ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/18822827

Specificity of interaction of haptoglobin with mammalian hemoglobin

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 1972

Impact Factor: 3.02 · DOI: 10.1021/bi00771a004 · Source: PubMed

CITATIONS
22

READS
17

3 AUTHORS, INCLUDING:

Marvin William Makinen
University of Chicago
95 PUBLICATIONS **2,106** CITATIONS
SEE PROFILE

Julie B Milstien
103 PUBLICATIONS **1,884** CITATIONS
SEE PROFILE

Available from: Marvin William Makinen
Retrieved on: 26 January 2016

Stengle, T. R., and Baldeschwieler, J. D. (1967), *J. Amer. Chem. Soc. 89*, 3045.
Sudmeier, J. L., and Pesek, J. J. (1971), *Inorg. Chem. 10*, 860.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Warren, J. C., Stowring, L., and Morales, M. F. (1966), *J. Biol. Chem. 244*, 309.
Weeds, A. G. (1966), *Biochem. J. 104*, 44p.
Weeds, A. G. (1967), *Biochem. J. 105*, 25c.
Weeds, A. G., and Hartley, B. S. (1968), *Biochem. J. 107*, 531.
Weeds, A. G., and Lowey, S. (1971), *J. Mol. Biol. 61*, 701.

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.

spectroscopic approach has permitted an investigation of the structural properties associated with specific interactions of protein surfaces which may be responsible for alterations in heme reactivity. On the basis of these results a model for the structure of the haptoglobin–hemoglobin complex is proposed and the regions of the hemoglobin molecule probably involved in binding haptoglobin are indicated.

Methods and Materials

Human Hp[1] was prepared according to Connell and Shaw (1961). $Hp_{1\text{-}1}$ was not used in this study because of its limited supply. HbO_2 was prepared according to Perutz (1968) from freshly drawn heparinized blood. The Hb binding capacity of stock solutions of Hp was determined either according to Connell and Smithies (1959) or Roy *et al.* (1969) with use of freshly prepared human ferriHb only. Preparation of Hp–Hb complexes with Hb of other mammalian species used in this investigation was based always on the binding capacity determined with human ferriHb. Electrophoresis (Brown and Johnson, 1970) of $Hp_{2\text{-}1}$ with Hb of all mammalian species in 1:1 molar ratio indicated no detectable free Hb upon complex formation.

Enhancement of peroxidatic activity was determined at heme concentrations of $1\text{–}5 \times 10^{-6}$ M and at 1:1 molar ratios of $Hp_{2\text{-}1}$ to ferriHb (Connell and Smithies, 1959). The activity of the unbound ferriHb was always compared to that of the complex at identical heme concentration immediately upon addition of the ferriHb to the reaction mixture.

The determination of circular dichroism,[2] electron paramagnetic resonance (epr) absorption properties, and associated preparative procedures for Hb and Hp–Hb derivatives have been previously described (Makinen and Kon, 1971). ORD studies were carried out with use of a Cary Model 60 spectropolarimeter programmed for constant illumination. Molar rotations were calculated using the relationship: $[M]_\lambda = (\alpha/lc)(M_w/100)$, where α is the observed rotation in degrees at wavelength λ, l is the path length in decimeters, c is the concentration in grams per milliliter, and M_w is the molecular weight. The molecular weight of $Hp_{2\text{-}1}$ used for calculating molar rotations was that based on the amino acid content (Black *et al.*, 1970), and 64,500 was used for all species of mammalian Hb studied.

Electric dichroism and electric birefingence properties were determined with use of an instrument as described by Yamaoka and Charney (personal communication of unpublished results, 1971). The birefringence, δ, in degrees, is reported according to Yamaoka (1964). The Kerr constant, B, was determined as described in the text, as was the specific Kerr constant, B/C, expressed in units of $(\text{rad}\cdot\text{cm}^4)/(\text{g}\cdot\text{statvolt}^2)$. The reduced dichroism, $\Delta\epsilon/\epsilon$, is defined as $(\epsilon_\parallel - \epsilon_\perp)/\epsilon$, where $\epsilon_\parallel$ is the extinction coefficient with light polarized parallel to the electric field direction; $\epsilon_\perp$ is the extinction coefficient with light polarized perpendicular to the electric field direction; and ϵ is the isotropic extinction coefficient. Determination of the reduced dichroism has been previously described (Charney *et al.*, 1970).

Only ferriHb and Hp–ferriHb were utilized for electrooptic studies to assure no change in oxidation state of the heme iron after the extensive periods of dialysis necessary to achieve solvent conditions of low ionic strength (<0.0005 M sodium 2-(*N*-morpholino)ethanesulfonate). Spectral properties of ferriHb and Hp–ferriHb after dialysis were in agreement with those previously reported (Makinen and Kon, 1971), and the Hb binding capacity of Hp solutions had not been altered through dialysis at neutral pH. The pH was adjusted as necessary after dialysis with small amounts of dilute HCl or aqueous, carbonate-free NaOH. Hp prepared according to the method of Connell and Shaw (1961) contains a variable amount of other contaminant serum proteins consisting primarily of serum albumin. Purified human serum albumin (Mann Research Laboratories) exhibited no birefringence under the applied electric field strength conditions employed for the study of Hp and Hp–ferriHb solutions. Possible contributions to the birefringence signals of Hp and Hp–ferriHb solutions from other contaminant proteins such as α_2-globulins were similarly ruled out with use of a purified preparation of human α_2-globulin fraction obtained from Mann Research Laboratories.

Birefringence was determined at 308 nm since at this wavelength neither ferriHb nor Hp–ferriHb exhibited dichroism signals.[3] Furthermore, unbound ferriHb exhibited no birefringence at this wavelength under the applied field strength conditions employed for the Hp–ferriHb complex. All electrooptic measurements were made at 5–10° at a Hp concentration of $1\text{–}10 \times 10^{-6}$ M. The pulse length was adjusted to achieve orientation of macromolecules with minimal heating of solutions. Human ferriHb was employed for all electrooptic studies unless otherwise indicated.

Results

Electric Birefringence and Dichroism Studies on the Haptoglobin–Hemoglobin Complex. When a solution of molecules is subjected to a homogeneous electric field, polar or electrically asymmetric molecules become partially oriented with respect to the electric field vector (O'Konski, 1968). This effect gives rise in the case of optically asymmetric molecules to induced optical retardation or electric birefringence measured with incident plane-polarized light parallel or perpendicular to the direction of the applied, homogeneous electric field. If the molecule exhibits electromagnetic transitions in an accessible region of the spectrum, these transitions can give rise to linear dichroism related to their orientation with respect to the direction of the electric field vector. This effect is known as electric dichroism.

The orienting force of a molecule in the electric field is due to interaction of the applied electric field with the electric dipole moment of the molecule. The latter is dependent upon two molecular properties: (a) the anisotropy of polarizability, which includes effects of ion migration of

[1] The following nonstandard abbreviations are used in this text: dHb, reduced or deoxygenated hemoglobin; ferriHb, methemoglobin; Hb, hemoglobin; Hp, haptoglobin, with subscripts 1-1, 2-1, or 2-2 to indicate the phenotypic variant; Hp–Hb, haptoglobin–hemoglobin; *i.e.*, haptoglobin bound to hemoglobin; Hp–dHb, haptoglobin bound to deoxyHb; Hp–HbO_2 haptoglobin bound to HbO_2; analogously, Hp–ferriHb, haptoglobin bound to ferriHb.

[2] Circular dichroism will be defined as $\epsilon_l - \epsilon_r$, where ϵ_l and ϵ_r are the decadic molar extinction coefficients on a heme basis for left and right circularly polarized light, respectively, in units of $M^{-1}\ cm^{-1}$. Conversion into the alternate measure of circular dichroism in terms of molecular ellipticity is achieved through the formula $[\theta] \approx 2.303(4500/\pi)(\epsilon_l - \epsilon_r)$, where $[\theta]$ is in units of $(\text{deg}\ \text{cm}^2)/\text{dmole}$ (Moscowitz, 1960).

[3] In this region the absorption properties of the heme group appear to be nearly isotropic with respect to in-plane and out-of-plane polarization directions on the basis of single crystal spectroscopic studies of horse ferriHb (M. W. Makinen and W. A. Eaton, unpublished observations, 1971). The electric field induced birefringence should on this basis reflect primarily the optical anisotropy due to the protein moiety.

TABLE I: Comparison of the Specific Kerr Constants of Hp_{2-2} and Hp_{2-2}–ferriHb as a Function of pH.[a]

pH	B/C (rad·cm⁴/g·statvolt²)	
	Hp_{2-2}	Hp_{2-2}–ferriHb
6.0	0.15 ± 0.04	0.35 ± 0.05
6.8	0.22 ± 0.02	0.12 ± 0.01
8.0	0.28 ± 0.05	0.19 ± 0.01

[a] The Kerr constant for each molecular species was determined from the limiting slope of the plot of the birefringence, δ, *vs.* the square of the applied electric field strength, E^2, at three concentrations of protein.

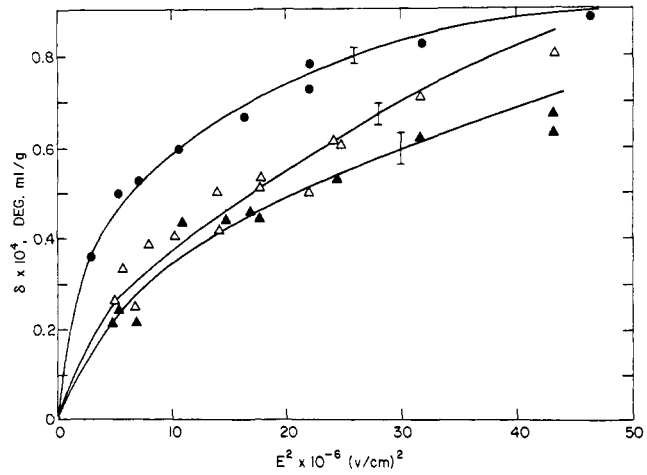

FIGURE 1: Dependence of the birefringence (δ) of Hp_{2-2} on the square of the applied electric field strength (E^2) compared to that of Hp_{2-2}–ferriHb complexes. The observed birefringence at 308 nm is indicated at constant Hp_{2-2} concentration for Hp_{2-2} (●), the fully saturated Hp_{2-2}–ferriHb complex (▲), and a mixture (△) of Hp_{2-2} and ferriHb added in 1:0.5 molar ratio at pH 6.8. Lines connecting experimental values are computer fitted, least-square curves determined according to the equation $\delta = aE^2 + bE + c$ as described by Milstien and Charney (1969). The bars indicating error limits represent a displacement of twice the standard deviation. The birefringence is indicated in (deg ml)/g of Hp_{2-2}.

polyelectrolytes; and (b) the permanent dipole moment resulting from the distribution of spatially separated, fixed charges. Thus, in principle, electric birefringence is capable of providing information about the orientation of the electric dipole moment with respect to overall molecular shape, and electric dichroism provides information about the configuration of chromophoric groups of a molecule with respect to the orientation of the electric dipole moment. In the case of polymeric macromolecules of biological interest, detailed structural information has been obtained on the basis of electric birefringence (*cf.* O'Konski, 1968; Haschemeyer, 1963; Cathou and O'Konski, 1970) and electric dichroism (Charney *et al.*, 1970; Allen and Van Holde, 1971) of proteins and polypeptides. Comprehensive reviews on the application and theoretical basis of these spectroscopic methods have been given by O'Konski (1968) and Charney (1971). In this investigation the methods have been applied to determine structural parameters of the Hp–Hb complex with particular emphasis on the orientation of the subunits of Hb upon binding to Hp.

The electric field induced birefringence of Hp_{2-2} and of Hp_{2-2}–ferriHb complexes[4] was measured at 308 nm to determine the orientation of these macromolecules as a function of increase in applied electric field strength. The general field strength behavior of the electric birefringence of C_0 and the C_{II} complex is illustrated in Figure 1. Comparison of their birefringence properties is made to a solution of Hp_{2-2} at equal concentration for which the total Hb binding capacity is half-saturated. The amount of birefringence depends on the applied electric field strength up to a point when saturation orientation is attained; *i.e.*, an increase in electric field gives no further increase in birefringence (Benoît, 1951).

The specific Kerr constant reflects the orienting force of a molecule (Peterlin and Stuart, 1939) and is calculated from the limiting slope of the plot of δ (in degrees) against the square of the electric field strength (in volts/centimeter) (Yamaoka, 1964). Estimates of Kerr constants were determined from data obtained at as low field strengths as possible for three concentrations of protein. The specific Kerr constants for Hp_{2-2} and for the completely saturated Hp_{2-2}–ferriHb complex determined as a function of pH are tabulated in Table I. The pH dependence of the Kerr constant of Hp_{2-2} appears to follow the general shape of its titration curve (Waks and Alfsen, 1966) within the limited pH range studied. For the C_{II} complex, on the other hand, the Kerr constant goes through a minimum near neutrality.

Electric birefringence studies initially carried out on Hp_{2-1} indicated that the birefringence properties were markedly dependent upon both aging and concentration of protein solutions. Further use of this phenotypic variant of human Hp in electrooptic studies was, therefore, terminated. On the other hand, experimental results with use of Hp_{2-2} were not influenced by these factors in the micromolar concentration range and low ionic strength employed. Determination of the specific Kerr constant for the C_0 and C_{II} species is a sensitive criterion of absence of change in aggregation state within the concentration range of Hp_{2-2} suitable for electrooptic studies.

The birefringence and dichroism properties of the Hp_{2-2}–ferriHb complex were not dependent upon the sequence of mixing of Hp_{2-2} and ferriHb components in preparing the complex with use of human ferriHb. On the other hand, the sequence of mixing with use of bovine or horse ferriHb produced a marked variation in specific Kerr constants of the resultant Hp_{2-2}–ferriHb complexes. Furthermore, though the Kerr constants for the complexes formed with bovine ferriHb were of the same order of magnitude as those containing human ferriHb, the horse ferriHb complexes gave Kerr constants which were decidedly concentration dependent and which extrapolated at zero protein concentration to values that were smaller by one order of magnitude. Further electrooptic studies with use of bovine or horse ferriHb complexed to Hp_{2-2} could not be carried out because of decreased signal-to-noise ratios. This change was probably caused by the

[4] Hp binds to Hb to form a complex of 1:1 molecular stoichiometry. Under conditions when the Hb binding capacity of a Hp solution is not saturated, binding results in formation of unsaturated (C_0), half-saturated (C_I), and fully saturated (C_{II}) molecules of Hp. These molecular species arise from the bivalent functional behavior of Hp characteristic of all three phenotypic variants (Malchy and Dixon, 1970) and correspond to the binding of 0, 0.5, and 1.0 mole of Hb per mole of Hp, respectively. In the case of Hp_{1-1}, the distribution of C_0, C_I, and C_{II} has been shown to conform to the expansion of $(p + q)^2$, where $p = 1 - q$ and q is the fractional saturation of Hb binding sites (Peacock *et al.*, 1970). Since polydispersity of Hp_{2-2} complexes was not observed under the conditions employed for electrooptic studies, we have applied the same relationship for the binding of Hb to Hp_{2-2}.

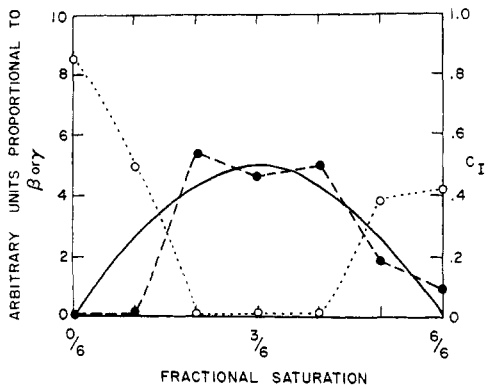

FIGURE 2: Comparison of the dependence of the permanent dipole moment and anisotropy of polarizability of Hp_{2-2} as a function of saturation of Hb binding sites at pH 6.8. The left-hand ordinate scale indicates values of β (●) and γ (○) in arbitrary units reflecting the change in the permanent dipole moment and anisotropy of polarizability, respectively. The solid trace represents the variation in concentration of the intermediate half-saturated complex C_I as a function of the molar ratio of ferriHb added to Hp_{2-2} (fractional saturation).

marked differences in macromolecular structure induced by binding of Hb of nonhuman origin to Hp_{2-2}.

Either a permanent or an induced dipole moment can cause orientation in an electric field, and assessment of the relative contribution of each to the orienting mechanism provides a sensitive parameter of molecular structure. O'Konski *et al.* (1959) have developed a convenient form of analysis of saturation data of the electric birefringence of axially symmetric macromolecules to determine the relative contributions of the induced dipole moment and of the permanent dipole moment to the orienting mechanism. If a molecule has a permanent dipole moment μ and an anisotropy of polarizability $\Delta\alpha$, the parameters $\beta = \mu E/kT$ and $\gamma = \Delta\alpha E^2/2kT$ are defined (O'Konski *et al.*, 1959), where E is the electric field strength, k is Boltzmann's constant, and T is the absolute temperature. The analysis then involves comparing a plot of $(\delta/E^2)/(\delta/E^2)_{E\to 0}$ *vs.* E^2 to a set of theoretical curves describing $(\delta/E^2)/(\delta/E^2)_{E\to 0}$ as a function of $\beta^2 + 2\gamma$

$$(\delta/E^2)/(\delta/E^2)_{E\to 0} = 15\Phi(\beta,\gamma)/(\beta^2 + 2\gamma)$$

where $\Phi(\beta,\gamma)$ is the orientation factor as defined by O'Konski *et al.* (1959). Comparison of the plot of experimental data against the curves constructed as functions of $\beta^2 + 2\gamma$ allows assessment of the relative contributions of each term to the orienting force of a molecule[5] (*cf.* Yamaoka, 1964, for a detailed discussion of the graphical method of analysis).

To this end the field strength behavior of the electric birefringence of Hp_{2-2} at 308 nm was studied as a function of applied electric field strength under conditions of varying the degree of saturation of Hb binding sites at pH 6.8. The experimental data were then analyzed according to O'Konski *et al.* (1959) to evaluate the change in β or γ of Hp_{2-2} as a function of saturation of Hb binding sites. The relative behavior of β and γ has been plotted in Figure 2 according to the change in fractional saturation of Hb binding sites. The direction of change of the contribution of γ (the anisotropy of polarizability) to the orienting force as a function of fractional saturation follows an approximate parallel course to the decrease in concentration of C_0 on the left-hand side of the plot in Figure 2 and to the increase in concentration of C_{II} on the right-hand side when these concentrations are calculated according to Peacock *et al.* (1970).[4] One would expect γ to be more important in the orienting mechanism of a globular protein (Krause and O'Konski, 1963), and this condition is observed for both the C_0 and C_{II} species at the extreme values of fractional saturation. Moreover, analysis (O'Konski *et al.*, 1959) of the contributions of β and γ to the orienting mechanism of the C_0 and C_{II} species as a function of pH indicates that the change in the magnitude of the specific Kerr constant (Table I) parallels the change in the contribution of γ to the orienting force. Such parallel behavior of these parameters indicates that the aggregation state of each macromolecular species has not changed within the pH range investigated (Yamaoka, 1964).

The permanent dipole moment is dependent upon the static charge distribution and is a vector quantity, in contrast to the anisotropy of polarizability. On this basis β would be the change upon complex formation such that the various vector components contributing to the permanent dipole moment of ferriHb would be added to that of Hp_{2-2} depending upon the symmetry and composition of the C_0, C_I, and C_{II} species. The most striking observation, thus, to be made in Figure 2 is that the magnitude of β and consequently of the permanent dipole moment follows the change in concentration of the C_I complex. For this reason the change in concentration of C_I with change in fractional saturation has been plotted in Figure 2 to underline this relationship.

Benoît (1951) has shown that the field-free time dependence of the decay of the birefringence signal is related to hydrodynamic properties of the molecule whereby the rotational diffusion constant θ can be estimated according to the relation: $\delta = \delta_0 e^{-6\theta t}$. The field-free decay of the electric birefringence from the steady-state level under saturating electric field strengths is plotted for Hp_{2-2} in Figure 3A according to the above relation. From this plot θ is calculated to be 18,600 ± 110 sec^{-1}. The rate of decay of the electric birefringence of the C_{II} complex at 308 nm is compared in Figure 3B to that of the electric dichroism at 405 nm, the position of maximum absorbance of the Soret band. Birefringence and dichroism changes give identical values of θ, 9510 ± 80 sec^{-1}, indicating that the bound subunits of Hb relax with the complex and have little separate rotational freedom. Also shown in Figure 3B is the time dependence of the decay of the dichroism signal for a solution of Hp_{2-2} in which only one-half of the Hb binding sites are saturated. This yields a value for θ of 10,700 sec^{-1}. Since this value is based on dichroism measurements only, it reflects primarily the lower molecular weight C_I complex with a contribution from C_{II}. The observed increase in rotational diffusion constant from that of the C_{II} complex is, thus, consistent with the direction of change in effective molecular weight of the dichroic species.

With the assumption that each molecular species has the same general shape, that of an elongated ellipsoid with an

[5] Application of the graphical method of O'Konski *et al.* (1959) is warranted for axially symmetric molecules. The analysis was carried out under the assumption that the Hp_{2-2} molecule approximates an ellipsoid of revolution with a ratio of major to minor semiaxes of 4:1. This assumption appears justified since low-angle X-ray scattering studies (Waks *et al.*, 1969) indicate that the Hp_{1-1} phenotype approximates this molecular shape and since the two molecules differ in molecular weight by only 20% (Black *et al.*, 1970).[6]

[6] The predominant portion of the molecular weight of both Hp_{1-1} and Hp_{2-2} is comprised by the two heavy subunits common to each phenotype and responsible for binding the subunits of Hb (*cf.* Sutton, 1970).

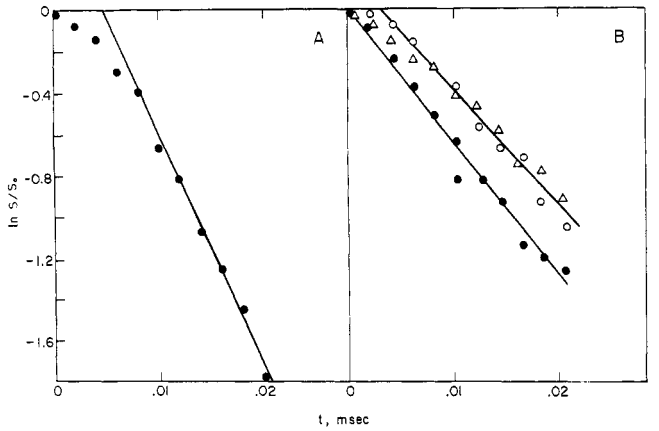

FIGURE 3: Time-dependent decay of birefringence and dichroism of Hp–ferriHb complexes. (A) Decay of the birefringence of Hp_{2-2} as a function of time after turning off of the applied electric field. (B) Comparison of the decay of the birefringence (△) and dichroism (○) of the fully saturated Hp_{2-2}–ferriHb complex after turning off the applied electric field. Solid circles represent the decay of the dichroism of a mixture of fully saturated (C_{II}) and half-saturated (C_I) Hp_{2-2}–ferriHb complexes achieved by addition of ferriHb to Hp_{2-2} in a 0.5:1 molar ratio. Since visualization of the kinetic decay was limited by the response time of the oscilloscope, each plot is the average of several determinations. The nonlinear portions at $t < 0.01$ msec are consequently neglected in determination of the rotational diffusion constant. The solid lines connecting data points represent a least-squares determination of the slope for each plot. S indicates the magnitude of either the birefringence or dichroism signal at time t; S_0, the corresponding initial value.

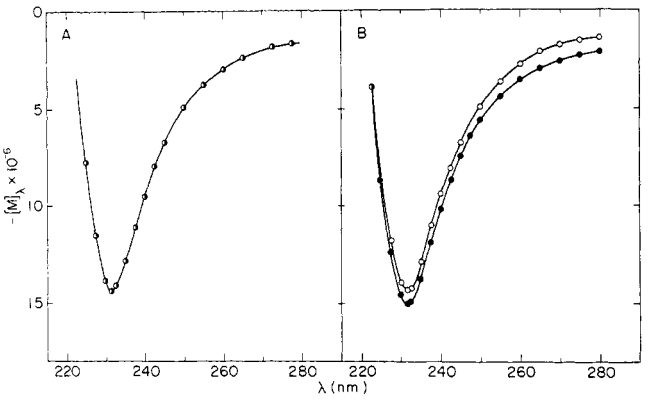

FIGURE 4: Comparison of the far-ultraviolet optical rotatory dispersion spectra of oxy (○) and deoxy (●) forms of Hp_{2-1}–Hb complexes formed with human (part A) and bovine (part B) HbO_2. The half-filled circles in part A indicate the superposition of spectra of both oxy and deoxy derivatives. HbO_2 was added in equimolar quantities to Hp_{2-1} at pH 7.0 in 0.05 M sodium phosphate buffer. The same solution of Hp_{2-1}–HbO_2 was employed to determine both spectra, deoxygenation being effected by addition of a minimum quantity of solid sodium dithionite. Cuvets were quickly sealed after dithionite addition for spectral recording.

axial ratio of 4:1, approximate dimensions of the C_0 and C_{II} complexes have been estimated from the rotational diffusion constants according to the relation

$$\theta = [3kT(\ln 2p - 0.5)]/8\pi\eta a^3$$

where k is Boltmann's constant, T is the absolute temperature, η is the viscosity of the solvent, a is the major semiaxis of rotation of the molecule, and p is the axial ratio (Perrin, 1934). The relationship between θ and a is relatively insensitive to the choice of p. A comparison of these structural parameters given in Table II implies that binding of the subunits of ferriHb to Hp_{2-2} is restricted to the terminal regions of the Hp_{2-2} molecule since an increase in the major semiaxis is observed upon complex formation.

Optical Rotatory Dispersion and Circular Dichroism of Haptoglobin–Hemoglobin Complexes. Optical rotatory dispersion (ORD) and circular dichroism (CD) have been widely applied in the study of protein conformation to characterize the nature of conformational changes induced by chemical or physical means. In view of the marked motional restriction of subunits of human Hb bound to Hp_{2-2} and the inequivalence of complex formation dependent upon the sequence of mixing bovine or horse ferriHb with Hp_{2-2}, we have applied these optical methods to investigate the interaction of Hp with Hb of different species origin.

The ORD spectra of mammalian Hb derivatives complexed to Hp are not comparable to those of the corresponding unbound Hb in the far-ultraviolet wavelength region. The ORD spectra of oxy and deoxy derivatives of human and bovine Hb complexed to Hp_{2-1} are illustrated in Figure 4. The spectra of the derivatives formed with Hb of these species are representative of Hb from all mammalian species utilized in this investigation. For human HbO_2 complexed to Hp_{2-1} (Figure 4A) the minimum in optical rotation is located at 231 nm. There is no change in the spectrum upon dithionite addition. Mouse and canine HbO_2 upon complex formation with Hp_{2-1} displayed comparable ORD properties. The absence of an observable change in the ORD spectrum upon deoxygenation stands in marked contrast to the spectral changes observed for free HbO_2 upon deoxygenation, reflecting structural alterations associated with the transition to the unliganded conformation (Brunori *et al.*, 1967).

The ORD spectrum of bovine HbO_2 complexed to Hp_{2-1} undergoes a general increase in optical activity upon oxygen removal (Figure 4B). This stands in contrast to the increase in optical activity associated with deoxygenation of HbO_2 observed only in the region of minimum rotation (Brunori *et al.*, 1967) under comparable solvent conditions. The position of minimum rotation is located at 232 nm, only slightly shifted from that of unbound Hb derivatives at 233 nm. The ORD behavior of rat and guinea pig HbO_2 complexed to Hp_{2-1} was identical with that observed for bovine HbO_2 with respect to the position of minimum rotation and increase in optical activity upon oxygen removal. Horse HbO_2 complexed to Hp_{2-1} displays a similar ORD spectrum. Addition of dithi-

TABLE II: Comparison of Structural Parameters of Hp_{2-2} and the Hp_{2-2}–ferriHb Complex.[a]

Molecular Species	Mol Wt	Rotational Diffusion Constant θ (sec^{-1})	Major Semiaxis (Å)
Hp_{2-2} (C_0)	120,050[b]	18,600 ± 110	73
Hp_{2-2}–ferriHb (C_{II})	184,550	9,510 ± 80	93

[a] The rotational diffusion constant, θ, was determined from the decay of the electric birefringence signal at 308 nm and was used to calculate a, the major semiaxis of revolution. See text for description. [b] Calculated on the basis of amino acid content (Black *et al.*, 1970).

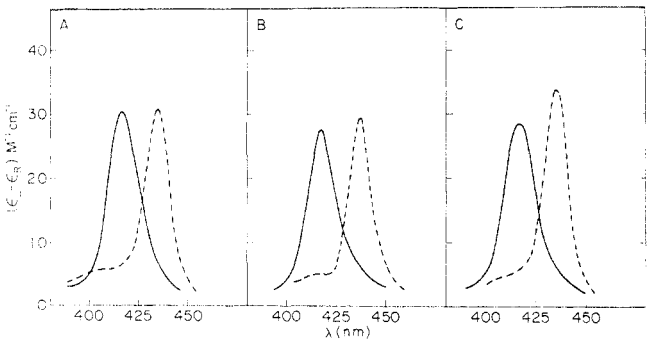

FIGURE 5: Comparison of Soret circular dichroism of oxy (—) and deoxy (- - -) forms of Hp_{2-1}–Hb complexes formed with human (A), mouse (B), and bovine (C) HbO_2. Conditions as in Figure 4.

onite, however, in the latter case resulted in an approximate 5% decrease in optical activity at 232 nm with an isosbestic point for oxy and deoxy spectra at 245 nm. Although the spectral changes of the horse Hb complex were not identical with those of the bovine Hb complex upon oxygen removal, the spectra of the oxy and deoxy complexes were clearly not superimposable, in marked contrast to the behavior of human HbO_2 bound to Hp_{2-1}. Reversal of the Hp_{2-1}–dHb spectrum to that observed for the corresponding Hp_{2-1}–HbO_2 complex was demonstrated by introduction of gaseous carbon monoxide into the solution of Hp_{2-1}–dHb in all cases.

Furthermore, the ORD spectral properties did not depend upon the sequence of mixing Hp_{2-1} and HbO_2 reactants. Changes in optical activity upon ligand removal for the bovine-like Hp_{2-1}–HbO_2 complexes were observed also under conditions of excess Hp_{2-1}. ORD spectra of oxy and deoxy derivatives of Hp–Hb complexes formed with all mammalian Hb molecules were identical in the spectral region below 220 nm.

Circular dichroism studies of the Hp–Hb complex formed with human Hb have demonstrated large changes in rotational strength of the Soret transition associated with the binding of Hb to Hp (Makinen and Kon, 1971), and comparable changes are associated with the binding of nonhuman Hb derivatives. Circular dichroism spectra of Hp_{2-1}–HbO_2 and Hp_{2-1}–dHb complexes formed with Hb of three mammalian species utilized in ORD studies are compared in Figure 5. Removal of oxygen by dithionite addition results in a change in position of apparent maximum circular dichroism from 417 nm for the oxy liganded complex to 435 nm for the deoxy derivative[7] in general, and each spectrum differs markedly from that observed for the corresponding free Hb derivative (Makinen and Kon, 1971). On the basis of theoretical studies of Hsu and Woody (1969, 1971), the marked changes in Soret circular dichroism for each Hb upon Hp–Hb complex formation are indicative of structural alterations in the heme

[7] The circular dichroism spectrum in the Soret region of Hp_{2-1}–HbO_2 formed with canine HbO_2 is identical with that with human HbO_2. Addition of dithionite, however, results in a spectrum markedly different from that observed for other Hp_{2-1}–dHb complexes in Figure 6. The apparent maximum $\epsilon_l - \epsilon_r$ is located near 410 nm with a less intense band near 435 nm. Free canine dHb exhibits maximum circular dichroism near 417 nm. These altered spectra in comparison to those of other Hb variants arise presumably from the markedly different content of aromatic amino acids of canine Hb (Jones *et al.*, 1971). Circular dichroism of the Soret transition arises predominantly from the interaction of the Soret transition with $\pi \rightarrow \pi^*$ transitions of nearby aromatic amino acid residues (Hsu and Woody, 1969, 1971).

TABLE III: Comparison of Peroxidatic Activity of Mammalian ferriHb and of Corresponding Hp_{2-1}–ferriHb Complexes.

Species of ferriHb	Peroxidatic Activity[a] ferriHb, pH 5.5	Hp–ferriHb, pH 4.0	Peroxidatic Enhancement[b]
Human	1.2	3.34	43.2
Mouse (C57BL)	1.9	2.86	28.0
Canine		3.56	5.7
Horse	2.8	4.32	3.5
Bovine	2.4	3.02	1.1
Guinea pig	3.3	4.24	1.1
Rat (Sprague Dawley)		4.44	1.1

[a] Calculated as [ΔOD_{470}/sec per nmole of heme) $\times 10^3$] for oxidation of guaiacol to tetraguaiacol as described by Connell and Smithies (1959). [b] Indicates the ratio of the peroxidatic activity of Hp–ferriHb to that of free ferriHb at pH 4.0.

environment induced through binding to Hp. Although there are small differences in apparent maximum circular dichroism of Hp_{2-1}–dHb complexes, depending on the species origin of mammalian Hb, these differences cannot be readily interpreted. It is probable that they reflect variations in amino acid residues included in the nearby heme environment and inequivalence of structural changes in the heme surroundings dependent upon the binding of each Hb to the Hp_{2-1} molecule.

Electron Paramagnetic Resonance Absorption and Enhancement of Peroxidatic Activity of Haptoglobin–Hemoglobin Complexes. Binding of Hb by Hp is associated with a marked change in heme reactivity, first observed as the enhancement of peroxidatic activity of Hb (Polonovski and Jayle, 1938). It has been established that the enhancement in peroxidatic activity arises in part from stabilization of the functional integrity of the prosthetic heme center against denaturation of Hb by guaiacol (Makinen and Kon, 1971). In view of the different optical properties of Hb variants upon binding to Hp suggesting nonequivalence of Hp–Hb complex formation, we have investigated the enhancement of peroxidatic activity of ferriHb upon binding to Hp_{2-1} dependent upon the species origin of Hb. These studies have been carried out in part with parallel investigations by epr techniques on the effect of guaiacol on Hb and Hp–Hb complexes.

Table III shows a comparison of the peroxidatic activity of each mammalian ferriHb at pH 5.5 and of the corresponding Hp_{2-1}–ferriHb complex at pH 4.0. Under conditions of tetraguaiacol formation, ferriHb appears to exhibit maximum activity at pH 5.5 and the Hp–ferriHb complex at pH 4.0 (Connell and Smithies, 1959). The ratio of the activity of the Hp–ferriHb complex to that of the unbound ferriHb at pH 4.0 is indicated as the peroxidatic enhancement. It is evident that although the absolute activity of the Hp–ferriHb complex at pH 4.0 is dependent upon the species origin of ferriHb, there is a clear gradation in the peroxidatic enhancement according to the order of species indicated in Table III. There is a corresponding but less pronounced gradation in the ratio of the maximum activity of the Hp_{2-1}–ferriHb complex at pH 4.0 to that of the unbound ferriHb at pH 5.5. The peroxidatic activity of each Hp–ferriHb complex is comparable

at pH 5.5 and at pH 7 to that of the corresponding unbound ferriHb.

The epr absorption spectrum of bovine HbNO and its corresponding Hp_{2-1}–HbNO complex in the presence of 0.030 M guaiacol is shown in Figure 6. At this concentration of guaiacol, peroxidatic activity of the Hp–ferriHb is near maximal and that of ferriHb is inhibited almost completely (Connell and Smithies, 1959). The spectra of corresponding horse HbNO complexes were similar and both types of mammalian HbNO exhibit behavior equivalent to that observed for human HbNO (Makinen and Kon, 1971) upon complex formation with Hp_{2-1}. The effect of guaiacol on all three types of HbNO in shifting the spectrum to a lower magnetic field, in decreasing the absorption at $g = 1.98$, and in inducing the three peaks due to ^{14}N hyperfine structure is, thus, comparable. These changes are indicative of protein denaturation which perturbs the rhombic structure of the nitric oxide liganded paramagnetic heme center (Kon, 1968). The complex formed with Hp_{2-1}, as is evident in Figure 6, retains to a much greater extent the inverted S-shaped region near $g = 2.030$ with bands near $g = 2.060$ and 1.986 as observed for HbNO in the absence of guaiacol. Since the effect of guaiacol on the nitric oxide liganded complexes of horse and bovine Hb is similar to that observed for human HbNO and complex formation with Hp results in inhibition of the spectral perturbations from guaiacol comparable to that observed for human HbNO, we have not examined the effect of guaiacol on Hp–HbNO complexes formed with Hb from other mammalian species listed in Table III. The results of this comparison of human, horse, and bovine HbNO imply that Hb of other mammalian species would behave similarly since human and bovine Hb represent the extremes in the spectrum of peroxidatic enhancement (Table III).

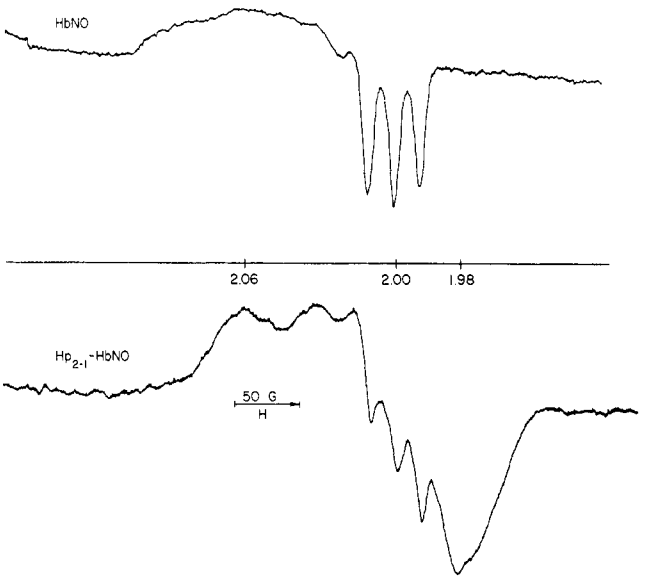

FIGURE 6: Electron paramagnetic resonance absorption of bovine HbNO (upper trace) compared to that of bovine HbNO complexed to Hp_{2-1} (lower trace) at 77°K and in the presence of 0.030 M guaiacol. HbNO and Hp_{2-1}–HbNO at a concentration of 10^{-4} M in heme were incubated in the presence of guaiacol in 0.1 M phosphate buffer (pH 7.0) for 5 min. The solutions were then frozen at the temperature of liquid nitrogen for spectral recording. The scales for identifying the positions of electron paramagnetic resonance absorption and for a displacement of 50 G apply to both spectra.

Discussion

Structural Implications of Electrooptic Studies. Electric birefringence and electric dichroism have been demonstrated to be powerful spectroscopic aids in studying conformational properties of biological proteins and polypeptides and configurational properties of their chromophores (for review *cf.* O'Konski, 1968; and Charney, 1971). These methods have been employed to gain information about structural properties of Hp and of the Hp–Hb complex. Analysis of the orienting mechanism of Hp complexes under the force of an applied electric field has required correlation of results with kinetic (Nagel and Gibson, 1967, 1971) and immunochemical (Kagiyama *et al.*, 1968) studies on Hp–Hb complex formation in order to deduce the binding arrangement of the Hb subunits on the Hp molecule.

From Figure 2 it is evident that the change in the permanent dipole moment parallels the change in the concentration of C_I, the complex in which only one pair of binding sites is occupied by Hb subunits. On the basis of the studies of Kagiyama *et al.* (1968), the C_I complex consists of a Hp molecule bound to an α and a β subunit of Hb. The change in the permanent dipole moment as a function of saturation of Hb binding sites of Hp_{2-2}, thus, indicates that the addition of the first pair of α and β subunits on the Hp_{2-2} molecule results in a substantial increase in the permanent dipole moment above that observed for Hp_{2-2} alone. This increase is in effect canceled through binding of the second pair of α and β subunits. The reduction in the permanent dipole moment upon saturation of the C_I complex probably reflects *symmetrical addition of the second half-molecule of Hb such that the vector components of the permanent dipole moment contribution from each bound $\alpha\beta$ pair cancel each other almost exactly.*

In Table II comparison of the change in the lengths of major semiaxis of the C_0 and of the C_{II} species shows an increase of approximately 20 Å upon binding of two $\alpha\beta$ dimers by Hp_{2-2}. Since the dimensions of the mammalian Hb molecule are of the order of 64 × 55 × 50 Å (Perutz, 1969), the increase in the length of the major semiaxis implies that the binding of Hb subunits does not consist simply of an end-on attachment of each $\alpha\beta$ dimer to the Hp_{2-2} molecule. The arguments outlined above for symmetrical binding of α and β in conjunction with the results tabulated in Table II suggest that *the subunits of Hb are bound not only symmetrically but also with each pair of α and β subunits located near the terminal regions of the Hp_{2-2} molecule.* The binding of Hb by Hp appears to be predominantly directed between the α subunit of Hb and the Hp molecule on the basis of fluorescence studies by Nagel and Gibson (1967) and Chiancone *et al.* (1968). Although separate binding sites for both α and β subunits are present, the binding site for the β subunit is induced by binding of the α subunit (Nagel and Gibson, 1971). The binding of Hb, therefore, may occur in a manner such that the α subunit yields more of its surface in binding to Hp than does the β subunit and that the β subunit is primarily responsible for the observed increase in length of the major semiaxis. A diagrammatic scheme of models of the Hp–Hb complex consistent with electrooptic results in Figure 7 illustrates the two possible symmetrical arrangements of the bound subunits of Hb. Both models would result in identical dichroism properties but differ in the arrangement of bound Hb subunits with respect to a plane containing the major axis of the Hp molecule. It is not possible to distinguish further between these models on the basis of electrooptic studies.

Waks *et al.* (1969) have reported the radii of gyration of the Hp_{1-1} and Hp_{1-1}–Hb molecules obtained from low-angle

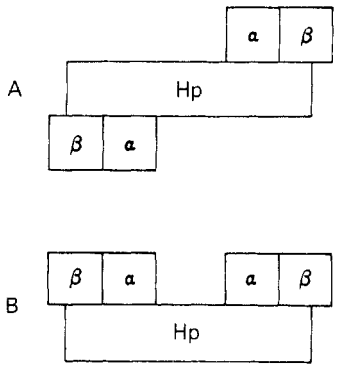

FIGURE 7: Schematic representation of the symmetrical binding of the α and β subunits of Hb to Hp. Both schemes A and B indicate that the bound subunits are related by a twofold axis of symmetry perpendicular to the long axis of the Hp and cannot be distinguished on the basis of electrooptic studies. It is not known whether the α and β subunits retain interchain amino acid contacts with each other upon Hp–Hb complex formation.

X-ray scattering studies. Calculations according to the relationship between the radius of gyration and the length of a cylindrical rod-shaped molecule (Tanford, 1961) suggest that the difference in length of the Hp_{1-1} and Hp_{1-1}–Hb molecules approximates 20 Å. Since all phenotypic variants of human Hp have a common heavy chain[8] which binds both subunits of Hb (Gordon and Bearn, 1966), comparison of the corresponding calculated structural parameters of Hp_{1-1} and Hp_{1-1}–Hb adds support to the interpretation of the results of our electrooptic studies on Hp_{2-2}. It is, therefore, likely that the mechanism of binding Hb for all phenotypic variants of Hp has a similar structural basis.

Enhancement of Peroxidatic Activity Dependent upon Species Origin of Hemoglobin. On the basis of circular dichroism, structural changes in the heme environment of Hb of all mammalian species utilized in this study have been demonstrated to occur upon complex formation in a manner similar to that described for human Hb (Makinen and Kon, 1971). These structural changes have little influence upon the electronic structure of the prosthetic heme groups, as is especially well demonstrated by the close identity of the epr absorption of Hp_{2-1}–HbNO complexes formed with human, horse, and bovine HbNO. The similar spectra of all Hp_{2-1}–HbNO complexes are indicative of equivalent electronic structures of the paramagnetic center, especially of the axially directed orbitals of the heme iron atom. These results suggest in agreement with earlier studies (Makinen and Kon, 1971) that the enhancement of peroxidatic activity induced by complex formation is not the result of perturbations on the electronic configuration of the iron d-orbital system. Furthermore, the comparable behavior of horse and bovine HbNO in the presence of guaiacol and Hp_{2-1} to that of human HbNO suggests that the high enhancement of peroxidatic activity observed for human ferriHb upon complex formation does not arise from a differential denaturing action of guaiacol dependent upon the species origin of Hb. The denaturing action of guaiacol on Hb structure appears to be equivalent toward the three types of Hb studied by epr techniques and appears to be hindered in an equivalent manner upon Hp–Hb complex formation.

The most important factor contributing to the enhancement in peroxidatic activity is probably related to the conditions used for the peroxidatic assay. The assay as described by Connell and Smithies (1959) is carried out in acetate buffer at pH 4.0. These are known denaturing conditions of horse and human ferriHb. It has been demonstrated that under these conditions the rate of denaturation of human ferriHb is greater than that of horse ferriHb (Steinhardt and Hiremath, 1967). Furthermore, on the basis of the rate of decrease in Soret absorption at 405 nm, there is a markedly slower rate of denaturation of bovine ferriHb than of human ferriHb under the conditions of the assay for peroxidatic enhancement (M. W. Makinen, unpublished observations, 1970). Thus, differences in enhancement of peroxidatic activity dependent upon the species origin of Hb would appear to arise primarily from the relative ease of denaturation of Hb under acidic solvent conditions.

Specificity of Hemoglobin Structure in Haptoglobin–Hemoglobin Complex Formation. The ORD spectra of Hp_{2-1}–HbO_2 and Hp_{2-1}–dHb complexes indicate that a conformational change of the protein moiety occurs upon deoxygenation of bovine, horse, guinea pig, and rat HbO_2 bound to Hp_{2-1} and that this change does not occur upon ligand removal from bound human HbO_2. The identity of the ORD spectra of complexes formed with human Hb (Figure 4A) indicates that *for human Hb bound to Hp equivalent conformational structures obtain for both liganded and unliganded states of the heme iron.* Conformational identity of both ligand states has been observed in a comparable manner for sperm whale myoglobin (Samejima and Yang, 1964) and the separated α subunit of human Hb (Brunori *et al.*, 1967). Since mouse and canine HbO_2 exhibited similar ORD properties upon complex formation, binding of these Hb derivatives by Hp is probably very similar to that of human Hb. The change in ORD properties of horse, bovine, guinea pig, and rat Hb bound to Hp upon loss of ligand suggests that binding does not occur in a manner equivalent to that of human Hb since conformational identity for both valence states of the heme iron is not observed. The markedly different electrooptic properties of horse and bovine ferriHb bound to Hp_{2-2} dependent upon the sequence of mixing reactants support this conclusion. On the basis of these results it is evident that *identical Hp–Hb complexes are formed with human Hb independent of the sequence of mixing of Hp and Hb components and that human Hb is specifically bound by Hp in a tight complex with markedly restricted movement of the bound α and β subunits.*

The results of our studies supply an explanation for the observations of Pavlíček and Jaenicke (1971) and Waks *et al.* (1969). These investigators have suggested that a series of Hp–Hb complexes of different geometry and composition are formed depending upon the Hp:Hb molar ratio and the sequence of mixing Hp and Hb components. However, they have used bovine (Pavlíček and Jaenicke, 1971) and horse (Waks *et al.*, 1969) Hb in their investigations. A comparable series of complexes formed with human Hb would not be expected on the basis of our results and, indeed, has not been detected.

From observations of the effect of chemical modifications of Hb on Hp–Hb complex formation, Lockhart and Smith (1971) have concluded that the Hp binding site of Hb is probably in the area of contact of the internal $\alpha\beta$ dimer–dimer interface regions and must be in a relatively lysine-poor region.

[8] The subunits of Hp are frequently labeled α and β in the literature. We have not employed this terminology to avoid confusion with designations for the subunits of Hb. The terminology *light* and *heavy chains* is made primarily on the basis of their comparative molecular weights and correspond to the α and β designations utilized by other investigators.

With this conclusion in mind we can consider implications about the probable Hp binding site of Hb on the basis of the results of our spectroscopic investigations and the amino acid sequences of mammalian Hb of several species utilized in this study.

On the basis of both electrooptic and ORD studies, human Hp binds specifically only human Hb with marked motional restriction of the bound subunits. Hp–Hb complexes formed with mouse and canine Hb appear to be more similar to that formed with human Hb in comparison to those formed with Hb of other mammalian sources. In this connection we note that human Hb readily forms hybrid molecules with both canine (Itano and Robinson, 1959) and mouse (Rosemeyer and Huehns, 1967) Hb but not with horse (Rosemeyer and Huehns, 1967) or bovine (Itano and Robinson, 1960) Hb. Comparison of the amino acid sequences of human, mouse, horse, bovine (*cf.* Dayhoff and Eck, 1968), and canine (Jones *et al.*, 1971) Hb indicates that certain regions of the sequences of Hb of nonhuman origin as a whole show frequent substitutions in comparison to the sequence of human Hb. These substitutions are concentrated largely near the 35, 104, and 108 amino acid positions in the α subunit and the 112 and 116 positions in in the β subunit. It is precisely these segments of the Hb protein chains which are involved in α_1–β_1 nonpolar contact regions (Perutz, 1969) and which may be involved in binding to Hp. Rosemeyer and Huehns (1967) have concluded in their studies on the mechanism of dissociation of Hb that the α_{104} and β_{112} regions are intimately associated with the cleavage plane of the molecule into α_1–β_1 dimers and that these regions are important in formation of hybrid molecules. Furthermore, the α_1–β_2 contact positions are invariant for all five species of Hb. It is probable that complementarity of contact surfaces similar to that necessary for hybridization of Hb subunits is also necessary for specific binding between Hb subunits and the Hp molecule. It would appear, therefore, that only if the stereochemical demands are satisfied for complementarity of binding surfaces can equivalent complexes be formed between Hp and Hb independent of the sequence of mixing and with marked motional restriction of the bound α and β subunits.

Since it is unlikely that the tertiary as well as the quaternary structures of Hb of different species origin differ greatly in view of the similar structures of horse and human Hb (Perutz, 1969), large conformational differences in Hb structure cannot account for the observed specificity of human Hp binding human Hb. It is well established through immunochemical studies of Hb (Reichlin, 1972) and of cytochrome *c* (Nisonoff *et al.*, 1970) that the binding of a globular protein by an antibody molecule is sensitive to single amino acid substitutions which affect only the local topological structure of the determinant region of the protein. It is, therefore, reasonable to conclude that the structural basis for the specificity of human Hp in binding human Hb is comparable to that observed in immunochemical reactions, that is, sensitive to topological changes in Hb structure. These results demonstrate the distinctive specificity in the interaction of two globular protein surfaces to form a tightly bound multicomponent complex.

Acknowledgment

We thank Mr. L. L. Taylor, Mr. R. S. Johnson, and Dr. A. C. Peacock for carrying out electrophoretic determinations of Hp phenotype. It is a pleasure to acknowledge the helpful discussions with Drs. E. Charney and W. A. Eaton. We deeply appreciate the encouragement and support given by Dr. J. E. Folk during the course of this investigation.

References

Allen, F. S., and Van Holde, K. E. (1971), *Biopolymers 10*, 865.
Barnett, D. R., Lee, T. H., and Bowman, B. H. (1970), *Nature (London) 225*, 938.
Benoît, H. (1951), *Ann. Phys. (Paris) 6*, 561.
Black, J. A., Chan, G. F. Q., Hew, C. L., and Dixon, G. H. (1970), *Can. J. Biochem. 48*, 123.
Black, J. A., and Dixon, G. H. (1968), *Nature (London) 218*, 736.
Brown, K. S., and Johnson, R. S. (1970), *Hum. Hered. 20*, 281.
Brunori, M., Engel, J., and Schuster, T. (1967), *J. Biol. Chem. 242*, 773.
Cathou, R., and O'Konski, C. T. (1970), *J. Mol. Biol. 48*, 125.
Charney, E. (1971), *Proc. Nucl. Acid Res. 2*, 176.
Charney, E., Milstien, J. B., and Yamaoka, K. (1970), *J. Amer. Chem. Soc. 92*, 2657.
Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finazzi Agrò, A., Wyman, J., and Antonini, E. (1968), *J. Mol. Biol. 34*, 247.
Connell, G. E., and Shaw, R. W. (1961), *Can. J. Biochem. Physiol. 39*, 1013.
Connell, G. E., and Smithies, O. (1959), *Biochem. J. 72*, 115.
Dayhoff, M. O., and Eck, R. V. (1968), Atlas of Protein Sequence and Structure, Silver Spring, Md., National Biomedical Research Foundation, pp 123–158.
Ebner, K. E. (1970), *Accounts Chem. Res. 3*, 41.
Gordon, S., and Bearn, A. G. (1966), *Proc. Soc. Exp. Biol. Med. 121*, 846.
Haschemeyer, A. E. V. (1963), *Biochemistry 2*, 251.
Hsu, M. C., and Woody, R. W. (1969), *J. Amer. Chem. Soc. 91*, 3679.
Hsu, M. C., and Woody, R. W. (1971), *J. Amer. Chem. Soc. 93*, 3515.
Itano, H. A., and Robinson, E. (1959), *Nature (London) 184*, 1468.
Itano, H. A., and Robinson, E. (1960), *Ann. N. Y. Acad. Sci. 88*, 642.
Jones, R. T., Brimhall, B., and Duerst, M. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1259.
Kagiyama, S., Ogawa, A., and Kawamura, K. (1968), *Proc. Jap. Acad. 44*, 1060.
Kon, H. (1968), *J. Biol. Chem. 243*, 4350.
Krause, S., and O'Konski, C. T. (1963), *Biopolymers 1*, 503.
Lockhart, W. L., and Smith, D. B. (1971), *Can. J. Biochem. 49*, 148.
Malchy, B., and Dixon, G. H. (1970), *Can. J. Biochem. 48*, 192.
Makinen, M., and Kon, H. (1971), *Biochemistry 10*, 43.
Milstien, J. B., and Charney, E. (1969), *Macromolecules 2*, 678.
Moscowitz, A. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., Ed., New York, N. Y., McGraw-Hill, Chapter 12, p 150.
Nagel, R. L., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 3428.
Nagel, R. L., and Gibson, Q. H. (1971), *J. Biol. Chem. 246*, 69.
Nisonoff, A., Reichlin, M., and Margoliash, E. (1970), *J. Biol. Chem. 245*, 940.
O'Konski, C. T. (1968), *Encycl. Polym. Sci. Technol. 9*, 551.
O'Konski, C. T., Yoshioka, K., and Orttung, W. H. (1959), *J. Phys. Chem. 63*, 1558.

Pavlíček, Z., and Jaenicke, R. (1971), *Eur. J. Biochem. 18*, 305.
Peacock, A. C., Pastewka, J. V., Reed, R. A., and Ness, A. T. (1970), *Biochemistry 9*, 2275.
Perrin, F. (1934), *J. Phys. Radium 5*, 497.
Perutz, M. F. (1968), *J. Crystal Growth 2*, 54.
Perutz, M. F. (1969), *Proc. Roy. Soc. B 173*, 113.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.
Peterlin, A., and Stuart, H. A. (1939), *Z. Physik 112*, 129.
Polonovski, M., and Jayle, M. F. (1938), *C. R. Soc. Biol. 129*, 457.
Reichlin, M. (1972), *J. Mol. Biol. 64*, 485.
Rosemeyer, M. A., and Huehns, E. R. (1967), *J. Mol. Biol. 25*, 253.
Roy, R. B., Shaw, R. W., and Connell, G. E. (1969), *J. Lab. Clin. Med. 74*, 698.
Samejima, T., and Yang, J. T. (1964), *J. Mol. Biol. 8*, 863.
Steinhardt, J., and Hiremath, C. B. (1967), *J. Biol. Chem. 242*, 1294.
Sutton, H. E. (1970), *Prog. Med. Genet. 7*, 163.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, Chapter 5, p 306.
Waks, M., and Alfsen, A. (1966), *Arch. Biochem. Biophys. 113*, 304.
Waks, M., Alfsen, A., Schwaiger, S., and Mayer, A. (1969), *Arch. Biochem. Biophys. 132*, 268.
Yamaoka, K. (1964), Ph.D. Dissertation, University of California, Berkeley, Calif.

Use of Resonance Interaction in the Study of the Chain Folding of Insulin in Solution†

D. A. Mercola,*‡ J. W. S. Morris,§ and Edward R. Arquilla

ABSTRACT: Certain relationships of the chain folding within the monomer of bovine insulin in solution have been studied by covalently labeling the three amino groups of insulin with an optical probe. Three purified derivatives containing from one to three groups of the symmetric dye fluorescein isothiocyanate bound to the B-1; B-1 and A-1; B-1, A-1, and B-29 residues (Bromer *et al.* (1967), *Biochemistry 6*, 2378) were examined by absorption and circular dichroism spectroscopy between 400 and 550 nm. The results were interpreted according to the simplified exciton model. This approach was confirmed by the examination of three model compounds prepared by the covalent addition of the dye group to models of the insulin sequence at the sites of substitution and a fourth model compound prepared by disubstitution of L-lysine. These monosubstituted model compounds had absorption spectra similar to the free dye, maxima at 489–492 nm, were nearly optically inactive (400–550 nm), and had similar probe group pK values, average 6.46 ± 0.03. In contrast, disubstituted lysine was 54% hypochromic, revealed two new absorption bands at ±866 cm^{-1} about the monosubstituted maximum (*ca.* 20,320 cm^{-1}) with molar circular dichroism values of +15.4 and −18.0 M^{-1} cm^{-1}, respectively, had a single pK of 7.28 ± 0.06, showed mutarotation, was poorly fluorescent and revealed a maximum probe separation of 7.4 Å all in agreement with a dye–dye complex involving exciton formation. The insulin derivatives were shown to be monomeric below 5×10^{-6} M by comparison of sedimentation (10^{-2}–10^{-3} M) and Beer's law behavior (10^{-6} to 4×10^{-2} M). Monomeric di- and trisubstituted insulins revealed circular dichroism maxima at about ±620 and ±230 cm^{-1} about the monosubstituted maximum with molar circular dichroism of −10.0, +21.5, −32.0, and +91.5, respectively. The results revealed that the B-1 to A-1 and A-1 to B-29 dye group separations were at most about 7.6 and 10.5 Å, respectively. These results support certain tertiary relationships previously postulated as necessary for high immunological and biological activity (Arquilla *et al.* (1969), *Diabetes 18*, 193) and it is argued that much of the crystalline structure of rhombohedral two-zinc porcine insulin remains invariant in solution.

Previous investigations of the structure–function relationships of insulin by immunochemical, biological and physical techniques (Arquilla *et al.*, 1967; Bromer *et al.*, 1967; Mercola *et al.*, 1967, 1969; Morris *et al.*, 1968–1970a,b) have led to a model of certain chain relationships and conformational features necessary for high immunological and biological activity (Arquilla *et al.*, 1969). In an effort to refine these relationships we have explored a new use of a spectroscopic technique involving exciton formation and have initiated its application to insulin. The stimulus for this approach was the availability of the techniques for the preparation and purification of three insulin derivatives covalently labeled with one

† From the Department of Biophysics, University of California, Los Angeles, California 90024, and from the Department of Pathology, California College of Medicine, University of California, Irvine, California 92664. *Received July 15, 1971.* Supported by a predoctoral fellowship from the Diabetes Association of Southern California and in part by a fellowship of the Pathology Department Training grant (GM 02049-01) to D. A. M. and by U. S. Public Health Service Grant AM 13441. Parts of these data were taken from the Ph.D. dissertation of D. A. M. (University of California, 1969) and a preliminary report was presented to the meeting of the Federation of American Societies for Experimental Biology, April 1969, Atlantic City, N. J. (*Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 508).

‡ Present address: Laboratory of Molecular Biophysics, Zoology Department, South Parks Road, Oxford.

§ Present address: School of Medicine, University of California, Irvine, Calif.