Fluorescent Probe Studies of Haptoglobin Type 1-1†

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ABSTRACT: Haptoglobin is an α_2 serum protein which forms an irreversible complex with hemoglobin. The combination between these two macromolecules resembles the binding of an antigen to its antibody except that the complex remains soluble. Haptoglobin is thought to possess two identical sites lying at two ends of the protein each of which is capable of binding the hemoglobin $\alpha\beta$ unit. This investigation was undertaken to determine the nature of the hydrophobic sites on haptoglobin and to determine whether they are important in the hemoglobin–haptoglobin reaction. The interaction of ANS with haptoglobin type 1-1 is characterized by a fluorescence maximum at 470 nm which would indicate a moderately nonpolar environment for the probe. There is a marked increase in fluorescence intensity in solutions containing ANS and haptoglobin as the pH is decreased from 7 to 4. This increase in fluorescence is not due to an enhanced binding since the dissociation constant at pH 4.0 is 9.8×10^{-5} M and at pH 7.0 it is 5.4×10^{-5} M. The fluorescence of ANS bound to haptoglobin type 1-1 is decreased by the addition of hemoglobin. However, competitive equilibrium dialysis experiments show that the binding of ANS is not affected by the addition of hemoglobin. Hence, the most probable interpretation is that ANS and hemoglobin do not compete for the same sites.

Haptoglobin (Hp)[1] exists as three phenotypes which have been designated by Smithies and Walker (1956) as Hp 1-1, Hp 2-1, and Hp 2-2. Hp 1-1 is a single molecule which shows a single band on starch gel electrophoresis while Hp 2-1 and Hp 2-2 are several molecules which show a series of bands. Hp 1-1 has two heavy chains and two light chains which are connected by disulfide bonds as determined by Malchy and Dixon (1973). Black and Dixon (1968) have shown a sequence homology between Hp light chain and some Bence-Jones proteins which suggests a common evolutionary origin for Hp and antibodies.

The combination between Hp 1-1 and Hb results in a soluble complex of molecular weight 163,000 (Jayle and Moretti, 1962) which is consistent with a 1:1 stoichiometry. Evidence from ultracentrifuge studies (Chiancone *et al.*, 1966) showed that an irreversible complex is formed between Hp and oxy Hb. Malchy and Dixon (1970) further investigated the number of binding sites in Hp by precipitation studies and acrylamide gel electrophoresis. Their data suggested that Hp is bivalent in its combination with Hb and reinforced the thesis that the Hb–Hp combination is similar to the antigen–antibody reaction.

The noncovalent attachment of fluorescent probes to proteins has been used to detect conformational changes in proteins by Weber and coworkers (Weber and Laurence, 1954; Weber and Young, 1964; Daniel and Weber, 1966; Weber and Daniel, 1966) and also by Stryer (1965, 1968), Laurence (1966), Brand *et al.*, (1967), McClure and Edelman (1966, 1967a,b), Parker *et al.*, (1967), and Kenner and Aboderin (1971). Fluorescent probes are useful since they exhibit a strong dependence on the polarity of the solvent and can be used as a probe of hydrophobic areas of proteins. This investigation was undertaken to determine the nature of the hydrophobic sites on Hp and whether they are important in the Hb–Hp reaction.

Experimental Section

Purification of Haptoglobin. Hp was prepared from human ascites fluid which had originally been collected from carcinoma patients at the Vancouver General Hospital and was stored in 500-ml polyethylene bottles at −20°. After thawing, white fibrous clots were removed by centrifugation at 8000*g* for 10 min followed by filtration through glass wool.

The purification of Hp 1-1 was done by the procedure devised by Dixon and coworkers (Chan, 1968). Crude Hp was obtained by addition of solid ammonium sulfate to ascites fluid at 0° until the ammonium sulfate concentration reached 55% saturation. The precipitate was collected by centrifugation at 13,200*g* for 30 min. It was then resuspended in 100 ml volume of 0.01 M sodium acetate buffer (pH 4.7) and next dialyzed against four changes of the same buffer in the cold room.

Hp was further purified by ion-exchange chromatography using DEAE-cellulose. The Hp containing dialysate was applied to a 5.0 × 15 cm DEAE column, followed by washing with 0.01 M sodium acetate buffer until the protein absorbance of the effluent was less than 0.02. In this procedure, many unwanted proteins are allowed to pass through the column while Hp is retained. The gradient was made up from 300 ml of 0.01 M sodium acetate–0.01 M sodium chloride (pH 4.7) in the first chamber and 300 ml of 0.01 M sodium acetate–0.3 M sodium chloride (pH 4.7) in the second. After the gradient, 200 ml of the second buffer was pumped through the column. The flow rate was maintained at 30–40 ml/hr. The contents of the tubes containing Hp were pooled and lyophilized.

This solid material was dissolved in 5 ml of 0.05 M ammonium bicarbonate (pH 8.5). The final step in the purifi-

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[1] Abbreviations used are: Hp, haptoglobin; Hb, hemoglobin; oxy Hb, oxyhemoglobin; Hb-Hp, hemoglobin–haptoglobin complex; DEAE-cellulose, diethylaminoethyl-cellulose; met Hb, methemoglobin; Tris, trishydroxymethylaminomethane; ANS, 8-anilinonaphthalene-1-sulfonate; Dnp, dinitrophenyl.

cation was gel filtration on a 2.5 × 186 cm column of Sephadex G-200 with a flow rate of 5-6 ml/hr. Fractions containing Hp were lyophilized directly.

Haptoglobin Activity. The activity of human Hp was determined by the peroxidase assay as described by Connell and Smithies (1959). The Hp content of the sample was estimated by a plot of peroxidase activity *vs.* the moles of Hp at constant moles of met Hb. The break in the curve was found to correspond to a 1:1 stoichiometry between purified Hp 1-1 and met Hb.

Polyacrylamide Disc Gel Electrophoresis. The Malchy and Dixon (1970) method for polyacrylamide disc gel electrophoresis was used. A 10-μl sample which had been thickened by addition of sucrose was applied to a 5% (w/v) gel and was then covered with electrophoresis buffer which contained 0.112 M Tris, 0.062 M boric acid, and 2.5 mM disodium ethylenedinitrilotetraacetate at pH 8.6. Electrophoresis was carried out at 200 V for 1 hr followed by staining in 0.1% (w/v) Amido Black in 10% (v/v) acetic acid. Only a single, strong band for purified Hp 1-1 was found which is in agreement with the behavior seen on starch gel electrophoresis (Connell and Smithies, 1959) corresponding to a single molecular species.

Fluorescence Intensity as a Function of pH. Fluorescence emission spectra were obtained on a Perkin Elmer MPF-2A fluorescence spectrophotometer operated at a slit width of 1.29 mm and 10-nm spectral band width. All reported wavelengths of maximum emission are uncorrected for variation in monochromator efficiency and phototube response with wavelength. Emission spectra were obtained at 25° as a function of pH using buffered systems with final ionic strength of 0.033 M. The concentration of protein was 10^{-4} M and ANS (Eastman) was 10^{-5} M. The exciting wavelength was 366 nm and analyzing wavelength was 470 nm.

Equilibrium Constant for the Interaction of ANS with Haptoglobin. Emission spectra were obtained by pipetting 2 ml of the Hp solution and 1 ml of buffer into a cuvet followed by the successive addition of 25 μl of ANS solution. Base line was taken before the addition of ANS solution. Only fresh solutions of ANS stored in low actinic volumetrie flasks were used. The concentration of Hp 1-1 varied slightly from 1.19 to 1.16 × 10^{-5} M during the titration. This molarity was determined by absorption using a specific absorptivity of E_{280} = 1.2 ml/(mg cm) (Hermann-Boussier *et al.*, 1960). ANS concentration varied from 2.71 × 10^{-5} to 1.06 × 10^{-4} M. The temperature of the fluorescence cell was maintained at 25° by circulating liquid from a water bath.

The fluorescence of ANS was corrected for self-absorption of incident light by using the equation of McClure and Edelman (1967a)

$$F = F_{obsd}(2.303E_{366}[\mathrm{ANS}]_t/(1 - 10^{-E_{366}[\mathrm{ANS}]_t})) \quad (1)$$

where E is the molar absorptivity of ANS at 366 nm and F and F_{obsd} refer to the corrected and observed intensities, respectively. The data were treated by the method described by McClure and Edelman (1967a)

$$F = F_{max} - K(F/[\mathrm{ANS}]_t) \quad (2)$$

where K is the apparent dissociation constant for the formation of the Hp-ANS complex, F is the corrected fluorescence intensity obtained from eq 1, F_{max} is the maximal fluorescence of the Hp-ANS complex, and $[\mathrm{ANS}]_t$ is the total concentration of ANS. Actually 2 is another form of the Scatchard equation (Scatchard, 1949; Klotz, 1953) for identical and independent binding sites as has been demonstrated by Russo (1969).

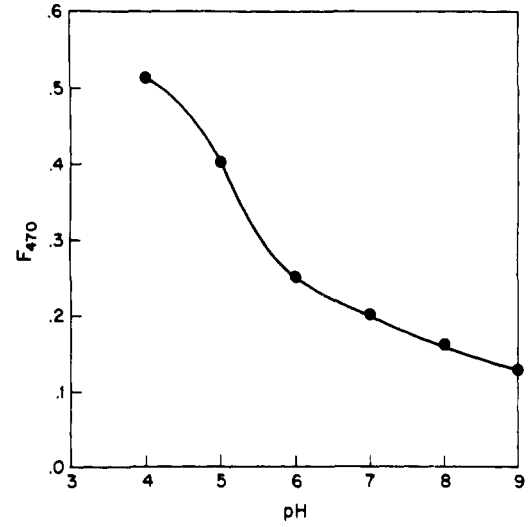

FIGURE 1: Fluorescence of the complex between ANS and Hp 1-1 as a function of pH. Hp 1-1, 1.23 × 10^{-4} M; ANS, 1.52 × 10^{-5} M. All buffers with final ionic strength 0.033 M at 25°. pH 4.0, formic acid-NaOH; pH 5.0, acetic acid-NaOH; pH 6.0, KH_2PO_4-Na_2HPO_4; pH 7.0, KH_2PO_4-Na_2HPO_4; pH 8.0, Tris-HCl; pH 9.0, Tris-HCl. Exciting wavelength, 366 nm; analyzing wavelength, 470 nm.

Equilibrium Dialysis. Cellulose casing of 0.25 in. diameter was used for equilibrium dialysis experiments (Klotz and Rosenberg, 1960). The tubing was heated in distilled water on a steam bath with three changes of fresh water in 4 hr, and was then left at room temperature for a few hours. The tubing was stored in a buffer solution in the refrigerator at least a half day before the tubing was used. Pyrex test tubes 20 × 150 mm were used as the dialysis vessel.

A 0.02% sodium azide solution was added in buffer solutions as a preservative to prevent bacterial growth. A pipet was used to transfer the sample into the casing with three knots tied in each end of tubing (Van Oss, 1967) followed by immersion in protein-free buffer. After equilibrium was reached in 24-48 hr, the concentration of ANS outside the bag was determined by absorption at 350 nm.

Results

Fluorescence Studies. Preliminary experiments were performed to determine whether there are any aromatic residues in Hp that are close enough and have the proper orientation to transfer energy to bound ANS. Energy transfer from the aromatic amino acids in Hp (1.13 × 10^{-7} M) to bound ANS (2.60 × 10^{-5} M) was demonstrated at pH 7.0. When a Hp solution was excited at 280 nm it showed a fluorescence maximum at 330 nm which is due to tryptophan (Teale, 1960). When ANS was added to the Hp solution, this tryptophan emission was quenched and an ANS emission in the visible was observed. This has been found in other ANS-protein systems (Stryer, 1965; Daniel and Weber, 1966) and is an example of singlet-singlet energy transfer which has an effective range of up to 65 Å (Stryer, 1968).

The intensity of fluorescence from the interaction of Hp 1-1 (in excess) with ANS was observed as a function of pH to determine whether there are changes in the ANS binding sites under these conditions. The data of Figure 1 show that higher fluorescence was observed as the pH was lowered.

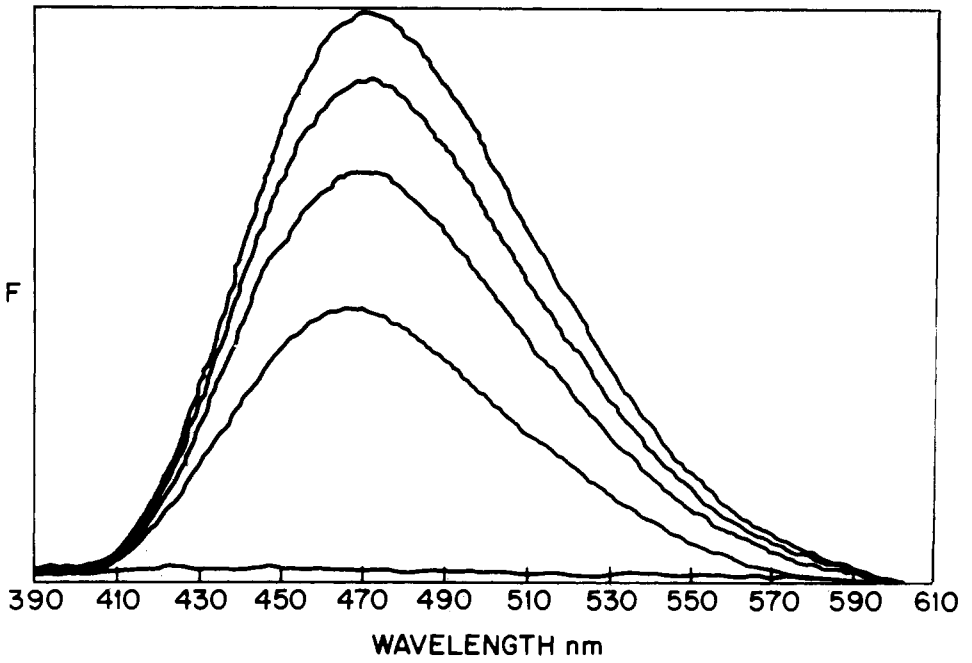

FIGURE 2: Fluorescence titration of Hp with ANS; 25 μl of ANS (1.98×10^{-3} M) was added consecutively to 3.0 ml of Hp 1-1 (1.17×10^{-5} M) at pH 4.0 and 25°. The buffer was formic acid–NaOH of ionic strength 0.033 M at 25°. The final concentrations of ANS are 1.64×10^{-5}, 3.27×10^{-5}, 4.83×10^{-5}, and 6.38×10^{-5} M.

The intensity at pH 4 was approximately fivefold greater than that at pH 9.

This variation in intensity with pH was further investigated by performing fluorescence titrations at several pH values. As an illustration the results at pH 4.0 are shown in Figure 2. Titrations at a specific pH were analyzed according to eq 2 and plots of F *vs.* $F/[\text{ANS}]_t$ were found to be linear. As shown in Figure 3A, F_{max} for the interaction of ANS with Hp 1-1 does not vary in the pH range 6–9 but increases greatly at values less than 6. In a system with identical and independent binding sites F_{max} is proportional to the number of these sites. The observed increase in F_{max} in an acid medium may reflect an increase in number of sites or it also may reflect a change in the proportionality constant (quantum yield of bound fluorophore). Figure 3B shows that in the pH range 6–9 K does not change but does increase at pH values less than 5. The value of K is 5.4×10^{-5} M in the neutral range and 9.8×10^{-5} M at pH 4.0 which means that there is less binding at pH 4 than at pH 6 for Hp 1-1.

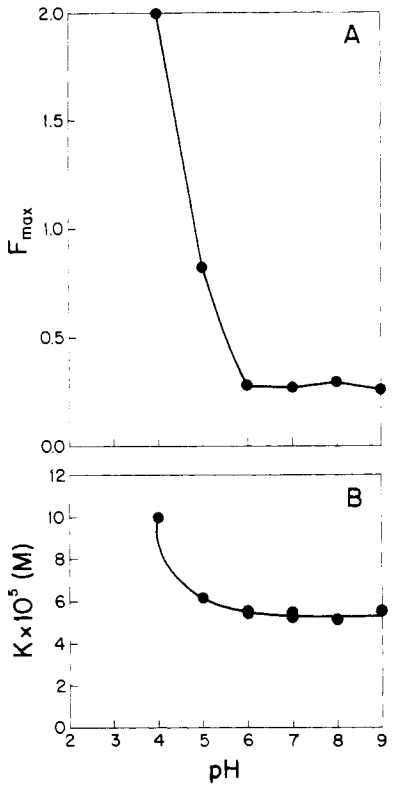

FIGURE 3: F_{max} (A) and K, the dissociation constant (B), vs. pH as determined from the titration of Hp 1-1 with ANS at 25°. Concentrations of Hp and Hb and buffer compositions as in Figure 2.

Since there are two sites for binding the $\alpha\beta$ subunits of hemoglobin the question of whether these functional sites are related to the ANS binding sites was investigated. Figure 4 shows that the fluorescence intensity is diminished by the addition of hemoglobin to the ANS-Hb complex. However, this is most probably due to the "inner-filter effect" (Ellis, 1966) rather than to any molecular interaction.

Equilibrium Dialysis. Hb and ANS both are bound to Hp. Equilibrium dialysis was used to investigate whether the binding sites are the same for Hb and ANS. Control dialysis sacks contained ANS and Hp, whereas solutions to be tested contained ANS, Hb, and Hp inside the dialysis sack. The experimental result obtained was that the absorbance at 350 nm outside the sack due to free ANS was 0.475 in both cases. The conditions of the experiment are summarized in Table I.

Discussion

ANS was used as a hydrophobic probe for Hp and the Hp–Hb complex. ANS fluoresced intensely at 470 nm when bound to Hp. It is known that the quantum yield increases and the maximum emission has a blue shift when the solvent polarity for ANS decreases (Stryer, 1965). The intensity of fluorescence from interaction of ANS with Hp 1-1 (in excess) was higher at lower pH, and this phenomenon

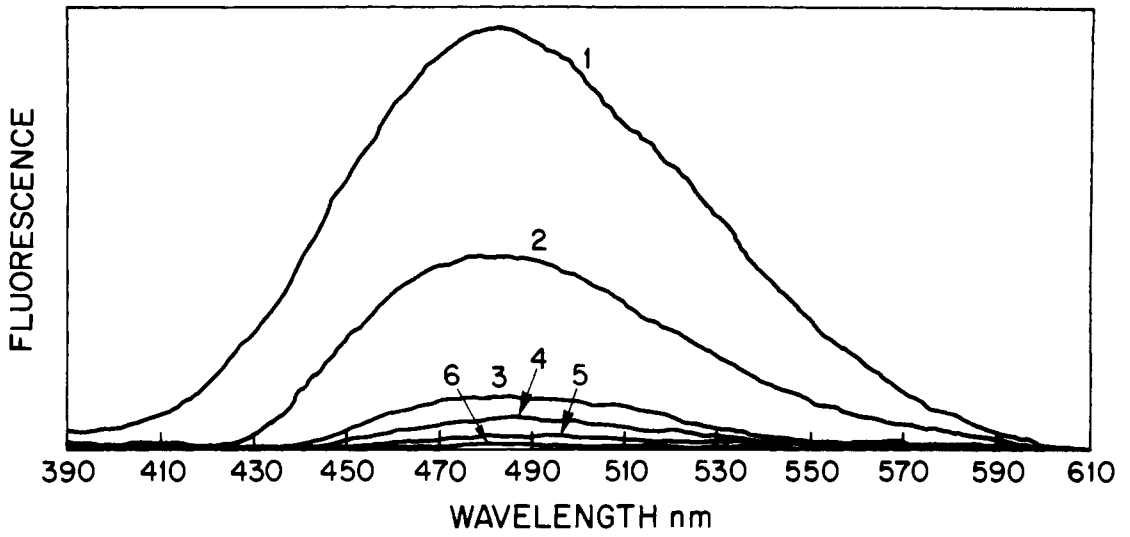

FIGURE 4: Effect of hemoglobin addition on the fluorescence of ANS–Hp. Hp, 10.6×10^{-5} mmol; ANS, 3.1×10^{-3} mmol. Solution 1, no Hb; 2, 2.4×10^{-5} mmol of Hb; 3, 6.0×10^{-5} mmol of Hb; 4, 8.4×10^{-5} mmol of Hb; 5, 10.6×10^{-5} mmol of Hb; 6, 15.0×10^{-5} mmol of Hb. Buffer composition KH_2PO_4–Na_2HPO_4 with ionic strength 0.066 M at 25°.

was further investigated by fluorescence titration between Hp and ANS (in excess). The values of K and F_{max} varied little between pH 6 and 9, but both increased at low pH. K, the dissociation constant, is inversely related to the binding affinity and increased from 5.4×10^{-5} M in the neutral range to 9.8×10^{-5} M at pH 4.0 for type 1-1 which means that the binding affinity decreased sharply on the acid side of pH 5. The interaction between ANS and Hp gave a dissociation constant of 5.4×10^{-5} M for type 1-1 at pH 7.0 which is a value similar to the dissociation constant 5.50×10^{-5} M for ANS and apohemoglobin (Stryer, 1965). In both cases there is a sharp decrease in affinity for ANS in acid solution which may be due to acid denaturation involving large changes in secondary and tertiary structure of proteins (Steinhardt and Zaiser, 1955; Beychok *et al.*, 1962). In addition, in the present work it was found that F_{max}, the maximal fluorescence of the Hp–ANS complex, changes from 0.3 at pH 6 to 2.0 at pH 4. This change with acidity is either due to a change in the ANS binding sites, the creation of new ones, or to a more complex combination of these factors.

It is known that Hp is tightly bound to free Hb with an association constant estimated at 10^{25} M^{-4} or 5×10^{6} M^{-1} for equivalent and independent sites (Chiacone *et al.*, 1968). Do ANS and Hb compete for the same sites? The ANS fluorescence with Hp decreased with increasing amount of Hb (see Figure 4) which is most probably due to the nonfluorescent Hb absorbing some of the exciting radiation. Because of this "inner-filter effect," the fluorescence vs. concentration curve can be significantly affected (Ellis, 1966). Equilibrium dialysis measurements were performed to further investigate the relationship between the ANS sites and the functional sites on Hp. The experimental result found was that the ANS absorption outside the dialysis bag was 0.475 at 350 nm for dialysate with or without Hb. This means that ANS concentration outside the bag was unaffected by the addition of Hb to an ANS–Hp complex which is consistent with Hb and ANS binding at different sites. An alternative explanation is that the addition of hemoglobin causes a conformational change in Hp. It is conceivable that if this is the case the same experimental results would have been observed for fluorescence and equilibrium dialysis measurements involving ANS.

Since there is sequence homology between Hp light chain and some Bence-Jones proteins the results obtained in this work should be compared to the binding of ANS to γ-G antibodies as studied by Parker and Osterland (1970). By means of equilibrium dialysis it was found that there were two binding sites for ANS. Antibodies which bind DNP tightly also tend to bind ANS tightly. Fluorescence studies showed that nearly 90% of the total fluorescence enhancement in anti-Dnp antibody was localized in the F_{ab} frag-

TABLE I: Dialysis Experiment.

	Test Sack		Control Sack	
	Outside of Bag	Inside of Bag	Outside of Bag	Inside of Bag
Vol of soln, ml	20.00	3.00	20.00	3.00
Moles of Hp type 1-1		0.78×10^{-6}		0.78×10^{-6}
Moles of Hb		1.43×10^{-6}		
Moles of ANS before dialysis		2.57×10^{-6}		2.57×10^{-6}
Moles of ANS after dialysis	2.18×10^{-6}		2.18×10^{-6}	
Moles of bound ANS		0.39×10^{-6}		0.39×10^{-6}
Bound ANS/total Hp		0.50		0.50
pH	7.00	7.00	7.00	7.00

ments rather than the F_c fragment which is significant in that the F_{ab} portion contains the antibody active site. The addition of homologous ligand to ANS–antibody complex greatly decreased the bound ANS fluorescence. For example, the addition of Dnp ligands interfere with bound ANS fluorescence in the ANS–anti-Dnp antibody complex. The results mentioned so far suggest that the antibody sites are hydrophobic. However, these systems were further investigated by competitive equilibrium dialysis. Since the association constant for Dnp ligand with anti-Dnp antibody is 10^6 M^{-1} and the constant for ANS with the same antibody is 10^4 M^{-1}, it should be possible to completely inhibit the fluorescence of the ANS–anti-Dnp antibody complex by addition of excess Dnp ligand if indeed the two ANS sites correspond to the antibody active sites. This was found not to be the case. If independent sites are involved the observed partial inhibition could occur by means of conformational changes induced by ligand. Parker could not state with certainty whether the ANS and DNP binding regions were the same or independent sites.

The data presented show that there are hydrophobic regions on Hp which do not correspond to the Hb binding sites. Calorimetric studies (Adams and Weiss, 1969) have shown that the enthalpy for formation of the Hb–Hp complex is −70.2 kcal/mol at 37° and −29.7 kcal/mol at 20°. It was concluded that hydrogen bonding reinforced by being in a hydrophobic environment is the most likely explanation for the type of bonding that keeps hemoglobin and haptoglobin together noncovalently. Another suggestion for the binding of the Hb and Hp has been that of ionic bonding (Robert *et al.*, (1956); Chiao and Bezkorovainy (1971)). However, Chan (1968) found stoichiometric complex formation throughout the range pH 4.0 to pH 11.0. At pH 7.5 it was found that the addition of 1 M salt had no effect on the binding which would indicate that the combination is not solely ionic in nature. It is highly likely that Hp–Hb complex formation is due to several types of interactions acting in a cooperative manner between protein surfaces of pronounced complementarity (Chan, 1968; Nagel and Ranney, 1964). Since crystallization of the complex has been successful (Waks *et al.*, 1968), direct verification from X-ray diffraction studies of the types of interaction present may be forthcoming in the near future.

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