparison between the State of Association of Hemoglobin prior to and after Spreading or Adsorption.

Exptl Conditions	State of assoc prior to Adsorption[a]	pH of the Subsolution	Mol Weight, M^1	State of assoc after Adsorption
Spreading from solution of pH 4.2	Dimeric	4.5	36 500 ± 1500 (n = 20)	Dimeric
Spreading from solution of pH 6.5	Tetrameric	4.5	37 300 ± 4200 (n = 5)	Dimeric
Adsorption from subsolution of pH 4.5	Dimeric	4.5	34 900 ± 1000 (n = 2)	Dimeric
Spreading from solution of pH 4.2	Dimeric	6.5	65 200 ± 3000 (n = 11)	Tetrameric
Spreading from solution of pH 6.5	Tetrameric	6.5	67 800 (n = 1)	Tetrameric
Adsorption from subsolution of pH 6.5	Dimeric	6.5	69 500 ± 2000 (n = 5)	Tetrameric

[a] Calculated from results in the preceding paper (Schroeder et al., 1976).

In applying eq 4 and 5 to the experimental data, assumptions concerning the numerical value of η are necessary. In measurements with horse myoglobin(III) we found no significant pH dependence of A_0 in the range between pH 4.5 and 7.0 (J. Kubicki, unpublished data). Therefore we consider A_{O2} and A_{O4} to be independent of pH also in the case of the analogous protein horse hemoglobin(III). Taking the hemoglobin molecule as a sphere and the surface area of the adsorbed molecule as the cross section of this sphere one obtains:

$$A_O = \text{const}\ M^{-1/3} \quad (6)$$

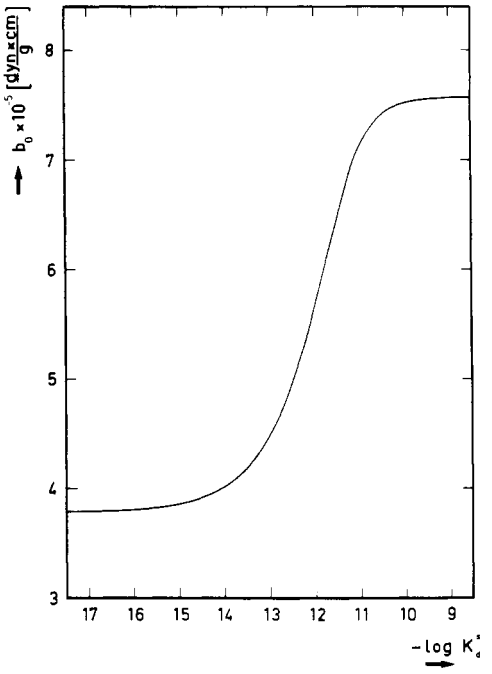

FIGURE 4: (Ordinate) Intercept b_0 of $(\pi/c^s)/\pi$ regression lines. The calculation procedure is described in the text. (Abscissa) Values of K_d^s (mol of heme/cm^2) used for calculation.

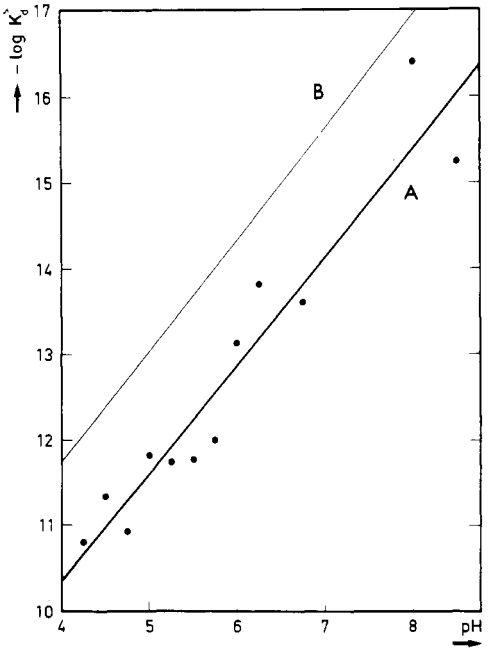

FIGURE 5: pH dependence of the dissociation equilibrium of hemoglobin (III) in monolayers. K_d^s (mol of heme/cm^2) derived from Figure 4 with average values of b_0 in Figure 2. (A) Regression line for the experimental points (●) of K_d^s. (B) Results from the analogous determination of the dissociation constant K_d^b (mol of heme/l.) in solution (Schroeder et al., 1976) transformed in applying eq 11.

This relation has been established experimentally by investigating more than 20 different proteins (J. Kubicki, in preparation). From eq 3c and 6 it follows that:

$$\eta = 0.26$$

Assuming hemoglobin(III) to be totally tetrameric in the pH range above 6.5 covered by our measurements, we obtain the following average values:

$$M_4 = 64\,900 \pm 1500\ (n = 36)^1$$

$$A_{O4} = 1.19 \times 10^7 \pm 0.03 \times 10^7\ (n = 36)^1$$

Using these values the following procedure has been adopted for the determination of K_d^s. Applying eq 4 π/c^s has been calculated as a function of π for distinct values of K_d^s given and represented by the corresponding regression line between 0.03 and 0.5 dyn/cm, the range in which measurements actually have been performed. The intercepts b_0 of the calculated regression lines are plotted in Figure 4 over $-\log K_d^s$. From this diagram the corresponding value of $\log K_d^s$ for a measured value of b_0 can be read. In Figure 5 the values of $-\log K_d^s$ which had been determined in this way are plotted over pH in the corresponding subsolution. The experimental points are fitted by a regression line (Figure 5, line A) with a slope of 1.26. As has been discussed elsewhere (Wyman, 1964), this value is identical with the number of protons bound upon dissociation.

In order to compare the dissociation constants found in solution with those in the monolayer, both sets of data are expressed in terms of volume and area fractions, respectively. As a first approximation for the volume fraction in the bulk (index b) of the subsolution, eq 7 holds:

$$\varphi_i^b = V_i c_i^b / M_i \qquad (7)$$

where V_i is the partial molal volume of species i. For the area fraction in the surface (index s):

$$\varphi_i^s = A_i c_i^s / M_i \qquad (7a)$$

where A_i is the partial molal area of species i. Using relations 7 and 7a and assuming spherical protein molecules

$$V_4 = 2V_2 \qquad (8)$$

$$A_4 = 2^{2/3} A_2 \qquad (8a)$$

we get:

$$K_\varphi^b = \frac{(\varphi_2^b)^2}{(\varphi_4^b)} = \frac{V_2^2 K_d^b}{V_4} = \frac{V_4 K_d^b}{4} \qquad (9)$$

$$K_\varphi^s = \frac{(\varphi_2^s)^2}{(\varphi_4^s)} = \frac{A_2^2}{(\varphi_4^s)} \frac{A_2^2 K_d^s}{A_4} = \frac{A_4 K_d^s}{2^{4/3}} \qquad (9a)$$

K_d^s is given in mol of heme/cm^2 (see eq 5) and K_d^b in mol of heme/l. as defined in the preceding paper. With a mean value of 30 Å for the radius of the hemoglobin molecule we get:

$$\frac{K_\varphi^b}{K_\varphi^s} = \frac{V_4 2^{4/3} K_d^b}{A_4 4 K_d^s} = 2.52 \times 10^{-10} (K_d^b / K_d^s) \qquad (10)$$

If the dissociation equilibrium in solution were not altered in the surface, i.e., if $K_\varphi^s = K_\varphi^b$, we obtain from eq 10 in logarithmical notation:

$$\log K_d^s = \log K_d^b - 9.60 \qquad (11)$$

Equation 11 has been used to express the values measured in solution (Schroeder et al., 1976) in terms of values found in the surface. These data are represented by line B in Figure 5. Comparison of the two lines in Figure 5 lends itself to the following statements. The relative small difference in the slope of the lines means that the number of protons bound upon dissociation is virtually the same both in the surface and in solution. The fact that the line for K_d^b is always found above that for K_d^s indicates that the dissociation is only slightly enhanced in the surface. From the distance between the two lines at pH 6.5, for instance, the difference in the free enthalpy

change of dissociation between bulk and surface amounts to 2.01 kcal/mol at 20 °C.

According to classical conceptions, protein adsorbed at the aqueous surface is unfolded. The results presented above, however, strongly suggest that hemoglobin(III) monolayers consist of globular molecules. From the unexpectedly close similarity of the numerical values of the dissociation constant for the surface and the bulk phase, it appears that the specific interactions governing the dissociation equilibrium and, hence, the overall native conformation are essentially retained. This is even more evident from the similarity of the pH dependence of the constant, indicating that the number of protons bound upon dissociation is the same in either phase.

Acknowledgment

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Further Studies on the Interaction of Actin with Heavy Meromyosin and Subfragment 1 in the Presence of ATP†

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ABSTRACT: It has been postulated that, during the hydrolysis of ATP, both normal and SH_1-blocked heavy meromyosin undergo a rate-limiting transition from a refractory state which cannot bind to actin to a nonrefractory state which can bind to actin. This model leads to several predictions which were studied in the present work. First, the fraction of heavy meromyosin or subfragment 1 which remains unbound to actin when the ATPase equals V_{max} should have the same properties as the original protein. In the present study it was determined that the unbound protein has normal ATPase activity which suggests that it is unbound to actin for a kinetic reason rather than because it is a permanently altered form of the myosin. Second, if the heavy meromyosin heads act independently half as much subfragment 1 as heavy meromyosin should bind to actin. Experiments in the ultracentrifuge demonstrate that about half as much subfragment 1 as heavy meromyosin sediments with the actin at V_{max}. Third, the ATP turnover rate per actin monomer at infinite heavy meromyosin concentration should be much higher than the ATP turnover rate per heavy meromyosin head at infinite actin concentration. This was found to be the case for SH_1-blocked heavy meromyosin since, even at very high concentrations of SH_1-blocked heavy meromyosin, in the presence of a fixed actin concentration, the actin-activated ATPase rate remained proportional to the SH_1-blocked heavy meromyosin concentration. All of these results tend to confirm the refractory state model for both SH_1-blocked heavy meromyosin and unmodified heavy meromyosin and subfragment 1. However, the nature of the small amount of heavy meromyosin which does bind to actin in the presence of ATP at high actin concentration remains unclear.

In muscle cells the key event in contraction is the interaction of myosin with actin and ATP at low ionic strength. In vitro kinetic studies of this interaction are difficult to interpret quantitatively because myosin aggregates at low ionic strength. However, heavy meromyosin and subfragment 1, which retain both the ATPase activity and actin-binding sites of myosin, are both soluble at low ionic strength. Their interaction with actin and ATP is, therefore, much more amenable to quanti-

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