Induced Conformational States in Human Apohemoglobin on Binding of Haptoglobin 1–1. Effect of Added Heme as a Probe of Frozen Structures†

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ABSTRACT: We report here studies of the conformation of the complex (haptoglobin–apohemoglobin, Hp-ApoHb) formed between human haptoglobin 1–1 (Hp 1–1) and human apohemoglobin, and that formed between Hp and an equimolar mixture of isolated α-globin (α^0) and β-globin (β^0) chains. As previously reported (Yip, Y. K., Waks, M., and Beychok, S. (1972), *J. Biol. Chem. 247*, 7237), the isolated globin chains do not measurably combine to form apohemoglobin. However, in the presence of haptoglobin, a stable complex is formed comprising one haptoglobin molecule and $2\alpha^0$ and $2\beta^0$ chains (Hp–$2\alpha^0 2\beta^0$). Circular dichroism (CD) studies of this complex reveal that the secondary structures of the component molecules do not change on binding. Thus, free and complexed α-globins are only slightly helical (20%), in contrast to the conformation of α-globin when bound to β-globin in apohemoglobin ($\alpha\beta$-globin dimer). The β-globin, which has the same secondary structure free or bound in apohemoglobin, retains its structure in complex formation. When apohemoglobin is bound to haptoglobin, a marked reduction in secondary structure occurs, the far-ultraviolet CD closely resembling that of Hp–$2\alpha^0 2\beta^0$. The reduction is attributed to unfolding of globin chains possibly resulting from partial separation on binding. In contrast to the complete recovery of structure of free globins when heme is added, binding of heme to the globin chains in Hp–apoHb leads to no recovery of backbone conformation. However, upon addition of 2 equiv of heme to the complex (one-half saturation with heme), the absorption and CD spectra of heme bands are identical with those in the haptoglobin–hemoglobin complex (Hp–Hb) in which the individual chain conformations are known to be preserved (Waks, M., Kahn, P. C., and Beychok, S. (1971), *Biochem. Biophys. Res. Commun. 45*, 1232). When the remaining two hemes are bound, the absorption and CD spectral characteristics of heme-containing subunits are not recovered. Thus, the finally reconstituted molecules are substantially different from the Hp–Hb complex. Similarities and distinctions between these haptoglobin complexes and those formed between antibodies and antigens are discussed, and the occurrence and roles in these protein–protein interactions of frozen conformational states are considered.

The complexes formed between hemoglobin and haptoglobin are interesting examples of protein–protein interactions that are not reversible under accessible conditions of concentration, temperature, pH, and other variables. Oxyhemoglobin is bound by haptoglobin, but deoxyhemoglobin is not (Nagel *et al.*, 1965), at least in the presence of phosphate ions or 2,3-DPG (Nagel and Gibson, 1972). However, deoxygenation of a complex between haptoglobin and oxyhemoglobin leads to little or no release of hemoglobin (Alfsen *et al.*, 1970). Moreover, at least two different kinds of complex can be formed in which all binding sites for hemoglobin are filled, depending on whether haptoglobin or hemoglobin is initially present in excess. Once formed, these complexes do not equilibrate. In addition, when in excess, haptoglobin forms a stable intermediate with only half the binding sites filled and subsequent binding of hemoglobin leads to only one of these complexes (Waks *et al.*, 1969).

The reaction is sometimes considered analogous to that between antibody and antigen, but it differs in several important characteristics, the most notable being that lattice type precipitates (precipitin reaction) are generally not observed with the Hp–Hb[1] complexes, and there is no phenomenon comparable to the solubilizing effect of excess antigen. In terms of sequence, there are homologies between portions of the heavy chains of haptoglobin and proteolytic enzymes (Barnett *et al.*, 1972), but none between haptoglobin and immunoglobulin heavy chains.

Biologically, one of the functions of the haptoglobin–hemoglobin reaction is to ensure that any intravascular hemoglobin outside the erythrocyte is efficiently removed from circulating blood. In this sense, the reaction does have a functional similarity to the immobilization of antigen by circulating antibody, since in both cases the objective of the interaction is the eventual disposal of both participants in the reaction. Alteration of conformation during reaction may be an asset in terms of eventual processing. This interaction, therefore, is different in a fundamental way from that between an enzyme and its substrate, since the enzyme must free itself of product and be restored to its initial conformation directly after activity, whereas the hemoglobin remains tightly bound to haptoglobin even as the surrounding concentration of free hemoglobin is reduced to zero.

The irreversibility does not extend, however, to the reaction of haptoglobin with the separated α and β subunits, both of

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[1] The abbreviation Hp–Hb customarily denotes any haptoglobin–hemoglobin complex in a 1:1 molar ratio. When the complex is formed by addition of haptoglobin to excess hemoglobin, the complex is specified as Hp·Hb; when prepared in excess haptoglobin, the complex is specified Cx (Waks *et al.*, 1969). Other abbreviations used are: Hp 1–1, haptoglobin 1–1; Hp–apoHb, the haptoglobin–apohemoglobin complex; $\alpha^h\beta^h$–$\alpha^0\beta^0$, heme-containing and heme-free α- and β-globin chains, respectively; 2,3-DPG, 2,3-diphosphoglycerate.

which are reversibly bound in the oxy or deoxy form, but with quite different affinities (Chiancone *et al.*, 1968).

It has been shown that apohemoglobin binds to haptoglobin with the same stoichiometry as hemoglobin, in each case two $\alpha\beta$ dimers being associated with a haptoglobin molecule (Chiancone *et al.*, 1968). This could involve two $\alpha\beta$-dimer sites or the globin subunits within the dimer may bind to two separate sites. Spectral and related studies involving heme-containing chains do not readily distinguish between these possibilities since isolated α- and β-heme-containing chains have very similar conformations, and these do not change on re-forming hemoglobin. Thus, separation of the α- and β-heme chains on binding of haptoglobin would produce little observable change in absorption and circular dichroic (CD) spectra of these compounds. The finding of Waks *et al.* (1971) that the CD spectrum of the Hp–Hb complex is nearly the sum of the component spectra thus does not settle the question of whether the subunits separate on binding. Recently, however, Yip *et al.* (1972) and Waks *et al.* (1973) have shown that the isolated globin chains in contrast to the heme-containing subunits differ in conformation from each other when separated, but not when combined in apohemoglobin. These differences probably reflect the importance of the integrity of the α_1–β_1 interface in preserving the apohemoglobin conformation. Were such differences to develop on binding apohemoglobin to haptoglobin, they should be readily detected as spectral deviations of the resulting complex from additivity. If, moreover, the two proteins are in contact over a large surface area (Makinen *et al.*, 1972), then these contacts may limit any further change of structure of the globin within the complex. Addition of heme, then, may not lead to restoration of the native conformation, as it does upon binding to apohemoglobin and to the isolated α- and β-globin chains (Waks *et al.*, 1973).

In this paper we report studies on the alteration of conformation induced in human apohemoglobin on bonding to haptoglobin 1-1. Unlike the binding of hemoglobin to haptoglobin, the reaction produces a marked change in the globin structure. The addition, moreover, of first 2 and then 4 equiv of dicyanohemin fails to yield the secondary structure of a complex prepared by mixing hemoglobin with haptoglobin. The interaction of haptoglobin with apohemoglobin thus "freezes" certain conformational states and prevents the restoration of native structure usually brought about by heme attachment.

Materials and Methods

Human haptoglobin 1-1 was purified by the procedure of Waks and Alfsen (1966). Human hemoglobin was prepared as described by Geraci *et al.* (1969). Haptoglobin–hemoglobin complexes were prepared in the met form, and converted to the cyanmet form by the addition of sodium cyanide. Hp 1-1 and Hp–Hb complexes were generous gifts from Dr. A. Alfsen's group.

Human apohemoglobin was prepared as described by Rossi-Fanelli *et al.* (1958); α- and β-globins were separated according to Yip *et al.* (1972). The Hp–apoHb complex was obtained by mixing Hp 1-1 and apohemoglobin in excess. The mixture was then applied to a Bio-Gel P-150 column, 65 × 1.5 cm, and equilibrated with 0.1 M phosphate at pH 6.7. Fractions of 2.2 ml were collected at a rate of 18 ml/hr using a Perspex peristaltic pump and Ultrarac fraction collector (LKB).

Hemin (crystalline type I, Sigma) was dissolved in 0.1 N NaOH. The concentration was determined by measuring the absorbance at 385 nm, using 5×10^4 as the extinction coefficient (Gibson and Antonini, 1963). The hemin was converted to the dicyanide form by the addition of sodium cyanide in excess. Addition of dicyanohemin to the Hp–apoHb complex in 0.1 M phosphate (pH 6.7) was performed at room temperature using a Gilmont microburet. Increasing amounts of dicyanohemin were added to 4 ml of Hp–apoHb complex, to a final heme/protein ratio of 2 and 4. The pH change of the mixture due to addition of dicyanohemin in 0.1 N NaOH was less than 0.1 pH unit. No precipitate appeared at any time. The presence of free heme was checked by the Bio-Gel P-2 method (Waks *et al.*, 1973). The mixture was allowed to stand overnight for complete recovery of Soret absorption.

Optical Measurements. The heme to protein ratio was calculated from the absorption spectra, which were measured at room temperature on a Cary Model 14 spectrophotometer. It was assumed that the extinction coefficients of cyanmet heme-containing complexes and cyanmet hemoglobin were identical in the 600–400-mn range: 11×10^3 at 540 nm and 12×10^4 at 420 nm. The extinction coefficients used at 278 nm are shown in Table I. The extinction coefficient of the Hp–apoHb complex was calculated on a molar additivity basis. It was measured by mixing Hp 1-1 and dimeric apohemoglobin at known concentrations in a molar ratio of one haptoglobin to two dimers and measuring the absorption of the complex obtained. The calculated and the measured values usually agreed to within less than 5%. The extinction coefficient of the complexed prepared by addition of 2 equiv of dicyanohemin to Hp–apoHb ($\alpha^h\beta^h$–Hp–$\alpha^0\beta^0$) was also calculated on a molar additivity basis. The extinction coefficient at 278 nm for the chains in cyanmet hemoglobin is 2.9×10^3, which is also the average of values for the separated α and β subunits in the cyanmet forms (Waks *et al.*, 1973). In each experiment in which solutions were mixed to add heme to a complex, the measured absorbance agreed with the absorbance calculated from the known values for the component solutions and their dilution on mixing. The extinction coefficients of Hp·Hb and the complex obtained by addition of 4 equiv of dicyanohemin to Hp–apoHb ($\alpha^h\beta^h$–Hp–$\alpha^h\beta^h$) at 278 nm were assumed to be identical.

Circular dichroism measurements were performed on a Cary Model 60 spectropolarimeter equipped with a 6001 CD attachment, at room temperture for the complexes and at 4° for apohemoglobin. The protein solutions were filtered through a Millipore 0.45-μ filter before scanning. Ellipticity values are presented on a mean residue basis except in the Soret region, 450–390 nm, when they are calculated on a heme

TABLE I: Extinction Coefficients of Apohemoglobin, Haptoglobin, and Haptoglobin Complexes at 280 nm.

	$10^{-4}\epsilon_{280}$
Hp 1-1	10.20[a]
Hp·Hb	21.80[a]
Apohemoglobin dimer	2.62[b]
Hp–apoHb ($\alpha^0\beta^0$–Hp–$\alpha^0\beta^0$ [c])	15.50
Hp–apoHb + 2 equiv of heme (CN^-) ($\alpha^h\beta^h$–Hp–$\alpha^0\beta^0$ [c])	18.50

[a] From Herman-Boussier *et al.*, 1960. [b] From Gibson and Antonini, 1963. [c] The superscripts 0 and h denote heme-free and heme-containing chains, respectively.

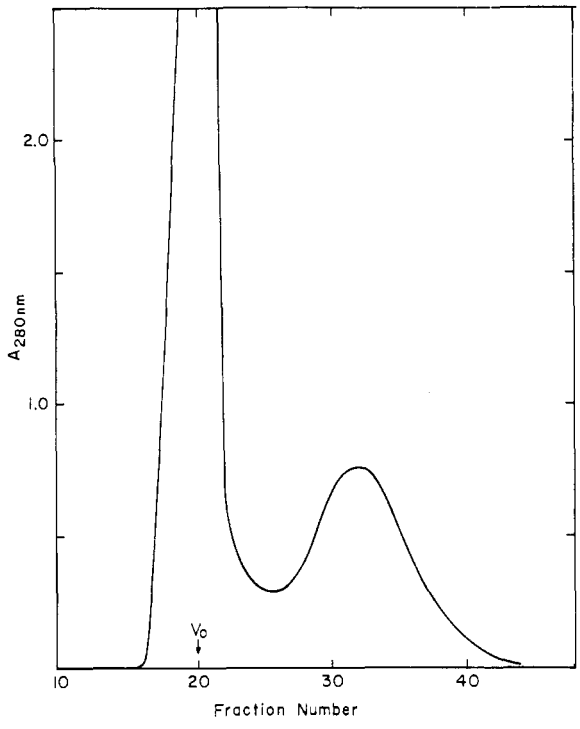

FIGURE 1: Elution profile, on a Bio-Gel P-150 column, of a mixture containing 2.24 × 10^{-7} mol of Hp 1-1 and 10^{-7} mol of apohemoglobin. The buffer is potassium phosphate (0.1 M, pH 6.7). The leading fraction eluted in the exclusion volume (V_0) is the Hp-apoHb complex. Apohemoglobin is eluted in a volume corresponding to a dimer.

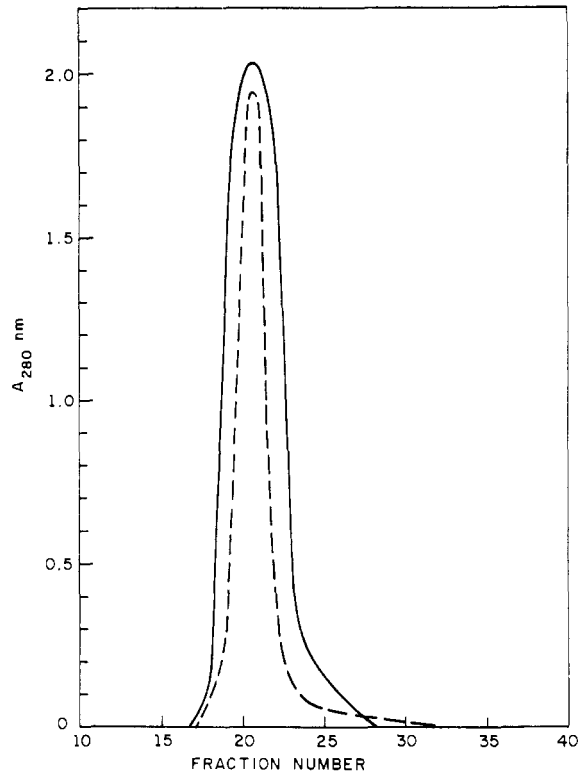

FIGURE 2: Elution profiles of: (–) a mixture containing 0.95 × 10^{-7} mol of Hp 1-1 and 1.9 × 10^{-7} mol of α- + β-globin chains and (- - -) a sample of Hp 1-1 alone at the same concentration. Experimental conditions are the same as in Figure 1.

basis. The mean residue weights used were 108 for apohemoglobin and hemoglobin and 113 for Hp 1-1 according to the amino acid composition (Cleve *et al.*, 1969; Black *et al.*, 1970). Hp 1-1 contains 15% of sugars by weight and on a weight basis a 1:1 complex contains 42% of hemoglobin and 58% of Hp 1-1.

Results

Haptoglobin–Apohemoglobin. Figure 1 shows the elution pattern of a mixture containing Hp 1-1 and apohemoglobin in a twofold molar excess. As expected, the Hp-apoHb complex (mol wt 150,000) is excluded from the Bio-Gel P-150 column while excess apohemoglobin (mol wt 30,000) is retained. Unlike apohemoglobin and α- and β-globins, the Hp-apoHb complexes were stable at room temperature without any apparent precipitate. Figure 2 shows the elution pattern of a mixture of Hp 1-1 and separated globin chains. In this experiment the molar ratio of Hp to globin chains was two α- and β-globin chains per Hp molecule. The elution pattern is made up of a single peak excluded in the same fraction number as the Hp-apoHb complex. The increased area under the peak of the complex, compared to the peak of Hp 1-1 alone, represents the added optical density of the bound globins. The figure demonstrates that the binding is complete, and that no unbound globin chains are eluted from the column.

Figure 3 shows the near-ultraviolet (uv) CD spectra of apohemoglobin, Hp 1-1, and the Hp-apoHb complex. All proteins display several negative bands in the 270-300-nm region. Apohemoglobin has a minimum at 284 nm with an ellipticity of −70 deg cm²/dmol. For Hp 1-1 there is a negative extremum at 281 nm with a molar ellipticity of −80 deg cm²/dmol. The Hp-apoHb complex exhibits negative bands located at the same wavelength as Hp 1-1: 289 and 281 nm. The negative extremum at 281 nm has a value of −60 deg cm²/dmol.

The 270-250-nm region shows a large positive band at 252 nm and a shoulder at 259 nm for apohemoglobin. The extremum at 252 nm has a value of 70 deg cm²/dmol. Hp 1-1 and the Hp-apoHb complex exhibit a negative minimum at 255 nm with intensities of −40 and −8 deg cm²/dmol, respectively.

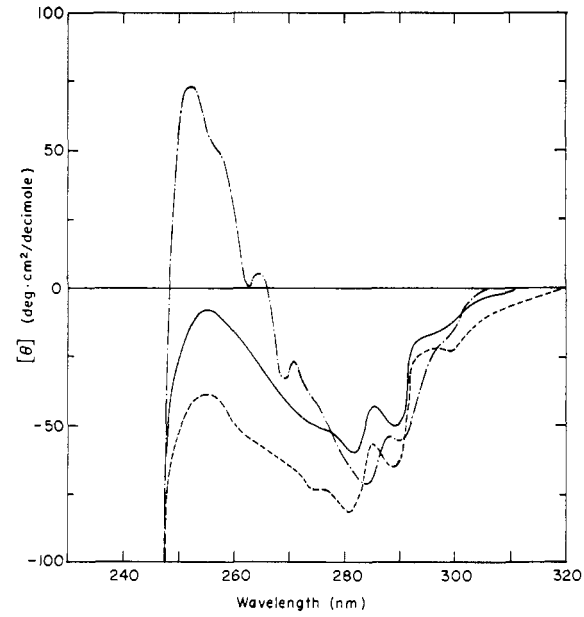

FIGURE 3: Near-ultraviolet CD spectra: (-·-) apohemoglobin, (- - -) Hp 1-1, and (–) Hp-apoHb; 0.1 M potassium phosphate (pH 6.7); temperature, 4° for apohemoglobin and 20° for Hp 1-1 and Hp-apoHb.

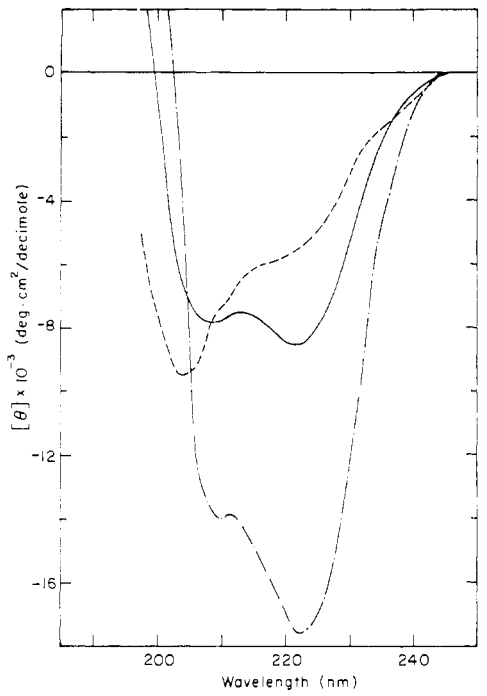

FIGURE 4: Far-ultraviolet CD spectra: (-·-) apohemoglobin, (- - - -) Hp 1-1, and (—) Hp-apoHb. Same experimental conditions as in Figure 3.

Figure 4 compares the far-uv CD spectra of Hp 1-1, apohemoglobin, and Hp-apoHb complex. The major features of the CD spectrum of Hp 1-1 are the negative extremum at 203 nm with an ellipticity of −9500 deg cm^2/dmol and the shoulder in the 215-225-nm interval. At 222 nm the measured ellipticity of Hp 1-1 is − 5800. The CD spectra of apohemoglobin and Hp-apoHb display a similar overall shape with a negative extremum at 222 nm. There is also a negative band at 210 nm for apohemoglobin and at 208 nm for the complex. At 222 nm the molar ellipticities are −17,750 for apohemoglobin and −8500 deg cm^2/dmol for the Hp-apoHb complex.

The additivity of the molar ellipticities of Hp 1-1 and apohemoglobin was investigated in both the near- and far-uv. At 281 and 255 nm the calculated values are −74 and 0 deg cm^2/dmol, respectively. In the Hp-apoHb complex the measured values are −60 and −8 deg cm^2/dmol. Table II summarizes additional parameters of the complexes. At 222 nm the theoretical sum for Hp-apoHb is −10,950 deg cm^2/dmol and the measured value −8500. Thus, the calculated and the measured values differ by −2450 deg cm^2/dmol. Furthermore, at 222 nm the experimental values obtained for the Hp-apoHb complex and the Hp-$2\alpha^0 2\beta^0$ complex are in approximate agreement. In addition, the experimental value for Hp-apoHb, while sharply different from its calculated value, is in excellent agreement with the calculated value of Hp·$2\alpha_0 2\beta_0$, which assumes residue ellipticities of the separated globins rather than intact apohemoglobin (see footnotes to Table II).

Addition of Dicyanohemin. The addition of 2 equiv of dicyanoheme to the Hp-apoHb complex generates absorption bands in the 300-600-nm region due to the bound prosthetic group. Figure 5 shows the typical absorption spectrum of such a complex: the maxima are located at 540, 420, and 360 nm as in cyanmethemoglobin. The heme/protein ratio, calculated from the absorption at 420 and 278 nm, equals 1.80 (0.9 heme equivalent per chain) and the extinction coefficient is 11×10^4. Figure 6 shows the near-uv CD spectrum of this complex. Addition of 2 equiv of dicyanohemin to Hp-apoHb results in the decrease of the intensities of the CD bands located at 281 and 289 nm. The major change, however, is the appearance of

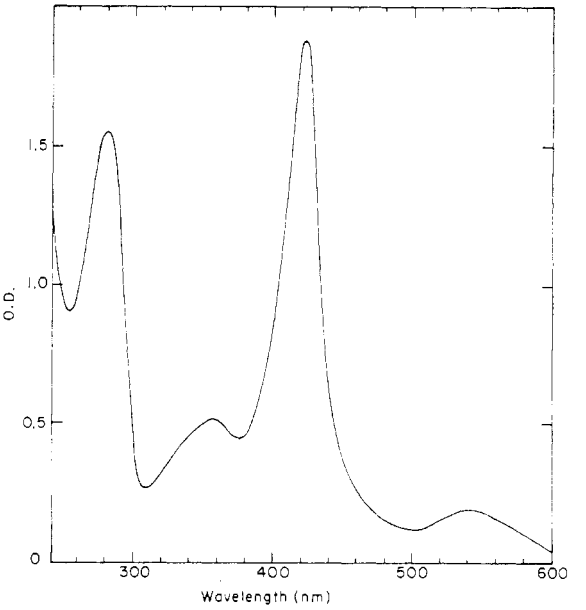

FIGURE 5: Absorption spectrum of the complex prepared by addition of 2 equiv of dicyanohemin to Hp-apoHb in 0.1 M potassium phosphate (pH 6.7).

TABLE II: Experimental and Calculated Ellipticities of Haptoglobin Complexes.[a]

Complexes	$[\theta]_{222}$ Exptl (Residue Basis)	$[\theta]_{222}$ Calcd for Additivity	$[\theta]_{420}$ Exptl (Heme Basis)	$[\theta]_{255}$ Exptl (Residue Basis)
Hp·Hb	−11,800	−12,800[b]	86,000	120
Hp-apoHb ($\alpha^0\beta^0$-Hp-$\alpha^0\beta^0$)	−8,500	−10,950[c]		−8
Hp + 2α-globin + 2β-globin (Hp-$2\alpha^0 2\beta^0$)	−7,700	−8,850[d]		−5
Hp-apoHb + 2 equiv of heme (CN^-) ($\alpha^h\beta^h$-Hp-$\alpha^0\beta^0$)	−8,850	−11,900[e]	88,000	80
Hp-apoHb + 4 equiv of heme (CN^-) ($\alpha^h\beta^h$-Hp-$\alpha^h\beta^h$)	−8,850	−12,800[f]	70,000	90

[a] [θ] is expressed in units of deg cm^2/dmol. [b] Calculated using the value of −5800 for Hp and −22,000 for human Hb. [c] Calculated using a value of −17,750 for apohemoglobin. Hp as in footnote *a*. [d] Calculated using a value of −7750 for α-globin and −17,750 for β-globin (Yip *et al.*, 1972); Hp as in footnote *a*. [e] Calculated using a value of −22,000 for $\alpha^h\beta^h$ (Hb value) and −17,750 for $\alpha^0\beta^0$; Hp as in footnote *a*. [f] Calculated using a value of −22,000 for $\alpha^h\beta^h$ (Hb value); Hp as in footnote *a*.

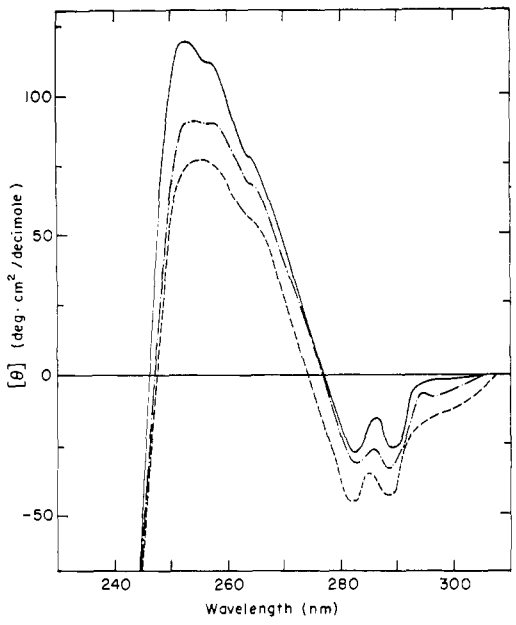

FIGURE 6: The near-ultraviolet CD spectra of: (–) Hp·Hb, (- - -) the Hp–apoHb complex after addition of 2 equiv of dicyanohemin, (–·–) the Hp–apoHb complex after addition of 4 equiv of dicyanohemin; 0.1 M potassium phosphate (pH 6.7), 20°.

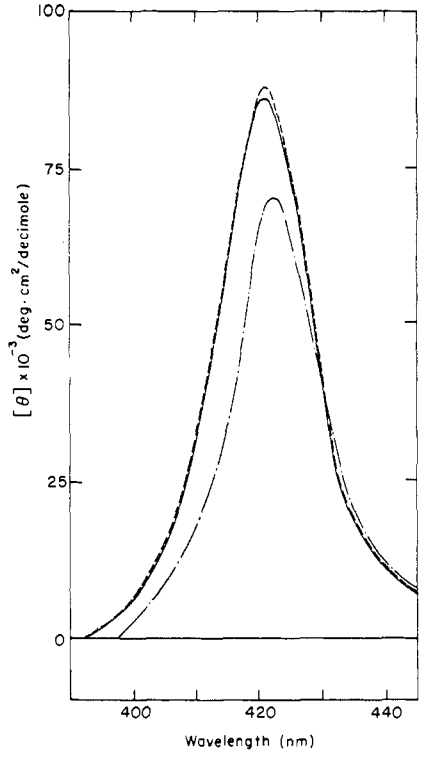

FIGURE 7: The Soret CD spectra of: (–) Hp·Hb, (- - -) the Hp–apoHb complex after addition of 2 equiv of dicyanohemin, (–·–) the Hp–apoHb complex after addition of 4 equiv of dicyanohemin; 0.1 M potassium phosphate (pH 6.7), 20°.

a large positive band at 255 nm. This band is generated by the binding of dicyanohemin in the heme pocket (Beychok *et al.*, 1967), and the observed ellipticity (on a molar residue basis) has a value of 77 deg cm²/dmol. Additivity calculations depend on assumptions about the distribution of heme on α- and β-globin chains, since the residue ellipticities at 255 nm are different for heme-containing α and β subunits. If it is assumed that the hemes are equally distributed, then the calculated ellipticity is 72 deg cm²/dmol (see Discussion).

Addition of two further equivalents of heme brings about another decrease of the CD bands located at 281 and 289 nm and an increase in the intensity of the heme band located at 255 nm, which reaches a value of 93 deg cm²/dmol. Comparison with the CD spectrum of the Hp·Hb complex shows that addition of 4 equiv of heme to the Hp–apoHb complex does not lead to recovery of the 120-deg cm²/dmol intensity of the native Hp·Hb complex. This failure is also found in the CD spectrum of both the Soret region and the far-uv. In the Soret (390–440 nm) the reconstituted complex (Hp–apoHb + 4 equiv of dicyanohemin) yields a maximum with a value of only 70,000 deg cm²/dmol at 420 nm, on a heme basis, compared to the value of 86,000 obtained for the Hp·Hb complex and the Hp–apoHb complex after addition of 2 equiv of dicyanohemin (Figure 7). Moreover, the maximum is shifted to 421 nm and the bandwidth significantly decreased.

Figure 8 compares the far-uv CD of the Hp–Hb complex, the reconstituted complex, and the Hp–apoHb complex after the addition of 2 equiv of dicyanohemin. At 222 nm the ellipticity of the Hp–Hb complex is equal to −11,800 deg cm²/dmol while both reconstituted complexes display a value of −9000 only. This value exceeds that of the Hp–apoHb complex by only 500 deg cm²/dmol. However, the heme/protein ratio calculated from the absorption spectrum at 420 nm is only 3.2 ± 0.1 although 4 equiv of dicyanohemin/mol of protein was added. Filtration through a Bio-Gel P-2 column indicated that all heme present in the sample was bound to the protein. When excess dicyanohemin was added, the heme/protein ratio remained at 3.2 after filtration through a Bio-Gel P-2 column.

Discussion

Haptoglobin–Apohemoglobin Complex. Separated α- and β-globins do not measurably combine with each other in an equimolar mixture (Yip *et al.*, 1972), but are bound by haptoglobin to form a stable complex comprising one molecule of Hp, $2\alpha^0$, and $2\beta^0$ chains. This suggests that the α-globin chain, though largely unfolded, nevertheless retains the ability to bind Hp 1–1, if β-globin is also available for binding. Moreover, the Hp–apoHb complex as well as the complex obtained by mixing 2α- and 2β-globin chains with Hp 1–1 are stable at room temperature, although apohemoglobin, and isolated α- and β-globin chains, are not. This is, in itself, a strong indication of a change of conformation of apohemoglobin on binding to Hp 1–1.

In the near-uv, differences between calculated and measured ellipticities are observed but cannot be estimated precisely. However, far-uv CD studies of the secondary structure of the Hp–apoHb complex confirm that a change in conformation accompanies complex formation. The molar ellipticity at 222 nm of this complex is more positive by 2400 deg cm²/dmol than the sum of ellipticities of Hp 1–1 and apohemoglobin. That this difference reflects secondary structure changes predominantly or entirely in the globin moieties, rather than in the haptoglobin, may be demonstrated as follows: at 203-nm, apohemoglobin and isolated α- and isolated β-globins are isodichroic, the ellipticities of all three passing through zero at that wavelength (Yip *et al.*, 1972). However, that wavelength corresponds to the only far-uv extremum in haptoglobin (in the interval 200–230 nm), with a residue ellipticity of −9500 deg cm²/dmol. If this value is preserved in the complex, then

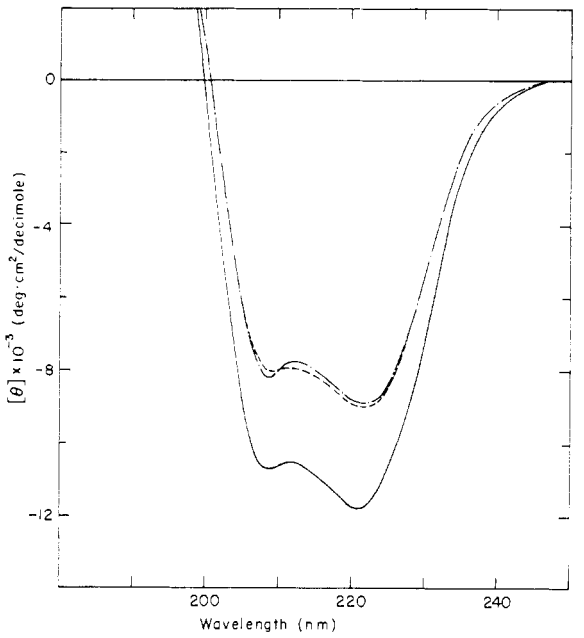

FIGURE 8: The far-uv CD spectra of: (–) Hp·Hb, (- - -) the Hp–apoHb complex after addition of 2 equiv of dicyanohemin, (– · –) the Hp–apoHb complex after addition of 4 equiv of dicyanohemin; 0.1 M potassium phosphate (pH 6.7), 20°.

the expected ellipticity is −5700 deg cm²/dmol. The observed value is within 3% of the calculated one, which is quite good agreement, at that wavelength. The departure from additivity at all other wavelengths is due to the different contributions to the CD of apohemoglobin, when free and bound. Thus both α- and β-globin chains unfold when apohemoglobin binds to Hp, or one of them does.

It has been recently demonstrated that isolated α-globin is substantially unfolded, with an α-helix content of about 20%, as compared to its helicity of 75% in hemoglobin and 50–60% in apohemoglobin (Yip *et al.*, 1972). Were dissociation and unfolding to occur upon binding of haptoglobin to apohemoglobin the calculated additivity of molar ellipticities at 222 nm would be −8870 deg cm²/dmol instead of −10,950, obtained by assuming the value for undissociated apohemoglobin. Therefore, the measured value of −8500 deg cm²/dmol is in good agreement with the ellipticity to be expected if the α-globin is unfolded when bound. Furthermore, a complex made up of 2α- and 2β-globin chains and one Hp 1–1 molecule yields, at 222 nm, a value of −7700 deg cm²/dmol. Although this result deviates by −1170 from the calculated one, the molar ellipticity is still within the same magnitude as the Hp–apoHb complex. Of course, it cannot be ruled out that in a complex made with isolated α- and β-globin chains further unfolding of α-, β-, or both globin chains occurs.

Comparison of Binding of Hemoglobin and Apohemoglobin to Haptoglobin. Although the detailed conformational differences between hemoglobin and apohemoglobin are still incompletely understood, recent investigations allow plausible inferences about differences in secondary structure. In an X-ray study of Hb M Hyde Park, Greer (1971) showed that loss of heme from β chains results in collapse of residues adjacent to the heme binding site into the empty heme pocket. The residues involved are mainly those either directly involved in the α_1–β_2 interface (Perutz *et al.*, 1968) or those neighboring such residues. While no comparable study exists for heme-free α chains, it is reasonable to expect similar changes. The changes are mainly restricted, in Hb M Hyde Park, to residues in the F, FG, CD, C, and G regions of the chains. Waks *et al.* (1973), moreover, have recently suggested that the entire C helix and the N-terminal segment of the G helix appear to owe their conformational stability in hemoglobin to the α_1–β_2 interface and to stabilizing heme contacts.

The dissociation of the tetramer of hemoglobin, which appears to be necessary for binding (Nagel and Gibson, 1971), exposes this interface and thus indirectly suggests involvement of these regions. The fact that hemoglobin and apohemoglobin are both tightly bound indicates, however, in view of the discussions above, that binding does not require the specific helical conformation of hemoglobin. Moreover, binding of apohemoglobin does not regenerate the hemoglobin conformation, according to the results of this paper, but rather induces still further disordering.

Subsites for Binding and Chain Separation. The fact that the isolated heme-containing α subunits, which are monomeric in solution, are bound by haptoglobin (Chiancone *et al.*, 1968; Nagel and Gibson, 1971) may be taken as evidence that preexisting dimers are not required, at least for reversible association. The same conclusion may be reached with respect to globins. In a mixture of noninteracting isolated α- and β-globins tight binding in correct proportions occurs (Figure 2). Preexisting apohemoglobin dimers (in measureable quantities) are accordingly not required. A dimer site thus accepts two monomer chains, but there is little reason to expect that apohemoglobin dissociates prior to binding. Once bound, however, unfolding of apohemoglobin, or of the α-globin chain in apohemoglobin, occurs. The most plausible explanation is that the dimer site is composed of two subsites, as strongly indicated by studies of isolated subunits with Hp (Chiancone *et al.*, 1968; Nagel and Gibson, 1971), and that binding separates the α and β chains at the α_1–β_1 interface to an extent which leads to unfolding of the α-globin (in apohemoglobin). The separation, however, may be incomplete, some of the α_1–β_1 surface suffering rupture and the rest remaining intact. This would point to involvement of some of the residues of that surface in binding to the haptoglobin, in addition to the regions of the α_1–β_2 interface referred to above.

Restoration of Structure upon Addition of Dicyanohemin. Addition of dicyanohemin to Hp–apoHb was studied in two steps: 2 and 4 equiv. Since it has been shown by Javaherian and Beychok (1968) that addition of 2 equiv of heme to horse apohemoglobin is able to restore more than half the helix content lost in its preparation, experiments were carried out to determine whether such a phenomenon occurs in the Hp–apoHb complex. The far-uv spectra of the complex containing only two bound hemes reveals not only the absence of such a recovery but surprisingly enough the absence of any recovery at all in the helicity of the apohemoglobin. Nevertheless, the absorption spectra as well as the near-uv CD spectra indicate that upon addition of 2 equiv of dicyanohemin to the Hp–apoHb complex, the characteristic features of the heme pocket are restored. The extinction coefficient of this molecule at 420 nm calculated from the spectrum of Figure 5 is 11×10^4, while it is 12×10^4 for cyanmetHb. The CD spectrum of the Soret band (450–390 nm) shows a recovery of the optical activity of heme, identical with that of the Hp·Hb complex.

Since it has been shown that α- and β-heme-containing chains have very different ellipticities at 255 nm (Beychok *et al.*, 1967) we have tried to identify whether dicyanohemin would preferentially bind to one of the chains or would be equally distributed between α- and β-globins. Calculations of additivity of molar ellipticity at 255 nm involving α- or β-heme chains and

Hp show that if dicyanohemin were bound predominantly to α-globin chains, the expected value (125) is much greater than observed; if bound to β-globin preferentially the expected experimental value (26) would be demonstrably lower than measured. If we assume that the two dicyanohemin equivalents are equally distributed between one α- and one β-globin, then the calculated ellipticity value would be 72 deg cm^2/dmol; the experimental result is 77. Therefore, the first 2 equiv of heme per mol of complex added to Hp-apoHb are equally distributed among α and β chains. The experimental results, which were reproduced several times, must be interpreted with some caution, however, since it is difficult to rule out preferential binding if the preferred chain exhibits heme ellipticity very distinctive from that observed in the subunits. There was no indication of this in the Soret CD. Alternatively, there may be preferred binding followed by redistribution. No recovery of secondary structure in either chain is observed (in sharp contrast to the full recovery when heme is added to α-, β-, or α,β-globin); however, recovery of characteristic absorption and CD spectra in heme bands suggests local restoration of the conformation of the heme sites.

Addition of two more equivalents of heme has no measurable effect on the secondary structure of the complex since the molar ellipticity at 222 nm remains unchanged at -9000 deg cm^2/dmol. Now, however, the native heme crevice cannot be reconstituted either, although the added heme is fully bound. The extinction coefficient at 420 nm drops to a value of 8×10^4. The molar ellipticity at 420 nm calculated on a heme basis falls, as well, from 88,000 to 70,000 deg cm^2/dmol, and the decrease of the Soret bandwidths also indicates incomplete restoration of the sites in which the two remaining hemes are bound.

The difference just described in the ability of the first two and the last two hemes bound to restore the sites in which they are attached is an intriguing, but at the same time puzzling, result of this investigation. The apparent heterogeneity in sites suggested by the observation has a counterpart in the distinctive complexes which can be formed from haptoglobin and hemoglobin (Waks *et al.*, 1969), the types formed depending on whether one or the other is present in excess during complex formation.[1]

The mechanisms by which more than one kind of complex are generated are not known. In addition to preexisting heterogeneity, there are a number of alternatives which might be considered. For example, two equally probable modes of binding might lead to two different states of bound apohemoglobin, neither of which can be restored in secondary structure but one of which can be sufficiently rebuilt by heme binding to regain characteristic heme spectral and CD parameters, or is less altered at the site during binding. If a sharp preference existed for heme binding to the restorable sites, this would account for the results. Alternatively, all bound apohemoglobin may be in the same conformational state, but binding of heme to one set of subsites in an Hp-apoHb molecule would then change the conformation at the remaining subsites.

The experimental results thus far available do not distinguish these possibilities. However, specific experimental tests are possible and are in progress.

Relation to Antibody–Antigen Complexes. In the introductory statement, we noted several important similarities and contrasts in antibody–antigen reactions, on the one hand, and haptoglobin–hemoglobin, on the other. Here, because of analogies to the Hp-apoHb complex, we discuss some results of studies on conformational change in antigens induced by antibodies elicited to them. Crumpton has recently reviewed a number of such induced conformational changes wrought by antibodies, with particular reference to the interaction of antibodies to apomyoglobin (Crumpton, 1972). In connection with the present work, the main finding is that antibodies formed in response to apomyoglobin combine with metmyoglobin with quantitative release of the bound heme (Crumpton, 1966). The anti-apomyoglobin–apomyoglobin precipitate, which formed after heme displacement, does not bind heme. Crumpton proposes that the most probable explanation of this phenomenon is that the antibody selects a transiently occurring conformation of metmyoglobin which resembles apomyoglobin with respect to conformation of a particular determinant. This combination results in "freezing of this conformation... followed by more extensive conformational changes elsewhere in the molecule." The final state achieved leads to discharge of heme. The implied constraint on flexibility of the bound antigen is supported in this view by stabilization to denaturation and increased resistance to proteolytic digestion of a number of enzymes bound to antibodies, as well as restoration of activity of inactive enzymes on binding (for references and specific examples, see Crumpton, 1972).

Many of these features are highly similar to what we have observed in the Hp–apoHb complex, *i.e.*, unfolding during binding and stabilization to denaturation. In particular, the ability of specific antibodies and of haptoglobin to freeze certain conformational states of their respective bound proteins is an important common characteristic connecting these systems. The analogy may not, however, be drawn too far, since there are important differences between the systems. To begin with, the specificities of antibodies and of haptoglobin are of altogether different kinds. The haptoglobin–hemoglobin interaction has no aspect of self-protein recognition, there being little or no species specificity over a range of hemoglobins of considerable diversity in sequence. At the same time, haptoglobin is uniquely specific for hemoglobin (or, better, the apoprotein of hemoglobin), showing no cross-reactivity whatever for example to a protein as closely related in conformation as myoglobin (Javid *et al.*, 1959). The association constant of the haptoglobin–hemoglobin reaction is many orders of magnitude greater than those of antibody–antigen reactions and the reversibility in the latter, signalled by the response to antigen excess, is absent in haptoglobin–hemoglobin. By taking advantage of multiple determinants on an antigen and gradation in binding constants, the antibody system overcomes a binding affinity of only moderate range and dissociation pressure. Haptoglobin binds hemoglobin with such extraordinary avidity that multiple determinancy is not required. In consequence, the complex remains soluble, at least in the circulating blood, where formation of precipitate during a hemolytic event might prove disastrous. Thus, two protein systems, both capable of immobilizing substrate in preparation for disposal, and possessing certain architectural similarities, have evolved along substantially different routes in order to carry out the distinctive biological function for which each is responsible in the organism.

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Surface Glycoproteins of Mouse L Cells†

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ABSTRACT: The surface glycoproteins of mammalian cells may be distinguished from internal glycoproteins by the fact that they are: (a) isolated with the smooth membrane fraction of cell homogenates, (b) degraded when intact cells are treated with proteolytic enzymes, and (c) labeled with reactive chemical groups which do not penetrate the cell membrane. By these criteria two molecular weight classes of glycoproteins, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, have been identified as components of the plasma membrane of mouse L-929 cells. Both are isolated with the smooth membrane fraction of cells lysed by Dounce homogenization. both are digested when cells are exposed to trypsin, and both are labeled by two methods, the pyridoxal phosphate–sodium borotritide system and the galactose oxidase–potassium borotritide method, which are designed to introduce radioactive label specifically into cell surface structures. The two glycoprotein classes have apparent molecular weights of approximately 100,000 and 50,000, respectively, on sodium dodecyl sulfate–polyacrylamide gels and both may contain more than one species of glycopolypeptide chain.

Current research on the surface glycoproteins of mammalian cells has been directed toward answering very basic questions. One wants to know, for instance, how many species of glycoproteins are found on the surface of particular cell types and how different cell types compare in their content and distribution of glycoprotein. How is the carbohydrate portion of these glycoproteins arranged on the polypeptide backbone and how is the whole structure associated with the lipid portion of the plasma membrane?

Studies of this type have been most thorough in the case of human erythrocytes which are found to have a single major glycoprotein species associated with the plasma membrane. This glycoprotein has been isolated in pure form and found to contain M and N human blood group specificities (Winzler, 1969; Marchesi *et al.*, 1972). The N-terminal portion of this glycopolypeptide contains all the carbohydrate and is located on the external surface of the cell while the C-terminal portion is devoid of carbohydrate and is thought to extend through the lipid bilayer to the interior surface of the cell (Segrest *et al.*, 1973; Marchesi *et al.*, 1972; Bretscher, 1971). Similar, but less extensive, studies have been carried out on other cell types. For example, glycoprotein components have now been characterized from the plasma membranes of human platelet (Phillips, 1972; Nachman *et al.*, 1973), rat liver (Glossmann and

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