Circular Dichroism and Electron Paramagnetic Resonance of the Haptoglobin–Hemoglobin Complex*

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ABSTRACT: Haptoglobin binds hemoglobin resulting in a complex of 1:1 stoichiometry and marked alteration of heme function with increased peroxidatic activity. This complex is investigated through a variety of spectroscopic methods in order to assess the influence of alterations in hemoglobin structure in changing the reactivity of the prosthetic group.

The electronic structure of the porphyrin group and iron d orbital system upon complex formation is demonstrated to remain unaltered on the basis of absorption and circular dichroic spectra and on the basis of electron paramagnetic resonance absorption of the nitric oxide liganded complex. Structural alterations in the heme environment upon complex formation are demonstrated by circular dichroism. The binding of haptoglobin prevents denaturation of hemoglobin by guaiacol. It is concluded that the changes in heme reactivity result from structural alterations in hemoglobin upon complex formation and protection against denaturation. Electron paramagnetic resonance of hemoglobin alkylated with *N*(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at the β93 cysteine residue indicates that in complex formation the βF helix assumes a deoxy-like conformation independent of oxidation and ligand state of the heme iron. These results in conjunction with the known crystallographic structure of deoxyhemoglobin explain the loss of the Bohr effect of hemoglobin upon binding to haptoglobin. Implications of the structure of haptoglobin–hemoglobin in relation to the fast-reacting species of hemoglobin are discussed.

Haptoglobin is an α_2-globulin isolated from serum (Connell and Smithies, 1959; Connell and Shaw, 1961) and combines with hemoglobin in an almost irreversible manner to form a complex of 1:1 molecular stoichiometry (*cf.* review by Jayle and Moretti, 1962). The marked enhancement of the peroxidatic activity of hemoglobin upon complex formation was first described by Polonovski and Jayle (1938) and shown to differ kinetically from the "pseudoperoxidatic" activity of hemoglobin described by Willstätter and Pollinger (1923). In addition, while hemoglobin in general is known not to catalyze the halogenation of organic molecules, a property of numerous peroxidases, halogenating ability of the haptoglobin–hemoglobin complex has been described (Dobryszycka, 1966). The binding of hemoglobin to haptoglobin is also associated with a marked increase in oxygen affinity (Nagel *et al.*, 1965a) and altered susceptibility of the heme groups to enzymatic degradation (Tenhunen *et al.*, 1969). These functional changes in heme reactivity strongly imply that marked structural changes in the heme environment occur as a result of complex formation.

In hemoproteins the environment of the heme group is generally considered as the predominant influence in determining enzymatic specificity and reactivity. However, it is difficult to make a direct assessment of the relative importance of factors which determine specificity and reactivity since hemoproteins have different primary and tertiary structures, electronic states, and bond types. In an effort to assess the influence of changes in the heme environment in modifying the normal function of a well-defined hemoprotein, we have investigated the haptoglobin–hemoglobin complex through a variety of spectroscopic methods.

The results of our studies define the electronic structure of the heme prosthetic group of the haptoglobin–hemoglobin complex and characterize structural changes in hemoglobin as a result of complex formation responsible for the marked alteration in heme reactivity. Furthermore, on the basis of the results of our investigations and the known crystallographic structure of hemoglobin (Perutz, 1965; Muirhead *et al.*, 1967; Perutz *et al.*, 1969) it is possible to provide an explanation for the loss of the Bohr effect of hemoglobin upon binding to haptoglobin (Nagel *et al.*, 1965a). In addition, implications of the results of our studies on the nature of the fast-reacting species of hemoglobin (Gibson, 1959) are discussed.

Materials and Methods

Human HbO_2 and ferriHb[1] were prepared according to Cameron and George (1969) except that lysis of erythrocytes was carried out with cold distilled water. HbCO was prepared by dialysis of freshly prepared HbO_2 against a CO-saturated 0.1 M phosphate buffer of neutral pH at 0–4°. HbNO was prepared as previously described (Kon, 1968). Alkaline ferriHb was prepared by titration of acid ferriHb to pH 10.1 with carbonate-free 0.3 N NaOH.

Heme concentration was estimated after conversion into the ferriHb cyanide derivative on the basis of the molar extinction coefficient of 11.0×10^3 at 5400 Å (Cameron, 1965). The ferriHb content of HbO_2 solutions immediately after final centrifugation of cellular stroma (Cameron and George, 1969) was found to be negligible since addition of cyanide caused no change in absorbance at 5400 and 6300 Å. These

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[1] Abbreviations used are: dHb, reduced or deoxygenated hemoglobin; ferriHb, met- or oxidized hemoglobin; Hb, hemoglobin; Hp, haptoglobin with subscripts 1-1, 2-1, 2-2 to indicate the phenotypic variant; Hp–Hb, haptoglobin–hemoglobin; *i.e.*, haptoglobin bound to hemoglobin; Hp–HbO_2, haptoglobin bound to HbO_2; analogously Hp–HbCO means haptoglobin bound to HbCO, etc.; Mb, myoglobin.

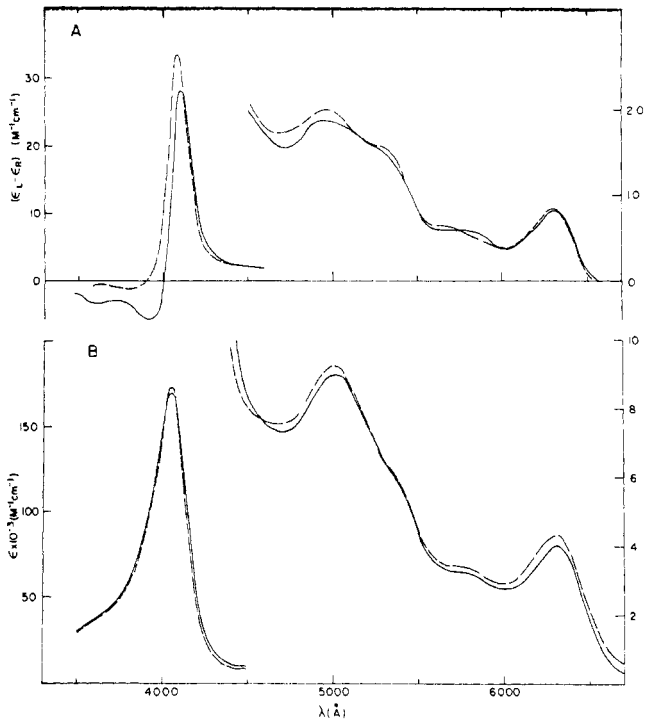

FIGURE 1: Circular dichroism and absorption spectra of the acid Hp_{2-2}–ferriHb (- - -) complex compared to those of acid ferriHb (—). Hb derivatives were buffered in sodium phosphate–sodium chloride buffer of ionic strength 0.05 at pH 6.0.

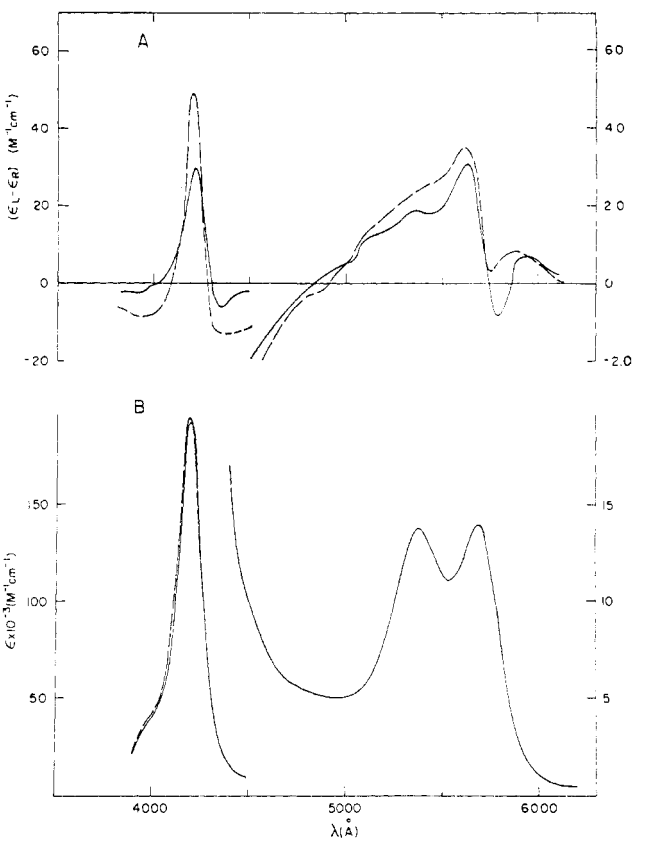

FIGURE 2: Circular dichroism and absorption spectra of Hp_{2-2}–HbCO (- - -) compared to those of HbCO (—). The absorption spectrum of Hp_{2-2}–HbCO in the visible region is entirely superimposable upon that of HbCO. The Hp_{2-2}–HbCO complex was prepared by addition of HbCO to a solution of Hp_{2-2} buffered in sodium phosphate–sodium chloride buffer as in Figure 1 but saturated with CO. The solution of HbCO for spectral characterization was prepared by similarly diluting an aliquot of a stock solution of HbCO but without Hp_{2-2}.

solutions were then used immediately for spectral characterization of O_2-liganded derivatives.

Hp was prepared from outdated human plasma according to Connell and Shaw (1961). The preparations were similar to a sample of purified Hp prepared from ascitic fluid in Dr. Connell's laboratory according to enhancement of peroxidatic activity and spectral properties. Hp_{1-1} was not used in this study because of its limited supply. Electrophoretic determination of Hp phenotype was carried out according to Brown and Johnson (1970). Peroxidatic activity of ferriHb and Hp–ferriHb was assayed according to Connell and Smithies (1959) with use of a Zeiss PMQ II spectrophotometer at 25°. Only freshly prepared ferriHb was used. Reagent grade 30% hydrogen peroxide was obtained from Industrial Chemicals, Morristown, N. J., and suitably diluted prior to use. Guaiacol was obtained from Eastman Chemical Co., Rochester, N. Y.

The equivalence of stock solutions of Hp to solutions of Hb was determined on the basis of the peroxidatic assay (Connell and Smithies, 1959). Solutions of Hp–Hb complexes were then prepared by addition of 5% excess Hp to the corresponding Hb derivative in buffered solution except as noted: Hp–HbNO was prepared from the Hp-HbCO analog; alkaline Hp–ferriHb was prepared by titration of the acid derivative; and Hp–dHb was prepared by addition of a small amount of sodium dithionite to the oxy derivative in nitrogen saturated buffer. The heme concentration of solutions of Hp–Hb complexes was determined on the basis of the pyridine hemochromogen content (Falk, 1964). The use of this method on stock solutions of ferriHb agreed to within 1% of the ferriHb cyanide content. For spectroscopic characterization of Hp–Hb complexes, the corresponding Hb derivatives were prepared only from solutions of HbO_2 which upon oxidation to ferriHb conformed to the spectral criteria of Cameron and George (1969) for absence of denatured protein.

Absorption spectra were recorded at 24–26° on a Cary Model 14 recording spectrophotometer. Circular dichroism spectra were obtained at ambient temperature (25–27°) with a Cary Model 60 spectropolarimeter equipped with a Model 6002 CD attachment. The instrument was programmed for constant illumination throughout the wavelength regions studied with band-pass-widths varying from 15 to 30 Å. The maximum absorbance did not exceed 2.0 in the wavelength region of interest. The standard used was an aqueous solution of *d*-10-camphorsulfonic acid with an $(\epsilon_l - \epsilon_r)$ of 2.20 at 2900 Å (Urry and Pettegrew, 1967).

Electron paramagnetic resonance spectra were recorded as first derivatives of the electron paramagnetic resonance absorption on a Varian V-4500 spectrometer as previously described (Kon, 1968). *N*-(1-*O*xyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide was obtained from Varian Associates, Palo Alto, Calif., and reacted with HbO_2 under the conditions described by Ogawa and McConnell (1967) for the pyrrolinidyl analog. The number of cysteine residues alkylated with spin label was estimated by SH group titrations carried out with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Connellan and Folk (1968). Solutions of spin-labeled HbO_2 and Hp–HbO_2 were deoxygenated in a specially constructed cuvet by alternate evacuation and flushing with high-purity

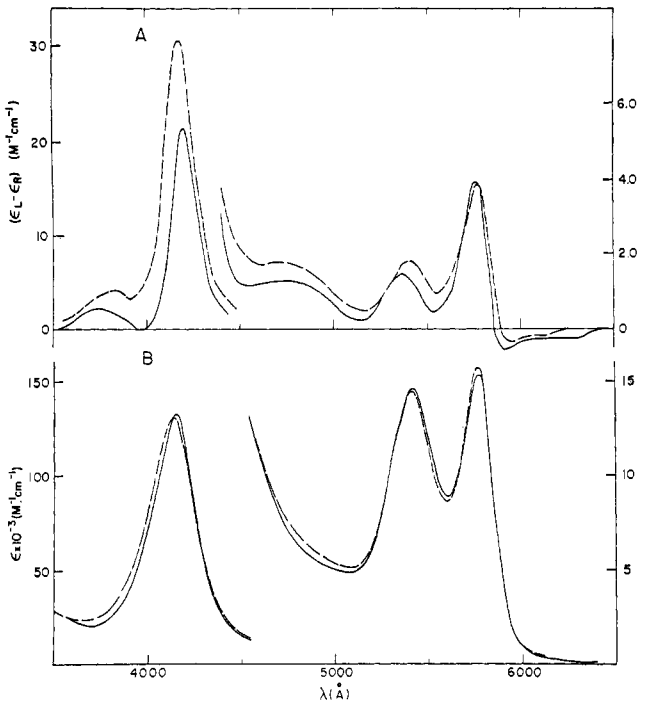

FIGURE 3: Circular dichroism and absorption spectra of Hp_{2-2}–HbO_2 (- - -) compared to those of HbO_2 (—). The solutions were prepared in an analogous manner to that indicated in Figure 2 but with buffer saturated with oxygen.

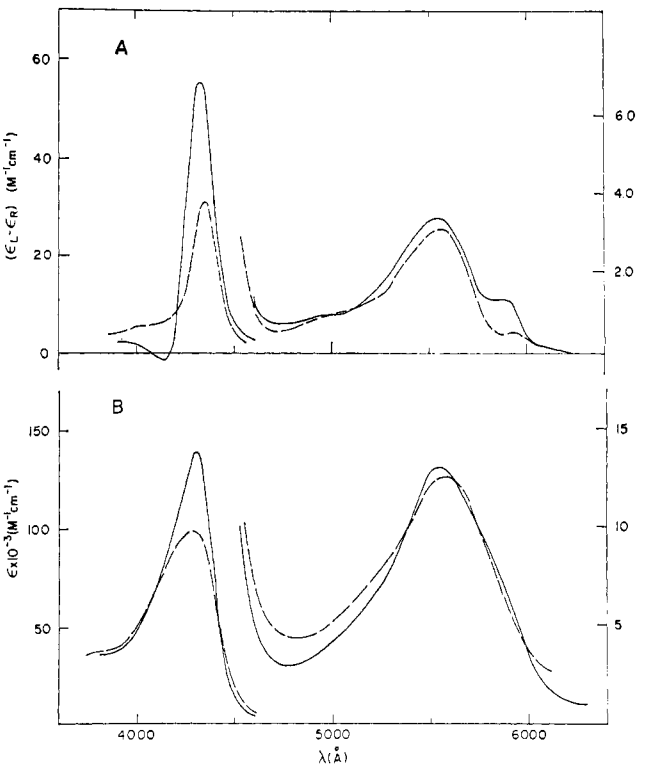

FIGURE 4: Circular dichroism and absorption spectra of Hp_{2-1}–dHb (- - -) compared to those of dHb (—). The sodium phosphate-sodium chloride buffered solution was saturated with nitrogen prior to addition of an aliquot of a stock solution of HbO_2, and solutions were prepared in an analogous manner as indicated in Figure 2. A small amount of solid sodium dithionite was then added to solutions in cuvets and the cuvets were quickly sealed.

nitrogen. The increase in ferriHb content after such treatment was negligible. Conditions for the study of NO-liganded derivatives by electron paramagnetic resonance have been described (Kon, 1968).

All inorganic chemicals were obtained as analytical grade reagents and used without further purification. Deionized distilled water was used throughout, and pH was measured with a Radiometer Model 22 electrometer equipped with a scale expander.

Results

Absorption and Circular Dichroism Spectra of Haptoglobin–Hemoglobin Complexes. The absorption properties of Hb derivatives illustrated in Figures 1B–4B are in excellent agreement with those known for ferriHb and HbO_2 (B. F. Cameron, to be submitted for publication, 1970), HbCO (Banerjee *et al.*, 1969), and dHb (Lemberg and Legge, 1949) prepared from human HbO_2. As evident through Figures 1B–3B the absorption properties of Hb derivatives do not change markedly upon complex formation with Hp. The spectra of the alkaline ferriHb derivatives (not shown) are practically identical. In addition, the spectra of Hp–Hb complexes formed with Hp of types 2-1 and 2-2 were identical in all respects. Spectral properties of Hp–dHb (Figure 4) will be discussed separately.

Circular dichroism spectra of Hp–Hb complexes differ markedly when compared to those of corresponding Hb derivatives (Figures 1A–3A). The changes in the apparent maximum $\Delta\epsilon$ in the region of the Q_0, Q_v, and B bands[2] of derivatives of Hb upon complex formation with Hp show several trends. For the derivatives in Figures 1–3, there is a prominent increase in the positive component of the Cotton effect associated with the B band and generally a blue shift in peak position. Comparison of these peak positions and maximum $\Delta\epsilon$ values of Hp–Hb derivatives is made in Table I with those of corresponding Hb derivatives presented in this communication and with those of separated α and β subunits. The differences between the circular dichroism[3] spectra of Hp–Hb complexes and of corresponding Hb derivatives are less pronounced in the visible region than in the Soret region. There is a proportionately greater increase in apparent maximum $\Delta\epsilon$ in the region of the Q_v band than for the Q_0 band for

[2] The Q_0, Q_v, and B bands are often referred to as the α, β, and γ or Soret band, respectively. In this communication we have continued the notation of Eaton and Hochstrasser (1967, 1968) based on the nomenclature used in the theoretical studies of Platt (1956). The identification of the Q_0 and Q_v bands for acid ferriHb at 5400 and 5000 Å, respectively, is based on the identification of these transitions at similar positions for acid ferrimyoglobin on the basis of the polarization ratio spectrum of single crystals of ferrimyoglobin (Eaton and Hochstrasser, 1968). This is in contradistinction to the assumption that these bands appear at 5800 and 5400 Å in analogy to those of HbO_2 (Brill and Williams, 1961). The identification of the Q_0 and Q_v bands of ferrous liganded derivatives of hemoglobin is based on the polarization ratio spectra of single crystals of corresponding hemoglobin and myoglobin crystals (W. A. Eaton, 1969, personal communication of unpublished observations).

[3] Circular dichroism will be defined as $\Delta\epsilon = (\epsilon_l - \epsilon_r)$, 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 $[\theta] \approx 2.303(4500/\pi)\Delta\epsilon$ (Moscowitz, 1960) where $[\theta]$ is in units of deg · cm^2/dmole.

TABLE I: Comparison of Peak Position and Circular Dichroism of Soret Band of Haptoglobin–Hemoglobin Complexes to Those of Corresponding Derivatives of Hemoglobin and Its Separated α and β Subunits.[a]

	Oxy		Deoxy		CO		Acid Ferri		Alkaline Ferri	
	λ_{max} (Å)	$\Delta\epsilon$	λ_{max} (Å)	$\Delta\epsilon$	λ_{max} (Å)	$\Delta\epsilon$	λ_{max} (Å)	$\Delta\epsilon$	λ_{max} (Å)	$\Delta\epsilon$
Hb	4200	21.3	4330	55.5	4220	29.9	4105	28.4	4190	14.4
Hp–Hb	4170	30.4	4350	30.4	4210	48.8	4085	32.8	4185	21.8
α	4140	29.1	4340	30.3	4170	53.3	4060	24.2		
β	4210	13.3	4350	21.8	4210	37.3	4140	19.4		

[a] Data for separated α and β subunits are those of Geraci and Li (1969). The peak positions of Hb and Hp–Hb derivatives are considered to be accurate to within 5 Å.

the derivatives in Figures 1–3, and this approximates the increase in apparent maximum $\Delta\epsilon$ associated with the B band. Similar differences in circular dichroism of the alkaline Hp–ferriHb complex compared to that of alkaline ferriHb are also observed.

No appreciable binding of dHb by Hp occurs upon mixing of the two protein moieties (Nagel *et al.*, 1956b). However, Hp–HbO_2 can be reversibly deoxygenated (Nagel *et al.*, 1965a) without detectable dissociation of the two components of the complex (Chiancone *et al.*, 1966). On this basis we have prepared the Hp–dHb complex for spectral characterization by dithionite reduction of $Hp_{2\text{-}1}$–HbO_2.

The absorption properties of $Hp_{2\text{-}1}$–dHb differ markedly from those of dHb (Figure 4B) throughout the visible and Soret regions in contrast to the small differences observed for other derivatives in Figures 1B–3B. The visible spectrum of $Hp_{2\text{-}1}$–dHb exhibits a broad band with maximum extinction at 5580 Å in contrast to the Q_v band of dHb at 5550 Å. The spectrum of $Hp_{2\text{-}1}$–dHb has no shoulder component corresponding to the Q_0 band seen in the dHb spectrum at about 5900 Å. The most marked difference occurs in the B band. The position of maximum extinction of the B band has shifted to 4280 Å in comparison to that of dHb located at 4300 Å. The maximum extinction, in addition, has markedly decreased. We have observed a maximum extinction coefficient of the B band of $Hp_{2\text{-}1}$–dHb of 98.3×10^3 M^{-1} cm^{-1} in good agreement with that of 104×10^3 M^{-1} cm^{-1} reported by Nagel and Gibson (1966). The widths of the B bands of $Hp_{2\text{-}1}$–dHb and dHb at half-maximum extinction are 420 and 300 Å, respectively.

The circular dichroism spectra of both deoxy derivatives (Figure 4A) show striking differences which correspond to those described in the absorption spectra. The circular dichroism band of $Hp_{2\text{-}1}$–dHb associated with the B transition is markedly decreased in apparent maximum $\Delta\epsilon$ and, in contrast to the changes in this region for other derivatives, shows a red shift in peak position when compared to dHb. In the visible region dHb exhibits enhanced optical activity associated with the Q_v and Q_0 bands over that observed for $Hp_{2\text{-}1}$–dHb.

When small increments were made in the HbO_2 concentration of a solution of $Hp_{2\text{-}1}$ under conditions described in Figure 3, the resulting circular dichroism spectrum in the vicinity of the B band was identical with that of $Hp_{2\text{-}1}$–HbO_2 until complete saturation of $Hp_{2\text{-}1}$ occurred. This result indicates that spectral properties of Hb are uniquely modified as a result of binding to Hp and are not determined according to the degree of saturation of the binding sites on the Hp molecule.[4] Furthermore, when HbO_2 was added to acid or heat denatured $Hp_{2\text{-}1}$ the resulting circular dichroism spectrum was entirely comparable to that of HbO_2 alone. Similarly, denatured $Hp_{2\text{-}1}$ showed no capacity to enhance the peroxidatic activity of ferriHb.

On the basis of the identification of heme iron d → d transitions in MbCO at about 5260 and 6250 Å (Eaton and Charney, 1969) by application of a relatively large anisotropy factor,[5] we conclude that the circular dichroism bands of HbCO and $Hp_{2\text{-}2}$–HbCO at 5100 Å (Figure 2A) have similar electronic origin. Li and Johnson (1969) have also noted that this circular dichroism band for HbCO is not of the same electronic origin as the Q_0 and Q_v bands. The identification of the small circular dichroism band at 6300 Å in the spectrum of HbO_2 (Figure 3A) as having its origin in the heme iron d orbital system can be similarly made on the same basis. The corresponding band for the $Hp_{2\text{-}1}$–HbO_2 complex is probably obscured by the general increase in positive circular dichroism in the region of the Q bands. Analogous to the spectra of the CO-liganded derivatives, however, both oxy derivatives exhibit circular dichroism bands at about 4750 Å with no corresponding band in the absorption spectrum. On the basis of their anistropy factor they may be similarly considered to have origin in the iron d orbital system. It is apparent from the spectra in Figures 3 and 4 that *the positions of bands associated with heme iron d → d transitions do not change for either HbCO or HbO_2 upon complex formation with Hp.*

Electron Paramagentic Resonance of Spin-Labeled Haptoglobin–Hemoglobin. The use of synthetic free radicals known as spin labels has found wide use in probing the structure, motion, and chemical reactions of biological macromolecules (Hamilton and McConnell, 1968). We have made use of this technique to assess changes in conformation of the β subunit of Hb upon Hp–Hb complex formation, for covalent attachment of the piperidinyl derivative (see Methods and Materials) is known to occur with at least 95% specificity at the β93 cysteine residue (McConnell *et al.*, 1969). Since spin

[4] Electrophoretic studies (Laurell, 1959; Peacock *et al.*, 1970) and kinetic investigations (Alfsen *et al.*, 1970) have shown that under conditions of excess Hp two types of complexes differing in molecular weight are formed.

[5] The anisotropy factor is defined as four times the rotational strength divided by the dipole strength or approximately $(\epsilon_l - \epsilon_r)/\epsilon$ (Condon, 1937), and criteria for its application to the identification of magnetic dipole allowed d → d transitions have been discussed by Mason (1963). A discussion of iron d → d transitions in hemoproteins is given by Eaton and Charney (1970).

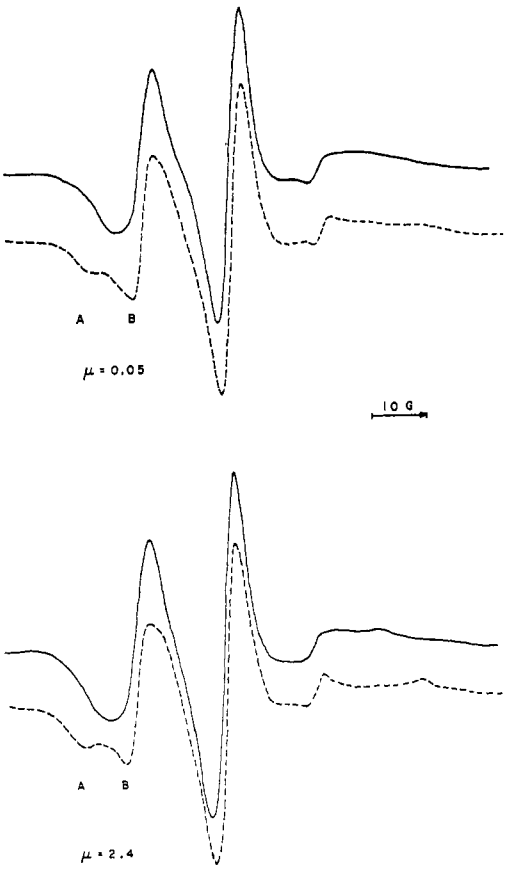

FIGURE 5: Electron paramagnetic resonance absorption of spin-labeled Hp_{2-1}–HbO_2 (solid trace) and spin-labeled HbO_2 (broken trace) under two conditions of ionic strength (μ). Electron paramagnetic resonance absorption spectra were obtained with spin-labeled derivatives in sodium phosphate buffered solutions to which sodium chloride was added to adjust the ionic strength at pH 7.0 and 25°. Heme concentration is approximately 5×10^{-5} M. The spectra are not normalized to the same integrated intensity.

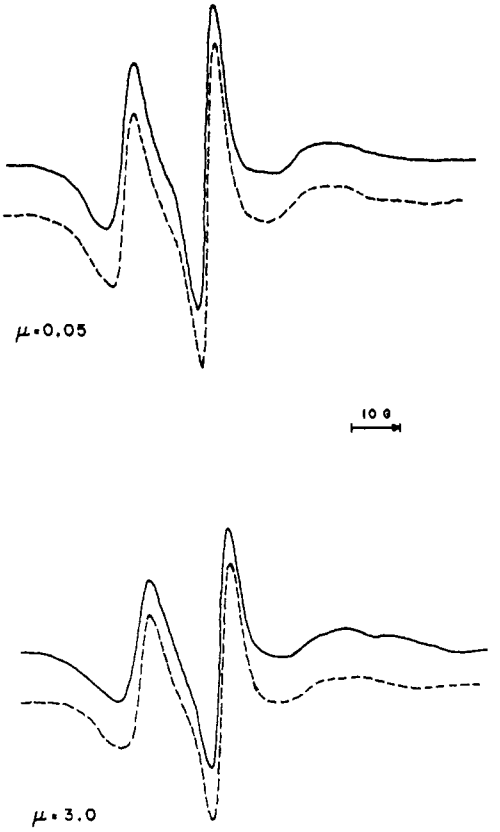

FIGURE 6: Electron paramagnetic resonance absorption of spin-labeled Hp_{2-1}–dHb (solid trace) compared to that of spin-labeled dHb (broken trace) under two conditions of ionic strength. Solutions of dHb derivatives were obtained by evacuation of the O_2-liganded species as described in Methods and Materials. Other conditions as in Figure 5.

label was reacted under conditions of a 1:1 ratio of equivalents with the β93 SH group, we assume that nonspecific labeling is negligible. Titrations of total SH group content of spin-labeled HbO_2 by the 5,5′-dithiobis(2-nitrobenzoic acid) method (Connellan and Folk, 1968) showed an SH:heme ratio of near unity.

In addition, the peroxidatic activity of spin-labeled ferriHb was equivalent to that of unmodified ferriHb on a heme basis. The enhancement of peroxidatic activity of spin-labeled ferriHb upon addition of Hp was also equivalent to that of unlabeled ferriHb in the presence of Hp. On this basis complex formation of Hp with Hb alkylated with the spin label can be considered equivalent to that with unmodified Hb.

The electron paramagnetic resonance absorption of spin-labeled Hp_{2-1}–HbO_2 is compared to that of the parent HbO_2 derivative under conditions of comparable ionic strength and pH in Figure 5. The two low-field signals observed for spin-labeled HbO_2 are designated A and B. McConnell *et al.* (1969) have shown that they correspond, respectively, to a highly and a weakly immobilized state of the spin label attached to the β93 position of HbO_2 and probably reflect an ionic strength dependent equilibrium of two conformational states in spin-labeled HbO_2 and ferriHb. This equilibrium is not observed for spin-labeled dHb (McConnell *et al.*, 1969).

The spectrum of spin-labeled HbO_2 after addition of sufficient Hp_{2-1} to complex all HbO_2 in solution shows loss of the equilibrium between the two states designated A and B, and the spectrum, in general, is characteristic of intermediate immobilization. There are smaller changes in the high-field component of spin-labeled HbO_2 after binding to Hp_{2-1} which are similarly indicative of a partial loss in immobilization of the label. Furthermore, addition of Hp_{2-1} to spin-labeled ferriHb under comparable conditions to those described in Figure 5 resulted in a spectrum identical with that of the Hp_{2-1}–HbO_2 complex. Neither the spin-labeled Hp_{2-1}–HbO_2 complex nor the Hp_{2-1}–ferriHb analog disclosed the appearance of signals comparable to A and B within a range of ionic strength of 0.05 to 2.4 M. Furthermore, the shape of the spectrum was not influenced when Hp was added to spin-labeled HbO_2 in excess of a 1:1 molar ratio.

The electron paramagnetic resonance absorption of spin-labeled Hp_{2-1}–dHb is similarly compared to that of spin-labeled dHb in Figure 6 under comparable conditions of ionic strength and pH. The shapes of the electron paramagnetic resonance spectra of the two spin-labeled derivatives are similar. Moreover, *the spectra of spin-labeled Hp_{2-1}–deoxyHb are similar to those of spin-labeled Hp_{2-1}–HbO_2 and Hp_{2-1}–ferriHb showing intermediate immobilization of the label and absence of the A $\rightleftarrows$ B equilibrium.*

Attempts to prepare spin-labeled Hp–Hb complexes by reaction of the spin label with the Hp–Hb complex resulted in a small fraction of label becoming covalently attached to

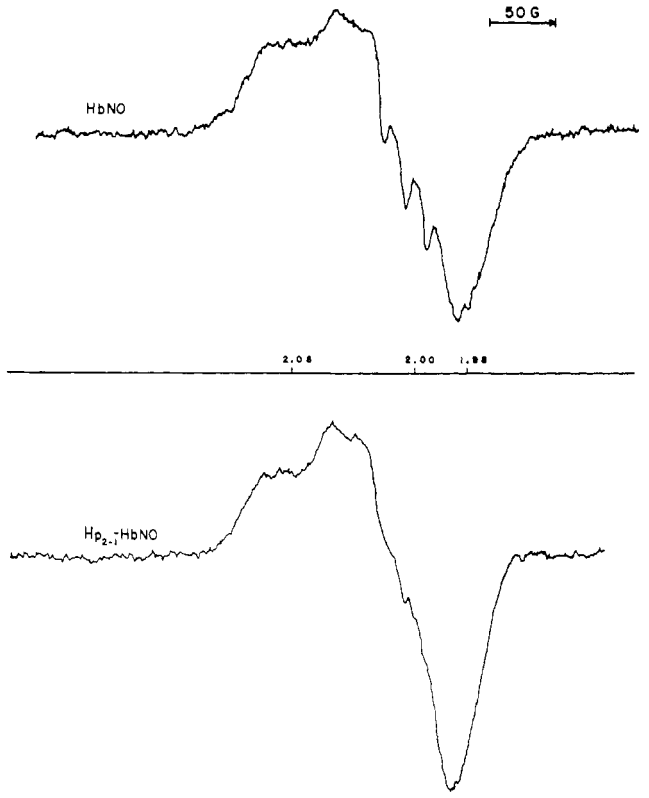

FIGURE 7: Electron paramagnetic resonance absorption of HbNO (upper trace) compared to that of Hp_{2-1}–HbNO (lower trace) at 77°K and in the presence of 0.009 M guaiacol. HbNO and Hp_{2-1}–HbNO at a concentration of 10^{-4} M in heme were incubated in the presence of 0.009 M guaiacol in 0.1 M sodium 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 positions of electron paramagnetic resonance absorption bands and for a displacement of 50 G apply to both spectra. The spectrum of Hp_{2-1}–HbNO and of HbNO in the absence of guaiacol is identical with that illustrated here in the lower trace for Hp_{2-1}–HbNO in 0.009 M guaiacol.

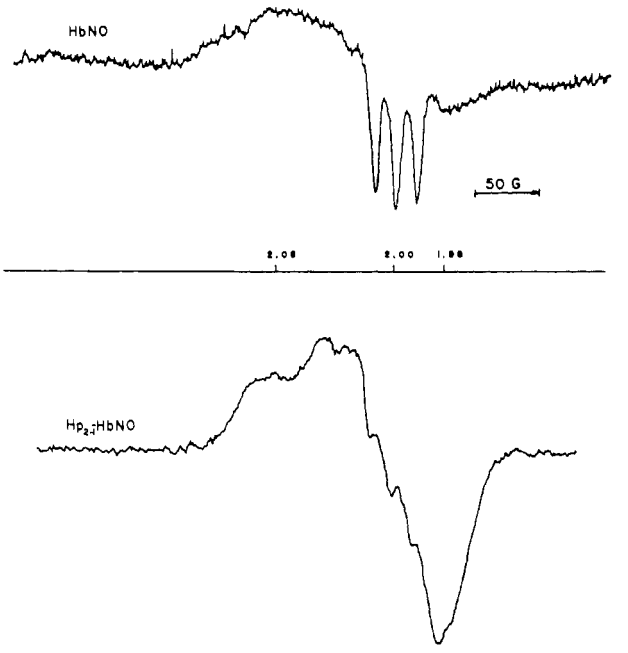

FIGURE 8: Electron paramagnetic resonance absorption of HbNO (upper trace) and Hp_{2-1}–HbNO (lower trace) in the presence of 0.027 M guaiacol. Conditions are as indicated in Figure 7.

the complex. The label gave a spectrum characteristic of very weak immobilization with intensities of the three hyperfine components of almost equal magnitude. This type of spectrum is not consistent with covalent attachment of the spin label to the β93 cysteine of the Hb moiety but rather reflects nonspecific labeling, possibly of the ε-amino groups of lysine residues. Markedly decreased reactivity of the β93 cysteine residues of HbO_2 toward iodoacetamide has been shown to occur as a result of Hp–HbO_2 complex formation (Malchy and Dixon, 1969), and undoubtedly reaction of the spin label as an iodoacetamide derivative with the β93 SH group of the Hp–Hb complex would be under even greater steric hindrance. *This decreased reactivity of the β93 cysteine residue upon Hp–Hb complex formation parallels the behavior toward alkylating reagents of the β93 SH group in dHb* described by Benesch and Benesch (1962).

Effect of Haptoglobin on Guaiacol–Hemoglobin Interaction. Guaiacol is used as an electron donor for assaying the activity of peroxidatic enzymes since it generally permits estimation of both kinetic constants in the peroxidase-mediated reduction of hydroperoxides (Chance and Maehly, 1955). It has been long noted, however, that the peroxidatic activity of Hb is low compared to that of horseradish peroxidase and that product formation is proportional in a logarithmic manner to the heme concentration (Willstätter and Pollinger, 1923). Since high concentrations of guaiacol markedly decrease the peroxidatic activity of ferriHb but exert relatively little influence on the activity of the Hp–ferriHb complex (Connell and Smithies, 1959), we have investigated the influence of this reagent on both spin-labeled ferriHb as well as on HbNO and the corresponding Hp–HbNO complex.

The electron paramagnetic resonance absorption of HbNO at 77°K exhibits three broad, partially superimposed bands indicative of rhombic symmetry of the paramagnetic heme iron center (Kon, 1968). Under comparable conditions the electron paramagnetic resonance absorption of the Hp_{2-1}–HbNO complex shows an identical spectrum exhibiting bands at about $g = 2.060$ and 1.986 with an inverted S-shaped region near $g = 2.030$. *This indicates that the characteristic electronic structure of the paramagnetic center of HbNO remains unchanged upon complex formation with Hp.* The general shape of the spectrum of Hp_{2-1}–HbNO is illustrated in the lower part of Figure 7.

The effect of incubation of HbNO in 0.009 M guaiacol for 5 min at pH 7.0 is apparent from the spectrum illustrated in Figure 7. At this concentration of guaiacol, the peroxidatic activity of the Hp–ferriHb complex approaches a maximum and that of ferriHb is markedly decreased (Connell and Smithies, 1959). The spectrum of HbNO is characterized by the appearance of three small bands centered at $g = 2.009$ and a slight decrease in the intensity of the band at $g = 1.986$. The appearance of the three bands is already evident in the presence of 0.001 M guaiacol.

The effect of increasing the concentration of guaiacol on both NO-liganded derivatives is evident in Figure 8. At 0.027 M guaiacol the three bands centered at $g = 2.009$ are more distinct for HbNO, the entire spectrum is shifted to a lower magnetic field, and the intensity of the band at $g = 1.986$ is markedly decreased. In contrast, the influence of this concentration of guaiacol on the Hp_{2-1}–HbNO complex is much less. At 0.027 M guaiacol the peroxidatic activity of

ferriHb is approximately only 50% of that in the presence of 0.009 M guaiacol, and that of the Hp–ferriHb complex has decreased by approximately 20% (Connell and Smithies, 1959). Further increase in guaiacol concentration to 0.080 M produced virtually no further change in the spectrum of either nitric oxide derivative. Moreover, addition of Hp_{2-1} to a solution of HbNO in 0.027 M guaiacol under the conditions described in Figure 8 resulted in restoration of the spectrum to that observed for the Hp_{2-1}–HbNO complex. The degree of restoration appeared to be a function of the time Hp_{2-1} was incubated with the HbNO–guaiacol mixture, and essentially complete reversal of the spectral shifts was attained after 15 min.

NO-liganded derivatives are unstable in acidic media where the peroxidatic activity of the Hp–ferriHb approaches a maximum. Here the effect of guaiacol could be observed by use of spin-labeled ferriHb. At pH 7 in the presence of comparable concentrations of guaiacol to those indicated in Figures 7 and 8, spin-labeled ferriHb showed weak immobilization in contrast to the moderate immobilization similar to that shown for HbO_2 in Figure 5. At pH 5.5 where the peroxidatic activity of ferriHb approaches a maximum (Connell and Smithies, 1959), electron paramagnetic resonance absorption of the spin-labeled derivative indicated a slight decrease in immobilization over that observed at pH 7.0. At pH 4 where the peroxidatic activity of the Hp–ferriHb complex approaches a maximum, electron paramagnetic resonance absorption of spin-labeled ferriHb showed very weak immobilization and a time-dependent decrease in signal intensity. The rate of decrease in signal intensity was dependent on both guaiacol concentration and pH. At pH 4 in the presence of 0.024 M guaiacol, the rate of decrease in signal intensity showed an approximate 50% diminution in peak-to-peak height in 15 min. In contrast the electron paramagnetic resonance absorption of spin-labeled Hp_{2-1}–ferriHb remained unchanged by the addition of guaiacol.

Discussion

Spectroscopic Considerations on the Haptoglobin–Hemoglobin Complex. The B and Q bands have their electronic origin in the π–π^* transitions of the porphyrin ring (Platt, 1956). The similar absorption properties of Hp–Hb complexes to those of the corresponding Hb derivative in Figures 1B–3B indicate that no significant alterations of the porphyrin pi electron structure have occurred as a result of Hb binding to Hp. The similar absorption spectrum of Hp_{2-1}–dHb (Figure 4B) in the Soret region to those of the separated deoxy subunits (Antonini *et al.*, 1965) probably reflects the loss of cooperativie ligand binding (Nagel *et al.*, 1965a; Antonini *et al.*, 1965).

There are several lines of evidence indicating that the electronic structure of the heme iron d orbital system has not been significantly altered upon Hp–Hb complex formation. Heme iron d $\rightarrow$ d transitions identified on the basis of anisotropy factors remain at comparable positions for HbO_2 and HbCO upon complex formation. Furthermore, in HbNO the unpaired electron of the paramagnetic center has been shown to be confined primarily to an orbital having d_{z^2} symmetry (Kon, 1968). The electron paramagnetic resonance absorption of the nitric oxide complex reflects the electronic structure of the (proximal) imidazole–heme iron–NO paramagnetic system (Kon, 1968; Kon and Kataoka, 1969), and, therefore, can be influenced by perturbations upon the $d\pi$–$p\pi$ orbital interaction. The identical electron paramagnetic resonance absorption of the two NO-liganded derivatives, thus, reflects equivalent electronic structures of the paramagnetic heme iron center, especially of the axial iron d orbital.

Furthermore, the absorption spectrum of alkaline Hp–ferriHb is practically identical with that of alkaline ferriHb. On the basis of the correlation between the spectroscopic properties of ferrihemoprotein hydroxides and magnetic susceptibility as outlined by George *et al.* (1961), we can conclude that the binding of Hb by Hp is not associated with an alteration of the spin state of the iron. This conclusion finds support in our observations that the electron paramagnetic resonance absorption at $g = 6$ of high-spin ferriHb derivatives is not altered upon binding to Hp (H. Kon, unpublished observations). It is, therefore, possible to conclude on the basis of these correlative spectroscopic observations that *the ligand field of the heme iron is not altered as a result of Hp–Hb complex formation.*

The rotational strength of the B transition in the circular dichroism of Mb has been described as arising predominantly from dipole–dipole interaction (Hsu and Woody, 1969). Since the intensities of the Q bands have related electronic origin with the B band (Platt, 1956; Gouterman, 1959), the optical activity associated with these transitions can be considered to be of similar origin. In view of the probable absence of significant mixing of iron d $\rightarrow$ d transitions with the B transition (Hsu and Woody, 1969) and the similarities in the heme environment of Hb and Mb (Perutz, 1965), it is likely that the rotational strength of the B transition in the circular dichroism of Hb and consequently of the Hp–Hb complex also arises predominantly through dipole–dipole interaction. In fact, the circular dichroism Soret spectra of Hp–Hb complexes are remarkably similar to those of Mb (Willick *et al.*, 1969) for corresponding oxidation and ligand states.

On this basis the large changes in the circular dichroism of the B band of Hb derivatives upon complex formation with Hp have their origin in a structural alteration of the amino acid residues relative to the central heme group of the α and β subunits. It is not possible to define with more precision the nature of the structural alterations in the environment of the heme groups except that they may involve primarily the interaction between the porphyrin group and nearby aromatic amino acid residues in analogy to that of Mb (Hsu and Woody, 1969).

Table I gives a comparison of the peak positions and magnitudes of apparent maximum $\Delta\epsilon$ for numerous Hp–Hb derivatives and corresponding forms of Hb and its isolated α and β subunits. Comparison of the circular dichroism spectra in Figures 1–5 to those of the corresponding isolated subunits (Geraci and Li, 1969) and the magnitudes of maximum $\Delta\epsilon$ in Table I indicates that the circular dichroism spectra of Hp–Hb are not equivalent to the mean of contributions arising from separated α and β subunits. It is, furthermore, apparent that the positions of maximum $\Delta\epsilon$ of the Hp–Hb complexes in each case appears generally displaced from that of the corresponding Hb derivative to that of the α subunit and that the maximum $\Delta\epsilon$ of the α subunit more nearly approximates that of the corresponding Hp–Hb complex than does the β subunit. Kinetic studies using separated subunits of Hb have indicated that the affinity of Hp for α chains is greater than that for β chains (Nagel and Gibson, 1967; Chiancone *et al.*, 1968). If a similar situation obtains in the binding of tetrameric Hb to Hp, the spectral trends indicated through Table I possibly may reflect that

the structural change in the heme environment of the α subunit makes a greater contribution to the increase in circular dichroism than that of the β subunit. The decreased optical activity associated with the Q_0 band of Hp–dHb (Figure 5) may similarly reflect a predominant contribution from the α subunit. Ueda *et al.* (1970) have observed that a positive circular dichroism band in this region is associated only with separated deoxy α chains.

Stability of the Haptoglobin–Hemoglobin Complex against Denaturation. The electron paramagnetic resonance absorption of HbNO in the presence of guaiacol is remarkably similar to that observed when HbNO is subjected to the denaturing effects of sodium dodecyl sulfate and sodium salicylate under comparable conditions (Kon, 1968). The influence of high concentrations of these reagents on the HbNO molecule results in similar spectral shifts to that shown in Figure 8 and finally in loss of rhombic symmetry and the appearance of complete randomization of the structural components surrounding the paramagnetic center (Kon, 1968). The spectral changes illustrated in Figures 7 and 8 for HbNO establish the similarity in influence of guaiacol to other known denaturing agents. The markedly decreased effect of guaiacol on the electron paramagnetic resonance spectrum of the $Hp_{2\text{-}1}$–HbNO complex indicates that denaturation of Hb structure is in some manner hindered and that only at much higher concentrations of guaiacol where the peroxidatic activity of the complex is decreased are significant changes in the spectrum evident. *This protection from denaturation must explain in part the marked enhancement of peroxidatic activity of Hb upon complex formation with Hp* (Polonovski and Jayle, 1938; Van Royen, 1950; Connell and Smithies, 1959).

The capacity of Hp to bind HbNO denatured by guaiacol restoring the spectrum to that normally observed for the Hp–HbNO complex probably reflects the ability of Hp to bind denatured Hb restoring the functional integrity of the prosthetic heme with its polypeptide environment. Smith and Beck (1967) have shown that the sequence of pH lowering from 7 to 4 with respect to addition of Hp to solutions of separated α or β subunits had little effect on the peroxidatic activity. This fact is readily explained by our observations that addition of Hp to denatured Hb restores the structural integrity of the functional heme iron–polypeptide system. Contrary to the conclusions of Smith and Beck (1967), however, our results indicate that binding of Hp to Hb prevents denaturation and in this manner is important in maintaining peroxidatic activity. It is probable that similar effects are the basis for the loss of peroxidatic activity of Hb in the oxidation of iodide (Van Royen, 1950) and in the presence of pyrogallol (Willstätter and Pollinger, 1923) during the catalysis of hydroperoxide reduction.

The exact mechanism of the time-dependent decrease in the signal intensities of the electron paramagnetic resonance absorption of spin-labeled ferriHb in the presence of guaiacol is unknown, but it could reflect drastic conformational change of the Hb polypeptide structure that results in loss of the free-radical nature of the label. Monitoring of the electron paramagnetic resonance signal at $g = 6$ of the high spin iron in ferriHb under comparable conditions indicates a rapid decay in signal intensity with time (H. Kon, 1969, unpublished observations). It is therefore probable that quenching of the electron paramagnetic resonance absorption of the spin label may be associated with structural changes which allow release of the heme group from its hydrophobic cavity or structural changes around the heme iron which cause a change in spin state. Bunn and Jandl (1968) have shown that exchange of heme in ferriHb is favored under acidic and alkaline pH conditions while exchange of heme does not occur in the Hp–ferriHb complex. It is possible that these phenomena are, therefore, related.

The Deoxy-Like Conformation of the β Subunit in Haptoglobin–Hemoglobin. The similar shape of the electron paramagnetic resonance spectrum of spin-labeled $Hp_{2\text{-}1}$–dHb to that of dHb labeled in the β93 position indicates that the label has attained comparable configurations in both molecules, and, thus, reflects a similar conformation of the proximal βF helix (Perutz, 1965) at the site of covalent attachment to the cysteine residue. This conclusion finds support in the similarity of our spectra to those of spin-labeled dHb reported by Ogawa and McConnell (1967) and in the absence of an equilibrium between the two states of immobilization, A and B, in spin-labeled Hp–Hb complexes as well as in spin-labeled dHb. Furthermore, the similarity in the reactivity of the β93 cysteine residue in the Hp–HbO_2 complex toward iodoacetamide (Malchy and Dixon, 1969) to the analogous behavior of the β93 cysteine in dHb (Benesch and Benesch, 1962) suggests that the region near the β93 position in both dHb and in Hp–Hb complexes assumes a comparable conformation. The results of our studies on spin-labeled Hp–Hb complexes provide direct structural evidence for the conclusion of Malchy and Dixon (1969) that a conformational change in HbO_2 occurs upon complex formation with Hp similar to that associated with deoxygenation. However, the high similarity of the electron paramagnetic resonance absorption of spin-labeled $Hp_{2\text{-}1}$–HbO_2 and $Hp_{2\text{-}1}$–ferriHb to that observed for both deoxy derivatives suggests that a similar configuration of the label with respect to the protein chain obtains in all three types of Hp–Hb complexes. It is, therefore, reasonable to assume that *the region of the F helix near the cysteine residue in the β93 position maintains similar deoxy-like conformations regardless of oxidation or ligand state of the heme iron in the Hp–Hb complex.*

The identification of the βF helix of Hb complexed to Hp as being in the deoxy conformation allows an immediate explanation in part for the absent or decreased Bohr effect of the Hp–Hb complex with O_2 binding (Nagel *et al.*, 1965a). X-Ray crystallographic studies (Perutz *et al.*, 1969) on Hb reacted at the β93 position with *N*-ethylsuccinimide indicate that in dHb the C-terminal histidine of the β subunit is hydrogen bonded with the carboxyl group of aspartate in the β94 position. The C-terminal histidines are, furthermore, responsible with the α-amino groups of the α subunits for most of the Bohr effect.[6] In the deoxy conformation the pK of the C-terminal histidines would be raised through the hydrogen bonding with the carboxyl group and its contribution to the Bohr effect diminished. Perutz *et al.* (1969) have, furthermore, pointed out that the free carboxyl group of the β94 aspartate residue may participate in the acid Bohr effect. A decrease in its contribution to the Bohr effect could be similarly explained by the resultant lowering of its associated pK upon hydrogen bonding. The analogous conformation of this portion of the β subunit of Hb in the Hp–Hb complex can, thus, explain in part the absent or decreased Bohr effect of the Hp–Hb complex (Nagel *et al.*, 1965a). Added support that the Hp–Hb complex assumes the hydrogen-bonded conformation associated with dHb is given through the decreased reactivity of the β93 cysteine

[6] For a discussion and review of the residues responsible for the Bohr effect, see Perutz *et al.* (1969).

toward iodoacetamide (Malchy and Dixon, 1969). Perutz *et al.* (1969) have shown that accessibility to the β93 SH group is more hindered in dHb and that this can explain its decreased reactivity toward alkylating reagents (Benesch and Benesch, 1962).

Similarities in the kinetic behavior of Hp–HbCO upon flash photolysis to that of the fast-reacting species of Hb (Gibson, 1959) have been outlined by Nagel and Gibson (1966). It is generally assumed that the fast-reacting species of Hb formed from flash photolysis of HbCO has the oxy conformation.[7] Our results that spin-labeled Hp–Hb maintains a deoxy-like conformation of the βF helix in both liganded and unliganded states and that the complex, nonetheless, behaves kinetically similar to the fast-reacting species (Nagel and Gibson, 1966) suggest that the heme environment associated with the oxy-liganded state need not be assumed as the only one compatible for displaying fast-reacting kinetic behavior. Added support for this conclusion is given through studies on HbH and carboxypeptidase A treated human Hb. Flash photolysis of the CO-liganded forms of HbH (Benesch *et al.*, 1964) and of carboxypeptidase A treated Hb (Antonini *et al.*, 1961) shows only kinetic behavior comparable to that of fast-reacting Hb. These modified forms of Hb do not have a conformation of the β subunit identical with that associated with HbO_2. X-Ray diffraction of HbH (Perutz and Mazzarella, 1963) indicates close similarity in quarternary structure to that of dHb, and electron paramagnetic resonance absorption of separated spin-labeled β subunits is quite different from that of spin-labeled Hb (Ogawa and McConnell, 1967). In addition, carboxypeptidase A treatment of spin-labeled HbO_2 results in a decrease in immobilization of the spin label (Boeyens and McConnell, 1966), indicative of a structural change of the β chain in the vicinity of the β93 cysteine residue.

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[7] For instance, see Anderson *et al.* (1970) and Perutz and Mazzarella (1963).

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Structural Features of Tropoelastin Related to the Sites of Cross-Links in Aortic Elastin*

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ABSTRACT: Tropoelastin is a soluble protein obtained from the aortas of copper-deficient swine. It is considered to be the precursor of insoluble elastin. Its isolation and purification has previously been described. When digested with trypsin, a number of small peptides are obtained. These have been isolated by ion-exchange chromatography and quantitated by amino acid analysis. Their amino acid sequence has been determined by the dansyl-Edman method and by mass spectrometry. Two of the peptides, Ala-Ala-Ala-Lys and Ala-Ala-Lys, appear to be repeated six times in the polypeptide chain. Because of this repeating structure, we propose that these peptides represent the areas of the chain involved in the formation of the desmosine and isodesmosine cross-links of insoluble elastin. We have proposed a linear folded model of tropoelastin which is compatible with these repeating structures.

We have recently isolated a protein from the aortas of copper-deficient swine which has elastin-like physical and chemical properties. The protein was given the name tropoelastin because it was considered to be the soluble precursor of insoluble elastin. Its molecular weight has been demonstrated to be approximately 68,000. Fingerprints of elastase-treated tropoelastin and insoluble elastin showed that there are many amino acid sequences common to both of these preparations. Edman degradation of tropoelastin has shown it to be a homogeneous protein with a unique N-terminal amino acid sequence (Sandberg *et al.*, 1969).

Tropoelastin does not have the desmosine cross-links present in insoluble elastin, but it does have a high content of lysine, more than sufficient for the production of the desmosine cross-links. During the process of cross-linking, the ϵ-amino groups of these lysine residues become altered by an enzyme, lysyl oxidase, to produce α-aminoadipic acid δ-semialdehyde (allysine) (Siegel and Martin, 1970; Pinnell and Martin, 1968). This latter substance is an intermediate in the formation of desmosine and other elastin cross-links (see review of Piez, 1968, and Miller *et al.*, 1967). About one-third of the lysine residues of tropoelastin is converted into desmosine cross-links. A large portion of the remainder of the lysine is also apparently oxidized to allysine and this then participates in cross-links which have not been completely elucidated. Some of these cross-links are of the aldol condensation type (Lent *et al.*, 1969). Lysinonorleucine and merodesmosine have also been identified as other possible cross-links (Franzblau *et al.*, 1969; Starcher *et al.*, 1967).

The present work is an evaluation of part of the structure of porcine aortic tropoelastin. It deals with portions of the molecule which are possibly destined to form the desmosine cross-links. These have been investigated as small lysine-containing peptides obtained by trypsin digestion of the tropoelastin molecule. Several peptides are present to the extent of two moles per mole of protein (68,000 molecular weight), suggesting some kind of duplicated structure. At present, it is not clear whether the duplication is an end-to-end

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