Nuclear Magnetic Resonance Studies of Heavy Metal Ion–Sulfhydryl Interactions in Myosin†

Robert G. Bryant,* Y. Legler,‡ and Moon H. Han

ABSTRACT: ^{35}Cl nuclear magnetic resonance measurements are reported for myosin preparations studied at pH 7 in 0.6 M KCl. Evidence is presented that a gross conformational change accompanies the loss of enzyme activity and addition of mercurials which substantially alters the environment of sulfhydryl groups in the molecule. This evidence is discussed in light of previously reported data on mercurial modification of sulfhydryl groups.

Studies of conformational changes in myosin are of great interest since they may provide some clue to the molecular mechanism of ATP energy transduction affected by the myosin adenosine triphosphatase. The progressive modification of sulfhydryl groups of myosin is known to cause modification of the calcium-stimulated adenosine triphosphatase of myosin and has been directly associated with conformational changes in the protein (Rainford *et al.*, 1964; Sekine and Kielly, 1964). The reaction of sulfhydryl reagents with myosin may have two effects on the adenosine triphosphatase activity. Reagents such as PCMB[1] initially stimulate the calcium-activated adenosine triphosphatase while other reagents or high concentrations of PCMB cause inhibition of the enzyme activity. Direct evidence for conformational changes in the myosin adenosine triphosphatase associated with sulfhydryl group modification has been obtained recently using various techniques such as fluorescence spectroscopy (Duke *et al.*, 1966; Cheung and Morales, 1969; Cheung, 1969) and electron spin resonance spectroscopy (Quinlivan *et al.*, 1969; Seidel *et al.*, 1970).

Nuclear magnetic resonance measurements have been used extensively to probe the structure of macromolecules, and ^{35}Cl nmr is particularly well suited for the study of protein sulfhydryl groups (Bryant, 1969; Stengle and Baldeschwieler, 1967; Bryant *et al.*, 1969; Sandberg *et al.*, 1969; Ellis *et al.*, 1969; Sudmeier and Pesek, 1971). Because the information obtained from the ^{35}Cl nmr experiment is different from that of the methds used previously to investigate myosin sulfhydryl groups, the present study was undertaken to gain additional information about the nature of the calcium-coupled adenosine triphosphatase activation.

Materials and Methods

Preparation of Myosin. Rabbit skeletal myosin was extracted and purified according to the method of Mueller *et al.* (1964) with some modification. Heavy metal contamination in the myosin preparations was mininized by addition of 0.1 mM EGTA to the first extraction medium. EGTA was omitted from the subsequent purification procedures. Average adenosine triphosphatase activity of myosin was approximately 0.5 μmole of P_i per mg per minute. The protein concentration was determined spectrophotometrically using an absorptivity of 0.543 ml mg^{-1} cm^{-1} at 279 nm (Josephs and Harrington, 1966). Myosin preparations were used within 3 days to avoid air oxidation of sulfhydryl groups.

Determination of Adenosine Triphosphatase Activity. Adenosine triphosphatase activity was determined using a pH-Stat at pH 8.0 and 25° (Green and Mommaerts, 1953). The reaction mixture contained 0.5 M KCl, 10 mM $CaCl_2$, and 1.2 mM ATP. The reaction was initiated by adding 0.05–0.1 mg of myosin. Studies of mercuric ion and PCMB effects on myosin adenosine triphosphatase activities were carried out with myosin preincubated with the mercurial reagents at 5° overnight.

NMR Experiments. Chlorine-35 nmr measurements were made on a Varian DP-60 nmr spectrometer system equipped with a Varian V-4210A variable frequency rf unit which operated at 5.7 MHz. The spectrometer system included a Princeton Applied Research lock-in amplifier which was operated at a frequency of 550 Hz with the phases adjusted to display the first audio side band as an absorption mode signal. The 5-ml samples were contained in 15 mm o.d. Pyrex test tubes and measured at the temperature of the magnet gap of 27 ± 2°. The nmr line widths are reported as the full width measured at half-height of the absorption mode signal. The errors reported are the standard deviations of the mean for at least five spectra.

Mercuric ion was added to the myosin solutions in microliter quantities employing a 10-μl pipet and an accurately known standard mercuric chloride solution. The nmr measurements were made within 10 min of each addition. The pH of the myosin solutions was maintained using 0.05 M Tris-maleate or 0.05 M phosphate buffers (pH. 7.0) in 0.6 M KCl. Tris was recrystallized before use. Other chemicals were reagent grade and used without further purification. The ratios of mercury(II) ion added to total myosin was calculated assuming a myosin molecular weight of 500,000.

Theory. The ^{35}Cl line width is usually dominated by quadrupole relaxation so that in the limit of extreme narrowing the line width for a nucleus of spin $^3/_2$ is given by

$$\Delta\nu = \frac{2\Pi}{5}(e^2qQ)^2\tau_c \qquad (1)$$

† From the Department of Chemistry and Laboratory for Muscle Research, Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minnesota 55455. *Received January 31, 1972.* Supported by Research Grant GY 8783 from the National Science Foundation, and by grants from Graduate School, University of Minnesota, and Minnesota Heart Association (M. H. H.).

‡ N.S.F. Undergraduate Fellow, Summer 1971.

[1] Abbreviations used are: PCMB, *p*-chloromercuribenzoate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

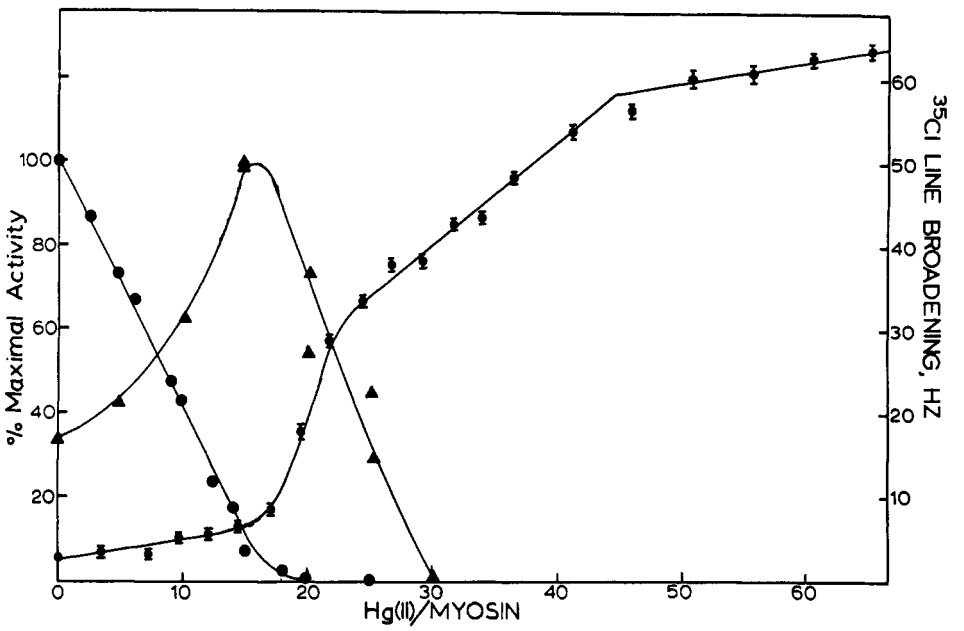

FIGURE 1: Adenosine triphosphatase activity as a function of mercurial concentrations. ▲ PCMB; ● $HgCl_4^{2-}$; ⧳ ^{35}Cl nmr line broadening, Hz.

where $\Delta\nu$ is the full line width in hertz at half-height, e is the unit charge, q is the electric field gradient at the nucleus of quadrupole moment Q, τ_c is the correlation time for reorientation of the field gradient, and the asymmetry parameter has been neglected (Abragam, 1961).

In 1 M aqueous sodium chloride solutions, the chloride ion is found in an approximately symmetric environment producing a field gradient at the nucleus approaching zero and a line width of about 13 Hz. If the quadrupolar chlorine nucleus can be found at environmentally different sites in solution, the line width will depend on the relative concentration of each site, the values of $(e^2qQ)^2$ and τ_c associated with each site, as well as the frequency with which the ^{35}Cl nucleus samples the various sites. In the case where exchange rate of the chloride is fast with respect to the width of each line in the spectrum, a single composite line is observed with the line width given by

$$\Delta\nu = \Delta\nu_a P_a + \Delta\nu_b P_b = \sum_{\text{sites}} \Delta\nu_i P_i \qquad (2)$$

where $\Delta\nu_a$ and $\Delta\nu_b$ are the contributions to the line width associated with sites a and b while P_a and P_b are the probabilities that the chlorine is at site a and b, respectively (Swift and Connick, 1962). The $\Delta\nu_i$ in eq 2 are given in each case by eq 1 where the variables may be the field gradient and the correlation time. If the chloride ion exchange rate were much faster than the rotational contributions to the correlation time, then the line broadening could be limited by the exchange rate contribution to the correlation time (Marshall, 1970). If the exchange rate is rapid, but not large compared with the broadest line in the spectrum, a large broadening of the chloride nmr may still be observed; however, the exchange rate again is the limiting factor in the relaxation equation (Swift and Connick, 1962). To summarize, a change in the contribution of a particular term in eq 2 to the total line width may arise from any of four sources: a change in concentration of sites, a change in the number of chloride nuclei associated with a particular site, a change in the electric field gradient at the chlorine nucleus at that site, or a change in the correlation time or exchange rate for that site. For any term of eq 2 to contribute to the total line width the chlorine site must exchange chloride ion rapidly with the bulk of chloride ion in the sample.

Results

The activity of the Ca^{2+}-activated adenosine triphosphatase is plotted as a function of concentration of mercurial reagents in Figure 1. PCMB clearly causes an activation of the adenosine triphosphatase activity as reported by other investigators (Rainford *et al.*, 1964; Kielly and Bradley, 1956; Azuma *et al.*, 1962). On the other hand, mercuric ion causes a marked decrease in enzyme activity beginning with the lowest concentrations (Figure 1). It is surprising that these reagents behave differently since they are both well-known sulfhydryl reagents.

A mercuric ion titration of a protein containing a sulfhydryl group normally causes a linear increase in the chlorine-35 nmr line width in neutral or acidic media until all the protein sulfhydryl groups have been titrated. The post-end-point region is also linear with a much smaller slope due to the formation of free $HgCl_4^{2-}$ ion in solution (Bryant, 1969). The mercuric ion titration of myosin at pH 7 in Tris-maleate buffer is also shown in Figure 1 and is clearly more complicated than the simple behavior expected. This titration curve is most easily discussed in terms of four different regions of the titration curve. The flat portion of the titration curve extending from zero to approximately 15 equiv will be called region I. The transition occurring between approximately 15 equiv and 22 equiv will be called region II. The linear portion of the titration curve from 22 equiv to approximately 44 equiv will be called region III. The post-end-point portion beyond 44 equiv will be called region IV.

The general features of this titration curve were reproduced with every myosin preparation studied. In some cases, however, the position of the transition in region II moved to lower ratios of mercury to myosin and appeared to be a function of the age of the preparation. The dotted curve in Figure 2 is the result of taking the same preparation (solid curve) exposed to a stream of oxygen for 20 min at room temperature prior to titration with mercury. The shift in the transition position is

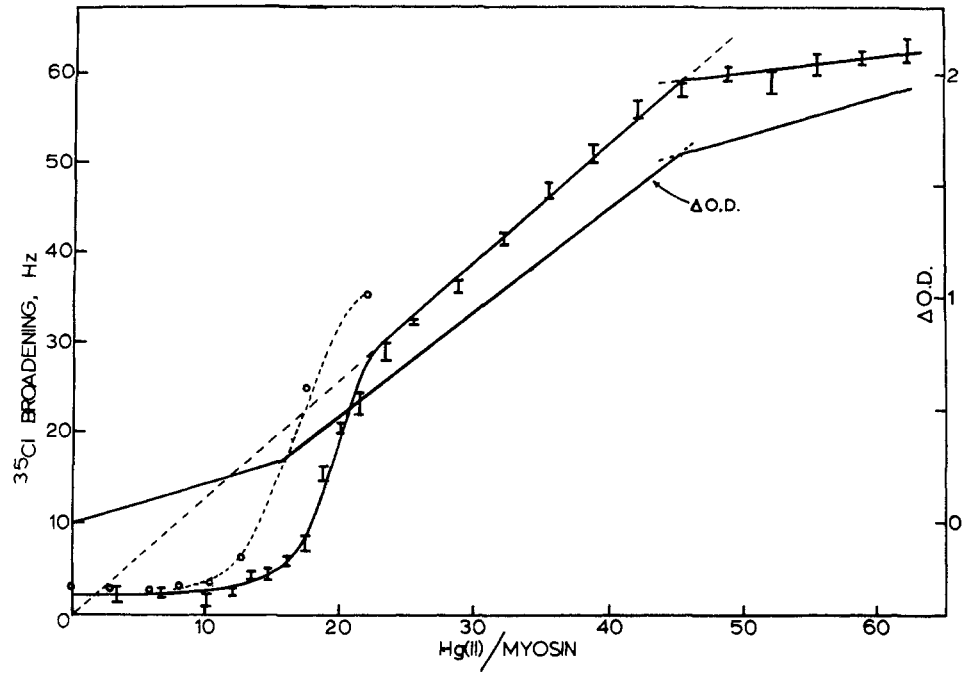

FIGURE 2: ^{35}Cl nmr line width for myosin solutions at pH 7.0 in 0.6 M potassium chloride. I, fresh myosin solution; O, myosin solution exposed to oxygen stream. Solid line is the optical difference spectrum recorded at 240 nm.

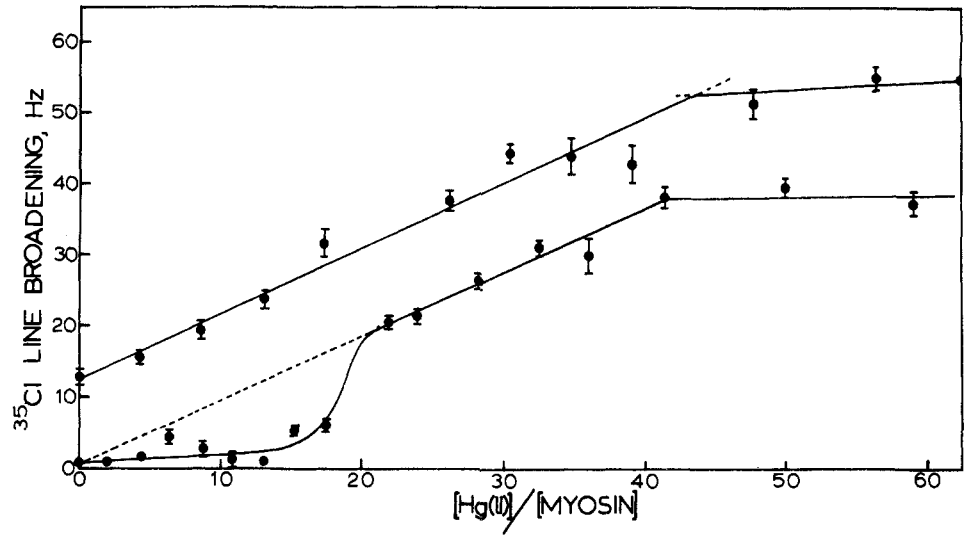

FIGURE 3: ^{35}Cl nmr line broadening for myosin solutions at pH 7.0 in 0.6 M potassium chloride. Bottom curve recorded on fresh myosin solution. Top curve is recorded for the same preparation in 10 M urea.

consistent of oxidation of about 4 sulfhydryl groups in myosin. Reproducible titration curves were obtained if all work was done within 48 hr after the myosin preparation was complete.

Although the transition midpoint is sensitive to exposure of the protein to oxygen, the transition could not be eliminated completely in 8 M urea. In 10 M urea, however, the transition is completely eliminated and the expected linear increase in ^{35}Cl line width is observed until the end point as shown in Figure 3. It is important to notice that the straight line portion of the curve in region III extrapolates to zero line broadening and that the slope of the curve in region III is very nearly identical with that in same region of the urea-denatured sample. The addition of calcium ion did not significantly alter the titration curve; however, the addition of Mg-ATP decreased the total line width slightly and Mg-ATP in millimolar concentrations stabilized the protein significantly against large line broadening by the mercuric ion.

Discussion

The lack of ^{35}Cl line broadening in region I of the titration curves may be explained by either of two hypotheses. (1) The product of the reaction with the first sulfhydryl groups of myosin is of the form PSHgCl but the rapid exchange of the chloride ion, which is necessary for observation of the protein-bound mercury species, is blocked in some way by the steric characteristics of the enzyme. (2) The product of the initial reaction of mercury with the protein is a coordinatively

saturated mercury species which does not include an exchanging chloride ion in the mercury primary coordination sphere. In this case the protein-bound mercury would not effect the chlorine nmr spectrum. In both cases the second region of the titration curve indicates that a conformational change in the protein generates products of the form PSHgCl and that this species is the only form of importance by the beginning of region III. For case 1 a conformational transition would remove the steric constraints on the chlorine exchange and expose the RSHgCl moiety to the aqueous chloride solution where the exchange produces the broadening of the nmr spectrum of the chloride ion. In the second case the conformational change must be such that at least one of the protein donated ligands is removed from the mercury and replaced by a chloride ion so that the product after the conformation change is identical with that for case 1.

The first alternative appears to be the simplest; however, it is quite unlikely that all of the sulfhydryl groups in myosin are located in such a way that the chloride ion exchange with the product PSHgCl is blocked at every site. Assuming that several sulfhydryl groups are on the protein surface where rapid chloride exchange is possible, then the first alternative must explain why these groups are not labeled in the initial stages of the mercury titration. In the absence of some unusual thermodynamic properties a random reaction of mercury with sulfhydryl groups is expected. Since there would be no preference for mercury reaction with exchange-blocked sites as opposed to non-exchange-blocked sites, a significant line broadening should occur in region I. This is not observed. Therefore, either all of the sulfhydryl groups in myosin are located in such a way that the chloride ion exchange is blocked, or there is a mechanism causing certain sulfhydryl groups at which chloride ion exchange is blocked to be thermodynamically favored in the binding of mercuric ion.

The second alternative provides a mechanism for the necessary thermodynamic selection by requiring that a protein-donated ligand displace the chloride ion at mercury. The overall reaction may be represented as shown in eq 3. There

$$PSH + HgCl_4^{2-} + P\text{–}XH = PSHgXP + 4Cl^- + 2H^+ \quad (3)$$

are at least two protein groups which may react in this way: sulfhydryl and histidine or other nitrogen functions.

If the second protein-donated mercury ligand is a second sulfhydryl group, then the product of the reaction must be a basically linear system about the mercury atom of the form PSHgSP (Bryant, 1972). The reaction proposed would therefore require that the sulfhydryl groups in the myosin molecule be located in such a way that as many as 16 of them could form such linear bonding systems. In light of the sensitivity of the protein to mild oxidation, this may not be a severe restriction. A second important consequence of two sulfhydryl groups per mercury site is that the free energy change for the reaction is very large. While this explains the thermodynamic preference of mercury for these sites, it is unlikely that a conformation change could break one of the sulfur–mercury bonds to regenerate a free sulfhydryl group and a molecule of the form RSHgCl. If region I is taken to represent the formation of RSHgSR then region II must represent eq 4.

$$RSHgSR + HgCl_4^{2-} = 2RSHgCl + 2Cl^- \quad (4)$$

The stoichiometry of this reaction requires that region II have a slope of the line width *vs.* mercury plot which is twice that of the slope in region III, which is then taken to represent the formation of the product RSHgCl from tetrachloromercurate ion and sulfhydryl group. The stoichiometry represented by eq 4 also requires that the width of region II equal that of region I. The slope of the line width plot in the transition region is much greater than twice that in region III and the width of the transition region, that is region II, is significantly less than that in region I. Therefore, it is quite unlikely that eq 4 accurately describes the chemistry of the mercury–myosin interaction (Figure 1).

Additional evidence is obtained from an optical difference spectrum drawn in Figure 2. If the product of the reaction of the protein with mercury is RSHgSR in region I, then one expects three types of change in the optical difference spectrum prior to the end point of the mercury titration: a change corresponding to the formation of the initial product RSHgSR from a mercuric ion and two sulfhydryl groups; a region corresponding to the reaction of RSHgSR with tetrachloromercurate ion to form 2 equiv of RSHgCl; and a region corresponding to the formation of RSHgCl from tetrachloromercurate ion and free sulfhydryl group. Only one break is observed in the optical difference spectrum which coincides with the beginning of region II in the nmr titration curve. It is therefore unlikely that an additional ligand at mercury is a second sulfur atom even though it may provide the necessary thermodynamic preference for the reaction of mercury at these sites.

These difficulties are avoided by suggesting that a different protein-donated ligand such as imidazole contributes to the chemistry of mercury binding in region I of the titration curve. Since there is a large number of histidine residues in heavy meromyosin (Barany *et al.*, 1964), this is not unreasonable. Such an equilibrium has been demonstrated in experiments on model compounds using histidine to displace chloride ion from a low molecular weight sulfur–mercury adduct. In this case the free energy change associated with placing a nonchlorine ligand on the mercury is expected to be small and a function of such parameters as the pH of the solution and the state of the protein. Because a protein conformational change could be such that it significantly shifts the equilibrium position of this ligation reaction, it is not possible to predict the width of the transition region. The added free energy for binding provided by the second protein-donated ligand provides the thermodynamic section of metal binding sites required. Since the product of the reaction of mercury with sulfur heavily favors a two coordinate mercury species in solution (Bryant, 1972) only one protein-donated mercury ligand in addition to sulfur is required in eq 3 to eliminate the rapid chloride ion exchange with the protein-bound mercury species. This hypothesis is thus capable of explaining the observed data without postulating any unusual properties of the protein sulfhydryl groups or any enormously energetic conformational changes in the protein.

These nmr experiments clearly demonstrate a marked conformational change in myosin modified by mercuric ions. A similar conformational transition has also been observed with ANS fluorescence and the dissociation of light-chain subunits in the presence of organic mercurials (Duke *et al.*, 1966; Samaha *et al.*, 1970). Organic mercurials initially activate the enzyme in region I while mercuric ion causes a linear inhibition. It appears likely that the reason for this difference is that mercury may react with two protein ligands while organic mercurials may react with only one. Chelation of two protein ligands could readily cause disruption of active conformation or directly block the catalytic amino acid residues such as imidazole or sulfhydryl groups in myosin.

TABLE I: Distribution of SH groups in Myosin.

Subfractions of Myosin	No. of SH Groups per Mole of Proteins	References
Myosin	44	Weeds and Hartley, 1968 Barany *et al.*, 1964
Light meromyosin	6	Weeds, 1966
Heavy meromyosin	38	(Estimated)
Subfragment 2	4	Weeds, 1967
Subfragment 1	34	(Estimated)
Light chains	4	Weeds and Lowey, 1971
Heavy-chain core	30	(Estimated)
DTNB light chain	2	Weeds and Lowey, 1971
Alkali LC 1	1	Weeds and Lowey, 1971
Alkali LC 2	1	Weeds and Lowey, 1971

The quantitative aspects of the nmr experiment are in good agreement with previously reported values for the sulfhydryl content in myosin (Barany *et al.*, 1964; Weeds and Hartley, 1968). Table I summarizes the distribution of sulfhydryl groups in myosin. Approximately 86% of the myosin sulfhydryl groups are located in the heavy meromyosin portion of the molecule and 80% located in subfragment I which carries the adenosine triphosphatase activity. Since light-chain components of myosin contain approximately 4 sulfhydryl groups at most (Weeds and Lowey, 1971), about 30 sulfhydryl groups are located in the heavy-chain portion of subfragment I which constitutes the globular heads of myosin molecule. Since the ^{35}Cl nmr experiments reported monitor the sulfhydryl groups only, the changes in conformation of myosin observed on addition of mercury may tentatively be ascribed to a conformational disruption of the heavy-chain core followed by the changes in the quaternary structure of subfragment I. This suggestion is supported by the mercurial-induced dissociation of light-chain subunits (Samaha *et al.*, 1970) and the absence of significant changes in optical rotatory strength of myosin on addition of PCMB (Warren *et al.*, 1966).

It has been recently demonstrated that myosin has two high affinity calcium sites on the light-chain subunits and about 50 nonspecific sites on the heavy-chain core (L. Emme, M. H. Han, and A. Rosenberg, to be published). Since little change in the nmr titration curve is observed in the presence of 5 mM Ca^{2+}, it appears that there is no direct relationship between calcium binding and modification of SH groups in myosin. However, addition of Mg-ADP does stabilize the protein against the conformation change observed by chlorine-35 nmr. Nucleotide-induced conformational changes in the myosin active site have been observed using difference spectra (Morita and Shimizu, 1969; Sekiya and Tonomura, 1967) and electron spin resonance (esr) techniques (Seidel *et al.*, 1970), and fluorescence probe methods (Cheung, 1969). It has also been demonstrated that salt-dependent dissociation of light-chain subunits can be stabilized by magnesium or calcium nucleotide complexes (Gershman *et al.*, 1969; Dreizen and Gershman, 1970).

In conclusion these experiments demonstrate that there are several types of sulfhydryl groups in myosin which may be distinguished by their reactivity with tetrachloromercurate ion. This result is consistent with earlier work using other sulfhydryl reagents such as PCMB; however, these experiments show in an addition that the dramatic conformation change in the globular portion of myosin induced by high levels of the mercury places all protein-bound mercury in a position where it may readily exchange chloride ion with the solution. The mercury interaction is different from the other modifiers of myosin in that the activity of the calcium-activated adenosine triphosphatase is not enhanced by reaction of the protein with mercuric ion although it is significantly stimulated by the reaction with PCMB and other sulfhydryl reagents. The cause for this difference may be that the two coordinate nature of the sulfur-bound mercuric ion causes extensive cross-linking of protein groups in the myosin molecule with resulting local denaturation and commensurate loss of enzymic activity.

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Specificity of Interaction of Haptoglobin with Mammalian Hemoglobin†

Marvin W. Makinen,* Julie B. Milstien,‡ and Hideo Kon

ABSTRACT: Complex formation of human haptoglobin with various mammalian hemoglobins is investigated as a model system of specific interaction of globular protein surfaces. Haptoglobin–hemoglobin complexes are examined through a variety of spectroscopic methods with regard to the arrangement of bound subunits of hemoglobin on the haptoglobin molecule, conformational properties of the complex with ligand binding by the heme iron, structural changes in the heme environment, and the electronic structure of the heme iron. Perturbations on hemoglobin structure as a result of haptoglobin binding are correlated with the enhancement of peroxidatic activity associated with complex formation and with the stabilizing influence of haptoglobin against denaturation of hemoglobin. On the basis of electric birefringence studies, the α and β subunits of hemoglobin are established to be bound symmetrically with respect to the long axis of the haptoglobin molecule. Electric birefringence and electric dichroism studies also suggest that binding of hemoglobin is restricted to the terminal regions of the haptoglobin molecule with marked restriction of motional freedom of bound hemoglobin subunits. Both electric birefringence and optical rotatory dispersion studies demonstrate that human haptoglobin binds human hemoglobin specifically. Hemoglobins of other mammalian species form complexes with human haptoglobin, the structural properties of which are dependent upon the sequence of mixing haptoglobin and hemoglobin and upon the amino acid sequence of the hemoglobin. The amino acid sequences of five mammalian hemoglobins correlated with spectroscopic properties of corresponding haptoglobin–hemoglobin complexes are discussed with reference to the probable haptoglobin binding site of hemoglobin.

Specific interaction of globular protein surfaces is basic to the organization and biological function of numerous organized macromolecular assemblies. For instance, several multicomponent enzyme systems (Ebner, 1970) exhibit modified catalytic activity not associated with the isolated component proteins and functionally depend upon a *specifier* protein binding to the *catalytic* protein. Antigen–antibody systems exhibit high specificity in interaction as is evident in the formation of hemoprotein–antihemoprotein antibody complexes in which complex formation is sensitive to single amino acid substitutions (Nisonoff *et al.*, 1970; Reichlin, 1972). These examples illustrate well the intricate stereochemical demands of interaction of protein surfaces to form organized macromolecular assemblies. Study of this structural specificity is clearly of importance in order to understand the chemical behavior of multicomponent assemblies prevalent in biological systems and the role of intermolecular interactions in modifying enzymatic reactivity.

Complex formation between haptoglobin and hemoglobin provides an unusual example of interacting protein surfaces, for it is well known that haptoglobin binds hemoglobin in an almost irreversible manner with high specificity and marked changes in heme reactivity (for review, *cf.* Sutton, 1970). This system may have value in the investigation of the structural basis of protein surface–surface interactions of multicomponent complexes. This is especially true in view of the large body of knowledge on the chemical and structural properties of mammalian hemoglobins (Dayhoff and Eck, 1968; Perutz *et al.*, 1968; Perutz, 1969) as well as on the chemical structure of human haptoglobin (Black and Dixon, 1968; Barnett *et al.*, 1970).

In this communication we report an investigation of the interaction of various mammalian hemoglobins with human haptoglobin. Through application of a variety of spectroscopic methods we have investigated the influence of perturbations resulting from interacting protein surfaces of the haptoglobin–hemoglobin complex on multiple levels of structural organization of the multicomponent system. These include the relationship of hemoglobin subunits to each other upon binding to haptoglobin, conformational properties of the complex with ligand binding, and structural changes induced in the heme environment upon complex formation. Functional changes in heme reactivity measured as the enhancement of peroxidatic activity have been examined with regard to the electronic structure of the heme iron prosthetic center and the stabilization of hemoglobin against denaturation. This multi-

† From the Enzyme Chemistry Section, Laboratory of Biochemistry, National Institute of Dental Research (M. W. M.), and the Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism, and Digestive Diseases (J. B. M. and H. K.), National Institutes of Health, Bethesda, Maryland 20014. *Received June 13, 1972.*

* Address correspondence to this author at Laboratory of Molecular Biophysics, Oxford University, Oxford, England.

‡ Present address: Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014.