Linkage between the Binding Sites for Zinc and Oxygen on Sperm Whale Myoglobin†

Joseph M. Rifkind, Melvin H. Keyes,‡ and Rufus Lumry*

ABSTRACT: The binding of zinc to sperm-whale myoglobin was shown to increase the affinity of myoglobin for oxygen and carbon monoxide. The increased affinity at the sixth-ligand position of the heme occurs in the presence and absence of substance K, which has previously been reported (Keyes, M., Falley, M., and Lumry, R. (1971), *J. Am. Chem. Soc. 93,* 2035) to increase the affinity at the sixth-ligand position of the heme in the absence of metal ions. There is a positive interaction between these two allosteric effectors. A threefold increase in the affinity constant for oxygen and carbon monoxide at saturating zinc concentrations is estimated from the effect of various nonsaturating zinc concentrations on the ligand affinity of myoglobin prepared by the method of Yamazaki et al. (Yamazaki, I., Yakota, K., and Shikama, K. (1964), *J. Biol. Chem. 239,* 4151), which does not remove substance K. Zn^{2+} association constants estimated from these studies are two orders of magnitude lower than the highest affinity zinc association constant determined by equilibrium dialysis, demonstrating that a relatively low affinity zinc site is involved. Ni^{2+} and Cd^{2+} also increase the affinity for oxygen or carbon monoxide, but to a lesser extent than Zn^{2+}. On the other hand, Mn^{2+}, Mg^{2+}, and Ca^{2+} produce a small decrease in the affinity at the sixth-ligand site.

The oxygenation of hemoglobin is affected (Wyman, 1964) by binding or chemical reaction of a number of small molecules with the protein (Antonini and Brunori, 1970). These effects generally have been linked to the demonstrated change in quaternary structure, i.e., the arrangement of the four subunits, which takes place when oxygen binds to hemoglobin (Perutz et al., 1968; Bolton and Perutz, 1970), and to a lesser extent to the changes which have been shown to take place within the hemoglobin subunits (Bolton and Perutz, 1970; Perutz, 1970; Perutz and TenEyck, 1971).

Myoglobin, on the other hand, exists as a monomer, and no change in tertiary structure was detectable by x-ray crystallography at a resolution of 2.8 Å (Nobbs et al., 1966). Nevertheless, NMR[1] evidence indicates that the binding of oxygen does produce conformational changes even at a considerable distance from the heme (Shulman et al., 1970; Patel et al., 1970; Jones et al., 1976). It is thus probable that the oxygenation of myoglobin produces conformational changes as occur on oxygenation of single chain hemoglobins (Huber et al., 1970) or within chains of tetrameric hemoglobin.

The relative simplicity of monomeric myoglobin as a model for linkage processes, in a single-subunit protein depending only on tertiary structure, has prompted our investigation of myoglobin.

Some factors such as pH which alter the oxygen affinity of hemoglobin have no effect on the oxygenation of myoglobin at least near 25 °C (Antonini et al., 1962). However, we previously described a complex linkage system in myoglobin involving the carbon monoxide site linked to two or more xenon sites only in the presence of a substance (substance K) which is found in many myoglobin preparations (Keyes and Lumry, 1968; Keyes et al., 1971). In this paper we report the effect of various divalent metal ions on the binding of both oxygen and carbon monoxide to myoglobin. A linkage system in the absence as well as the presence of substance K is observed which involves the oxygen binding site and one or more Zn^{2+}-binding sites.

Experimental Section

Sperm-whale myoglobin was prepared by the method of Yamazaki et al. (1964) modified according to the procedure of Keyes et al. (1971). This material (Yamazaki-myoglobin) was used directly for most of the myoglobin experiments. However, substance K was removed from some myoglobin preparations by rechromatography on Sephadex G-25 (Keyes et al., 1971). This material will be referred to as K-free myoglobin.

The method of Keyes et al. (1967) was used to study the binding of gaseous ligands to myoglobin. Zn^{2+} binding was determined by equilibrium dialysis, using the procedure previously described (Rifkind, 1974; Rifkind et al., 1976). The myoglobin was placed inside dialysis bags and equilibrated with an outside solution containing Zn^{2+}. The difference between the zinc ion concentration inside the bags and outside the bags after holdup correction gave the concentration of Zn^{2+} bound to the protein. The free Zn^{2+} concentration was determined from the outside concentration correcting for the binding to unprotonated Tris using a pK of 2.43 for the association of Zn^{2+} with tris(hydroxymethyl)aminomethane (Tris) and a pK_a of 8.1 for Tris (Hanlon et al., 1966). At the pH used for the binding studies no correction was necessary for the formation of hydroxylated Zn^{2+} species (Rifkind and Eichhorn, 1972). The free Zn^{2+} concentration for the oxygen and carbon monoxide binding experiments was determined by the same procedure.

Results

The Effect of Zinc on the Binding of Ligands to Myoglobin. In the absence of interactions involving other small molecules,

† From the Laboratory of Molecular Aging, National Institutes of Health, National Institute on Aging, Gerontology Research Center, Baltimore City Hospitals, Baltimore, Maryland 21224 (J.M.R.), and the Laboratory of Biophysical Chemistry (publication No. 69), Chemistry Department, University of Minnesota, Minneapolis, Minnesota 55455 (R.L.). *Received July 12, 1977.* The work done at University of Minnesota was supported by the Office of Naval Research, Department of Defense (Nonr 710(55)), The Air Force Office of Aerospace Research (AF AFOSR 1222 67), and the United States Public Health Service (A.M. 05853).

‡ Present address: Owens-Illinois, Inc., Technical Center, Toledo, Ohio 43607.

[1] Abbreviations used: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

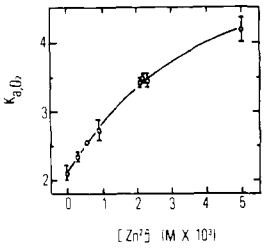

FIGURE 1: The apparent equilibrium constants for the reaction of O_2 with sperm-whale Yamazaki-myoglobin vs. the free concentration of Zn^{2+}. Conditions: 0.002 M Tris-Cl at pH 6.9 ± 0.01, 20 °C. The length of the error-bar is twice the standard error determined from the error in the fractional saturation, Y.

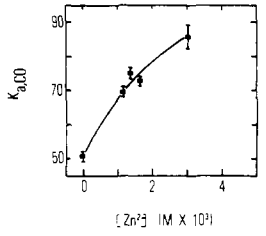

FIGURE 2: The apparent equilibrium constants for the reaction of CO with sperm-whale Yamazaki-myoglobin vs. the free concentration of Zn^{2+}. Conditions: 0.043 M Tris-Cl at pH 6.85, 20 °C. The length of the error-bar is twice the standard error determined from the error in the fractional saturation, Y.

the binding of ligands to the single heme group of myoglobin can be described by one constant defined by

$$K_{0,X} = \frac{[MbX]}{[Mb]} \frac{1}{P_X} \quad (1)$$

where [MbX] is equal to the concentration of myoglobin with ligand (CO or O_2) bound, [Mb] is the concentration of myoglobin without ligand bound, and P_X is the partial pressure of the ligand.

Interactions with other molecules can change the equilibrium constant for the binding of ligands by producing species other than Mb, e.g., MbZn, which have equilibrium constants for the binding of ligands different from $K_{0,X}$. It is therefore convenient to define an apparent equilibrium constant, $K_{a,X}$, given by the expression

$$K_{a,X} = \frac{Y}{1 - Y P_X} \frac{1}{P_X} \quad (2)$$

where Y is the fraction of all myoglobin species with ligand bound.

Figures 1 and 2 show that Zn^{2+} has an appreciable effect on $K_{a,X}$ for the binding of both oxygen and carbon monoxide to myoglobin purified by chromatography on DEAE-Sephadex (Yamazaki-myoglobin) (Yamazaki et al., 1964). Subsequent chromatography on Sephadex G-25 was shown (Keyes et al., 1971) to remove a low molecular weight contaminant referred to as substance K. Substance K interacts with myoglobin and the removal of this substance (K-free myoglobin) produces a significant change in the thermodynamics for the binding of CO and O_2 to myoglobin (Keyes et al., 1971). The results in Table I show that Zn^{2+} increases the oxygen affinity of myoglobin in the absence as well as in the presence of substance K, although a somewhat larger effect is observed in the presence of substance K (Yamazaki-myoglobin).

Assuming that only one Zn^{2+} binding site is linked to the binding of ligands:

TABLE I: Ligand-Affinity Enhancement of Different Myoglobin Preparations by Zinc.[d]

	$K_{Yamazaki}$ ($Torr^{-1}$)	$K_{K\text{-free}}$ ($Torr^{-1}$)	$\frac{K_{K\text{-free}}}{K_{Yamazaki}}$
K_{0,O_2}(no Zn^{2+})	2.09 ± 0.11[a]	1.89 ± 0.03[b]	0.90
K_{a,O_2} (1.78×10^{-3} M Zn^{2+})	3.24 ± 0.10[a]	2.48 ± 0.01[c]	0.89
$K_{a,O_2}/K_{0,O_2}$	1.55	1.32	

[a] Conditions: 0.002 M Tris-Cl at pH 6.9, 20.0 °C. [b] Conditions: 0.01 M Tris-Cl at pH 7.3, 22.3 °C; corrected to 20.0 °C using $\Delta H° = -18.1$ kcal/mol (Keyes et al., 1971). [c] Conditions: 0.01 M Tris-Cl at pH 6.8, 22.0 °C; corrected to 20.0 °C using $\Delta H° = -18.1$ kcal/mol (Keyes et al., 1971). [d] Myoglobin concentration about 5×10^{-5} M.

$$K_{a,X} = \frac{Y}{1 - Y P_X} \frac{1}{P_X} = \frac{[MbX] + [MbXZn]}{[Mb] + [MbZn]} \frac{1}{P_X} \quad (3)$$

It is thus apparent that the effect of zinc on $K_{a,X}$ will depend on the association constants for the binding of zinc to liganded and unliganded myoglobin. We can define L and N_X as the association constants for the binding of Zn^{2+} to unliganded myoglobin and to myoglobin with the ligand X bound to it, respectively. $K_{a,X}$ can then be written as:

$$K_{a,X} = \frac{K_{0,X}(1 + N_X[Zn^{2+}])}{1 + L[Zn^{2+}]} \quad (4)$$

Equation 4 can be rearranged to give:

$$\frac{K_{0,X}}{K_{a,X} - K_{0,X}} = \frac{1}{N_X - L} \frac{1}{[Zn^{2+}]} + \frac{L}{N_X - L} \quad (5)$$

The data of Figures 1 and 2 can then be plotted as $K_{0,X}/(K_{a,X} - K_{0,X})$ vs. $1/[Zn^{2+}]$, and estimates of L and N_X can be obtained from the slope and intercept of this linear plot (Figures 3 and 4). The experimental uncertainty in determining the concentration of myoglobin with ligand bound accounts for the largest error in $K_{0,X}/(K_{a,X} - K_{0,X})$, and accounts for the large uncertainty of some of the points in Figures 3 and 4. Least-squares fitting of the O_2-binding data in Figure 3 gives the values of $N_{O_2} = (6.5 \pm 0.3) \times 10^2$ M^{-1} and $L = (2.2 \pm 0.3) \times 10^2$ M^{-1}. The same analysis of the CO-binding data (Figure 4) gives the values $N_{CO} = (7.8 \pm 1.9) \times 10^2$ M^{-1} and $L = (3.2 \pm 1.6) \times 10^2$ M^{-1}. It is apparent that, although the values for both constants from the CO data are somewhat higher, they are within error the same as those obtained from the O_2 data.

The straight line fit of the data in Figures 3 and 4 indicates that the observed increased affinity for ligands in the presence of Zn^{2+} can be explained by a single Zn^{2+} binding site, but does not prove that only one such site is linked to ligand binding. In the case of multiple sites, the values of N_X and L obtained from the linear plots (Figures 3 and 4) are not equal to the intrinsic association constants, but will, nevertheless, approximate the actual association constants.

Direct binding of zinc to Yamazaki-oxymoglobin was studied by equilibrium dialysis in order to compare the total zinc binding with the oxygen-linked Zn^{2+} binding associated with the constant N_{O_2}. These experiments yield the value of the association constant for the highest affinity Zn^{2+} binding site on oxymyoglobin of $(6.8 \pm 0.3) \times 10^4$ M^{-1}. This value is two orders of magnitude higher than N_{O_2}, forcing the conclusion that the site for oxygen and carbon monoxide in myoglobin is linked to one or several sites (Zn^{2+} sites) which are much weaker than the highest affinity Zn^{2+} binding site de-

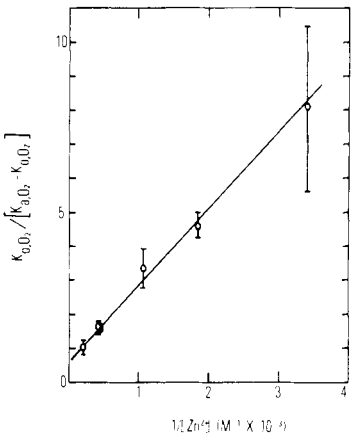

FIGURE 3: Plot of $K_{0,O_2}/(K_{a,O_2} - K_{0,O_2})$ vs. $[Zn^{2+}]^{-1}$. The same data as used in Figure 1 are plotted in this fashion in order to determine zinc binding constants (see text).

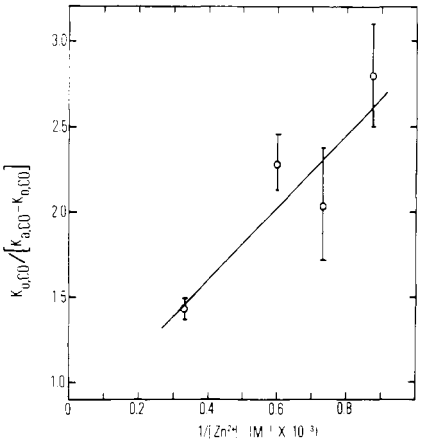

FIGURE 4: Plot of $K_{0,CO}/(K_{a,CO} - K_{0,CO})$ vs. $[Zn^{2+}]^{-1}$. The same data as used in Figure 2 are plotted in this fashion in order to determine zinc binding constants (see text).

termined by x-ray diffraction and direct-binding methods (vide infra).

The Effect of Other Divalent Metal Ions. In Table II is shown the effect of various other metal ions on the binding of oxygen or carbon monoxide to Yamazaki-myoglobin. It is found that while the effect of Zn^{2+} is greater than any of these metal ions, Cd^{2+} and Ni^{2+} also increase the equilibrium constant for oxygen or carbon monoxide. Since Cd^{2+} and Ni^{2+} are closest to Zn^{2+} in the usual order of metal-binding affinities (Irving and Williams, 1948), the one or more metal binding sites linked to the binding of oxygen and carbon monoxide may be general divalent metal-ion binding sites.

The small but significant decrease in the affinity of myoglobin for ligands in the presence of Mn^{2+}, Ca^{2+}, and Mg^{2+} (Table II) suggests a different type of interaction with these metal ions. The observation that this effect is approximately equivalent to the removal of substance K (Table I) suggests a possible competition between substance K and these metal ions. Such a competition could involve the binding of the metal ions to substance K or, alternatively, the binding of the metal ions at or in the region of the substance K site on myoglobin.

Discussion

The Binding Sites for Zn^{2+}. X-ray analysis of metmyoglobin has determined the location of a Zn^{2+} binding site in the region of histidine GH1, asparagine GH4, and lysine A14

TABLE II: Ligand-Affinity Enhancement or Reduction Produced by Metal Ions.[a]

Metal ion	Concn[b] ($M \times 10^3$)	Ligand[c]	pH	$K_{a,X}/K_{0,X}$
Ni^{2+}	3.0	CO	7.4	1.30
Cd^{2+}	5.0	O_2	7.0	1.15
Co^{2+}	6.69	CO	7.0	1.00
Mn^{2+}	6.7	CO	7.2	0.92
Mg^{2+}	1.43	O_2	6.7	0.93
Ca^{2+}	1.68	O_2	7.7	0.92

[a] Yamazaki-myoglobin in 0.002 M Tris-Cl, 20 °C. Concentration about 5×10^{-5} M. [b] Total metal ion concentration. [c] CO and O_2 appear to be equivalent in this experiment, although the comparison was not made with all cations.

(Banaszak et al., 1965). Breslow and Gurd (1963) have previously found that metmyoglobin binds a total of seven atoms of zinc. The Zn^{2+} association constant for the highest affinity site of metmyoglobin (Breslow, 1973) is very similar to the oxymyoglobin-Zn^{2+} association constant determined by equilibrium dialysis. Therefore, the x-ray site is probably the highest affinity Zn^{2+} binding site on oxymyoglobin as well as metmyoglobin. The failure to observe an affect on the oxygenation due to binding at this site may not be surprising considering the fact that this site is quite far from the heme.

Cann (1963, 1964a,b) has observed that Zn^{2+} produces a flattening of the visible absorption spectrum of myoglobin at a concentration of 9×10^{-3} M, which is somewhat higher than the concentrations used in our ligand binding experiments (Figures 1 and 2). Similar effects are also observed with Cu^{2+} when more than one Cu ion is bound to myoglobin (Cann, 1974a; Breslow and Gurd, 1963). It has been suggested that these spectral effects are produced by the binding of Zn^{2+} or Cu^{2+} to the proximal histidine, F8, which consequently unfolds part of the molecule denaturing the protein. At our Zn^{2+} concentration, particularly at a low ionic strength (Cann, 1964a), no such spectral effects are expected (Breslow and Gurd, 1963), and they were not observed. The effect of Zn^{2+} on the binding of ligands to myoglobin must therefore involve a binding site of intermediate affinity.

The association constant which we have determined for the zinc site which is linked to the ligand sites of myoglobin is very similar to that of a 1:1 Zn^{2+}:imidazole complex (Sillen and Martell, 1964). This suggests that simple coordination to isolated histidines on the surface of the molecule may be involved. Various studies indicate that of the 12 histidines in sperm-whale myoglobin six to eight are exposed to the solvent with the remaining masked at neutral pH (Breslow and Gurd, 1962; Banaszak et al., 1963). Therefore in addition to histidine GH1, involved in the high affinity Zn^{2+} binding, and histidine A10, located in the same region of the molecule and associated with the high affinity Cu^{2+} binding site (Banaszak et al., 1965), there are four to six readily available histidines in sperm-whale myoglobin.

Myoglobin Linkage Systems. The results shown in Figures 1 and 2 indicate that Zn^{2+} more than doubles the myoglobin binding constant for oxygen and carbon monoxide. From the values of N_X and L obtained from the linear plots of Figures 3 and 4, it is possible to calculate values for the binding of ligands to Yamazaki-myoglobin at saturating concentrations of zinc ($K_{1,X}$).

$$K_{a,X(Zn^{2+}\to\infty)} = K_{1,X} = \frac{[MbZNX]}{[MbZn]}\frac{1}{P_X} = \frac{K_{0,X}N_X}{L} \quad (6)$$

K_{1,O_2} for the binding of oxygen to myoglobin, with the Zn^{2+}

site linked to ligand binding saturated, is 6.2 $Torr^{-1}$. The increase in oxygen affinity produced by a saturating concentration of zinc, $K_{1,O_2}/K_{0,O_2}$, obtained from the ratio of N_{O_2}/L is equal to 3.0 ± 0.5. Similar values with somewhat larger errors can be calculated for $K_{1,CO}/K_{0,CO}$ from the CO data. This ratio, which is a measure of the linkage free energy between the zinc site(s) and the sixth ligand position of the heme, is smaller than most physiologically important linkage systems thus far studied with hemoglobin, at least under conditions of maximum linkage.

The results of Table I indicate that both allosteric effectors, Zn^{2+} and substance K, increase the affinity of myoglobin for oxygen and carbon monoxide. Furthermore, there is a positive interaction involving Zn^{2+} and substance K, such that Zn^{2+} has a greater effect on the sixth-ligand affinity in the presence of substance K ($K_{a,O_2}/K_{0,O_2}$ is greater for Yamazaki-myoglobin than K-free myoglobin) and substance K has a greater effect on the sixth-ligand affinity in the presence of zinc ($K_{\text{K-free}}/K_{\text{Yamazaki}}$ is less in the presence of zinc than in the absence of zinc). This positive interaction rules out the possibilities that Zn^{2+} is substance K or competes for the same myoglobin site as substance K.

There are two possible explanations for the positive interaction between Zn^{2+} and substance K. There may be a preferential increase in the affinity of the effector for MbX relative to that of Mb. This effect will increase the sixth-ligand affinity at saturating concentrations of the effectors, increasing the linkage free energy. Since the results shown in Table I are for nonsaturating levels of Zn^{2+} and substance K, the positive interaction between effectors may alternatively reflect an increased affinity of the effector for both MbX and Mb. This will not alter the linkage free energy or the sixth ligand affinity at saturating concentrations of the effectors, but only lower the concentration of effectors necessary to produce a given increase in ligand affinity.

Another linkage system has been reported for myoglobin involving the sixth-ligand site and xenon hydrophobic binding sites, which can also bind cyclopropane or nitrogen (Keyes and Lumry, 1968, 1971; Keyes, 1968). The xenon effect involves the cooperative binding of at least two xenon molecules. X-ray crystallographic studies (Schoenborn et al., 1965; Schoenborn and Nobbs, 1966) indicate that at least one of these xenon molecules binds at a hydrophobic binding site on the proximal side of the heme between histidine F8 and the pyrrole ring of the heme with the 2-vinyl substituent. Xenon produces a fivefold increase in the affinity for carbon monoxide of myoglobin saturated with substance K, but has no effect on the binding of oxygen to myoglobin. It has no effect on the binding of carbon monoxide in the absence of substance K. Thus far the maximum linkage system includes the sites for binding of CO, substance K, two Xe atoms and Zn^{2+}.

The presence of the xenon and zinc linkage systems both coupled to substance K, but qualitatively and quantitatively different from each other, demonstrates how varied allosteric linkage phenomena can be even in a single subunit protein like myoglobin for which no conformational changes have been detected by x-ray crystallography (Nobbs et al., 1966). It can thus be concluded that thermodynamically significant changes (probably in part conformational) take place when ligands bind to myoglobin. Perhaps the effects of various ligands on myoglobin NMR spectra (Shulman et al., 1970; Patel et al., 1970; Jones et al., 1976) reflect the conformational changes.

Comparison of the Zinc Linkage System in Myoglobin and Hemoglobin. Zn^{2+} has also been shown to increase the oxygen affinity of human (Oelshlegel et al., 1973; 1974; Rifkind and Heim, 1977) and horse hemoglobin (Rifkind, unpublished). For purified hemoglobin the reported 3–4 fold increase in oxygen affinity is similar to that found with myoglobin. As a consequence the free energy associated with the myoglobin Zn^{2+} linkage system is similar to that associated with the hemoglobin Zn^{2+} linkage system.

This observation is particularly interesting considering the fact that according to Nobbs et al. (1966) no changes in X-ray structure are observed when binding ligands to Fe(II)-myoglobin, whereas significant changes have been reported for both the tertiary and quaternary structure of hemoglobin when oxygen binds (Perutz et al., 1968; Bolton and Perutz, 1970). The Zn^{2+} system is the first reported case where the same substance has a quantitatively similar effect on both myoglobin and hemoglobin. Other factors such as pH, CO_2, and 2,3-DPG have dramatic effects on hemoglobin but are generally thought to have no effect on myoglobin (Antonini and Brunori, 1970).

The oxygenation of hemoglobin is affected by binding Zn^{2+} to the highest affinity site, for which the association constant is even larger than that of the high affinity Zn^{2+} binding site on myoglobin (Rifkind et al., 1976). Thus, a free Zn^{2+} concentration three orders of magnitude lower than that necessary to effect the binding of ligands to myoglobin (Figures 1 and 2) increases the oxygen affinity of hemoglobin (Rifkind and Heim, 1977). This difference suggests that under the correct conditions the binding of ligands to myoglobin may be found to be influenced by many of the substances now thought to affect only tetrameric hemoproteins.

If substance K has physiological significance as a linkage mediator, its effect does not appear to be large since the free energy of CO binding changes by only 0.6 kcal/mol on saturating myoglobin. However, from the point of view of mechanism, the larger changes in enthalpy, −4.5 kcal/mol, and entropy, −14 gibbs/mol, on saturating with the contaminant indicate a process of some significance (Keyes et al., 1971; Rudolph et al., 1972).

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Structural Study of Spectrin from Human Erythrocyte Membranes†

Z. Kam, R. Josephs, H. Eisenberg, and W. B. Gratzer*

ABSTRACT: Human erythrocyte spectrin prepared from fresh blood is a mixture of different association states. Depending on the manner of preparation, the two-chain dimer or the tetramer predominates. These forms are not in rapid thermodynamic equilibrium. The molecular weight of the dimer by sedimentation and diffusion and by light scattering is about 5×10^5. The frictional properties indicate a low or moderate asymmetry (axial ratio in the range 2–10), and from the angular dependence of light scattering intensity an upper limit of about 80 Å can be set for the radius of gyration. The tetramer similarly has a moderate asymmetry. Electron microscopy reveals that the dimer is a compact, slightly elongated molecule, and that the tetramer probably consists of two parallel dimers. On increasing the concentration of solutions containing spectrin dimers, oligomers are formed, which are not rapidly dissociated on dilution. At very low protein concentrations (below about 0.05 mg/mL) there is evidence of the onset of a rapid dissociation equilibrium between dimers and single chains. Other physical properties of the spectrin have been measured. The size and shape of the spectrin molecule would seem to rule out any major physical resemblance to myosin.

Spectrin is the major protein of the erythrocyte membrane, and is thought to play a critical role in determining its physical properties. In particular there is now an accumulation of evidence to suggest that it exerts a contractile control over the shape of the cell (Sheetz et al., 1976b). The presumption of a contractile function, as well as the subunit molecular weight, the presence of ATPase activity in the preparations, and the appearance of actin in the aqueous extracts from the membranes have led to the conjecture that spectrin may be structurally and functionally related to myosin (Guidotti, 1972; Brandon, 1975; Schechter et al., 1976). Arguments have been given (Gratzer and Beaven, 1975) against such a view, but there has been poor agreement between results from different laboratories, bearing on the size and shape of the molecule; one study (Schechter et al., 1976) indeed reports, in contrast to earlier work, frictional properties compatible with a very asymmetric, myosin-like particle. In addition Sheetz et al. (1976b) have reported a weak cross-reaction of spectrin with antibodies to smooth-muscle myosin (though not of myosin to antispectrin). We have undertaken a study of spectrin by hydrodynamic methods, light scattering, and electron microscopy

† From the Polymer Department, Weizmann Institute, Rehovot, Israel (Z.K., R.J., and H.E.), and Medical Research Council Cell Biophysics Unit, King's College, London WC2, England (W.B.G.). *Received June 29, 1977.*