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THE ASSOCIATION EQUILIBRIUM BETWEEN HAPTOGLOBIN AND APOHEMOGLOBIN

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SUMMARY

The binding of haptoglobin to apohemoglobin (hemoglobin devoid of heme) has been investigated. Haptoglobin of genetic type 1-1 labelled by 1-dimethylamino-naphthalene 5-sulfonyl chloride or 2-dimethylaminonaphthalene 5-sulfonyl chloride has been titrated with apohemoglobin in 20mM phosphate pH 6.8 or 5.7, at 5°C. The formation of the complex has been followed by the increase of the polarization fluorescence. The titration curves show that dansylated haptoglobin and apohemoglobin are in association equilibrium and that one haptoglobin molecule binds two molecules of apohemoglobin. The calculated association constant is $K = 4.6 \times 10^6$.

The high affinity of human haptoglobin for hemoglobin (1) provides an efficient mechanism for the removal of intravascular hemoglobin from circulation. The complex formed between these two proteins, which probably involves large surfaces of contact (2), is an unusual example of stability in a macromolecular assembly. However the association equilibrium between these two proteins has never been measured since the haptoglobin-hemoglobin complex cannot be dissociated under accessible conditions of temperature, pH and ionic strength in aqueous solvents. We have sought to explore aspects of this interaction by studying the binding of apohemoglobin to haptoglobin. While the conformation of apohemoglobin and apoHb chains is different from that of hemoglobin in a number of important respects (3), the stoichiometry of the interactions is the same: one dimer of apohemoglobin or hemoglobin per one half

Human hemoglobin: Hb; apohemoglobin: apoHb; haptoglobin 1-1: Hp. 1 or 2 dimethylaminonaphthalene 5-sulfonyl chloride: Dns-Cl. Dansylated Hp: Hp-Dns; Hp.Hb denotes an haptoglobin-hemoglobin complex in a 1:1 molar ratio.

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Hp molecule (one heavy and one light chain). Furthermore, both proteins probably bind to the same site in haptoglobin. It is hoped that as the conformational relationship between hemoglobin and apohemoglobin is elucidated, the specific surface complementarity required to achieve the very tight binding in the Hp·Hb complex may be inferred from comparative studies utilizing apohemoglobin.

In particular, the local conformation of the regions which comprise the $\alpha_1\beta_2$ interface in hemoglobin are altered in apohemoglobin (4). Since this interface is one of the presumed binding areas of hemoglobin to haptoglobin, it may be less extensively involved in apohemoglobin (5) and the complex less tightly bound.

In this paper we report for the first time the direct measurement of the association equilibrium between human haptoglobin 1-1 labelled by dansyl chloride and apohemoglobin. The stoichiometry and the association constant of the equilibrium reaction have been measured by polarization fluorescence.

MATERIALS AND METHODS

Human apoHb was prepared according to Rossi-Fanelli et.al. (6) with the modifications described by Yip et.al. (7). Human Hp was purified as previously described (8). The protein was covalently labelled with 1-dimethylamino-naphthalene 5-sulfonyl chloride (a generous gift of Dr. G. Weber) or with 2-dimethylaminonaphthalene 5-sulfonyl chloride (Molecular Probes. Roseville. MN U.S.A.) by the procedure of Weber (9). Excess of fluorescent dye was removed on a Bio-Gel P-2 column equilibrated with a 20 millimolar phosphate buffer pH 5.7 or 6.8. The number of moles of Dns-Cl bound per mole of protein was determined from absorption spectra measured on a Cary 118 spectrophotometer. For 1-dimethylaminonaphthalene 5-sulfonyl chloride molar extinction coefficients given by Weber (9) at 280 nm and 340 nm were used: $1.85\ 10^3\ M^{-1}cm^{-1}$ and $4.30\ 10^3\ M^{-1}cm^{-1}$ respectively. Protein concentrations were determined using molar extinction coefficients at 280 nm given by Waks and Beychok (4) $10.2\ 10^4\ M^{-1}cm^{-1}$ for Hp and $2.60\ 10^4\ M^{-1}cm^{-1}$ per apoHb dimer. Hp concentration was also measured by its Hb binding.

Excitation and emission fluorescence spectra of dansylated Hp and of complex were recorded on a FICA model 55 absolute differential spectrofluorometer (FICA. Le Mesnil Saint Denis. France.) All the spectra were automatically corrected for Raman scattering and photomultiplier response.

Fluorescence polarization measurements were performed on an SLM model 400 polarization fluorometer (SLM Instruments. Urbana. Ill. U.S.A.) using a 400 watt Xenon lamp. Measurements were carried out using 340 or 390 nm exciting light, 4 nm exciting bandwidth and Corning glass cut off filters CS 3-72 and CS-44 or CS 3-144 and CS 3-73, on the path of fluorescent light.

The titrations were performed in the fluorescence cuvette containing 2ml of the dansylated Hp in 20 millimolar phosphate pH 5.7 or 6.8. Increments of

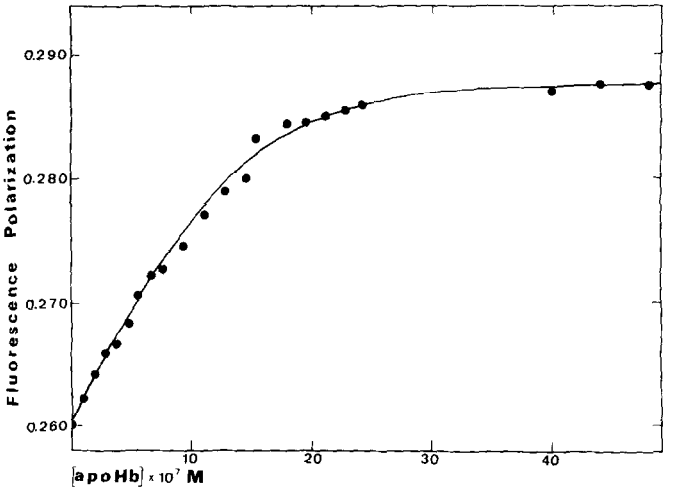

Figure 1. Titration curve of Hp 1-1 labelled by 2-dimethylaminonaphthalene 5-sulfonyl chloride with apohemoglobin at pH 5.7, in 20mM phosphate, at 5°C. The changes in polarization are plotted against the total concentration of apoHb expressed in dimers. The concentration of Hp expressed as a half Hp molecule (one heavy and one light chain) was 9.05 10^{-7} M.

10 or 20 microliters of apoHb were added, the cuvette was stirred and changes in polarization of complexes measured. The total concentration ranges for apoHb were from 5.10^{-8} to 2.0 10^{-6} molar and for Hp from 9.15 10^{-8} to 1.0 10^{-6} M

All the experiments were done at 5°C with the thermostated cell holder regulated within ±0.02°C by a Haake F2K thermostat.

RESULTS AND DISCUSSION

From spectrophotometric absorption measurements the extent of labelling of Hp by Dns-Cl was 1.10 i.e. one mole of dansyl chloride per half molecule of Hp. Since a molecule of Hp is made up of two heavy and two light chains this indicates that the fluorescent label is covalently bound only to one N terminus per half molecule. The hemoglobin binding of labelled Hp measured by static fluorescence quenching (3) and peroxidase activity (10), was identical within ±5% to unlabelled Hp.

The results of the titration of the dansyl conjugate of Hp with apoHb is shown in Fig. 1. The polarization increases with increased amounts of added apoHb. At high apoHb concentrations polarization reaches a plateau indicating

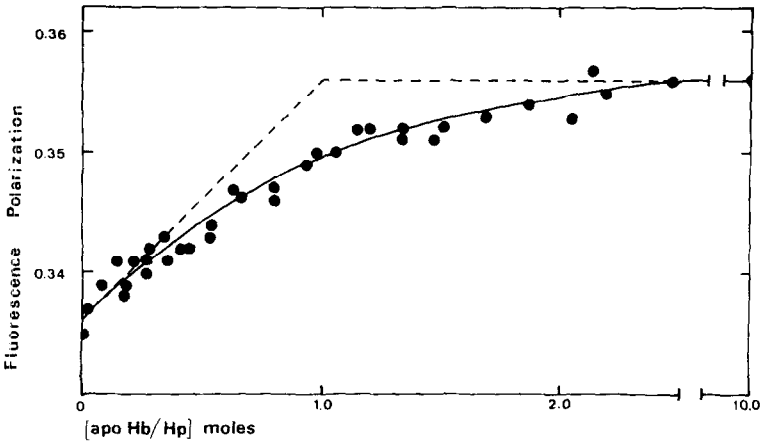

Figure 2. Dependence of fluorescence polarization on the formation of the Hp-apoHb complex. A molar ratio of one indicates a saturated complex made up of a dimer of apoHb per half Hp molecule (or a tetramer per Hp molecule). Hp was labelled with 1-dimethylaminonaphthalene 5-sulfonyl chloride. Titration was carried out at pH 6.8, in 20mM phosphate at 5°C. Concentration of Hp 5.0 10^{-7} M; concentration of the stock solution of apoHb 9.0 10^{-6} M.

saturation of Hp-Dns by apoHb. In Fig. 2 the increase in fluorescence polarization has been plotted against the molar ratio apoHb/Hp.

Application of fluorescence to the hydrodynamic properties of proteins has been described by Weber (11). At constant temperature, viscosity and fluorescent lifetime, fluorescence polarization can be used to follow changes in the hydrodynamic volume of a fluorescent particle. Since it is known that Hp and apoHb associate we can assume that the increase of fluorescence polarization is related to the formation of a complex of high molecular weight (147,000 daltons). Indeed, polyacrylamide gel electrophoresis reveals the formation of such a complex during titration. From extrapolation of the tangent at the origin of the titration curve in Fig. 2 and of the plateau, the stoichiometry of the reaction can be approximated. As expected, the two lines intersect at a molar ratio around one apoHb tetramer per Hp molecule.

As noted above, the assumption that the polarization is proportional to extent of interaction depends on constancy of the fluorescence lifetime of the probe when bound to the haptoglobin alone or in the complex with apohemoglobin.

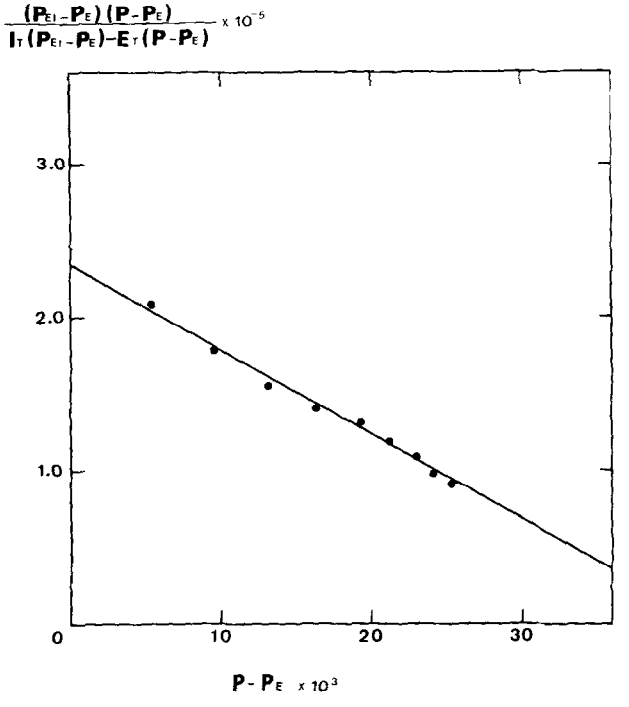

Figure 3. Scatchard plot of apoHb binding to Hp. The concentration of dansylated Hp was $9.45\ 10^{-8}$ M. The concentration of apoHb varied from $2.03\ 10^{-8}$ to $5.31\ 10^{-7}$ M. Other experimental conditions as in Fig. 1.

Dr. G. Weber (personal communication) has pointed out to us that if direct measurement of fluorescent lifetime was not available, comparison of fluorescent emission spectra of complexes formed during the titration at low and high apoHb concentrations could provide indirect evidence of a constant fluorescent lifetime. Fluorescence emission spectra of Hp labelled with 2-dimethylaminonaphthalene 5-sulfonyl chloride were recorded during the titration with apoHb. At low and high apoHb/Hp ratios the intensity and the maximum of emission spectra excited at 370 nm, was identical, indicating no change in the lifetime of the fluorescent label.

In Hp molecule the two binding sites for Hb dimers are independent as shown by Peacock et.al. (12). If this is also true for dimeric apoHb, an association constant can be calculated. The binding data are presented in Fig. 3 in the form of a derived Scatchard plot (S. Beychok, personal communication).

$$\frac{(P_{EI} - P_E)\ (P - P_E)}{I_T(P_{EI} - P_E) - E_T(P - P_E)} = K(P_{EI} - P_E) - K(P - P_E) \qquad (1)$$

where P_{EI} is the polarization of the complex at saturation, P_E the polarization of the free Hp, P the polarization at a given value of added apoHb, E_T total Hp, I_T total apoHb, K the association constant. (Equation (1), should be noted, does not require any approximation about the distribution of added ligand among free and bound states. In particular, it avoids the error introduced by assuming that all added ligand is bound.) Plotting the left hand term against $P-P_E$ (Fig.3) gives a straight line the slope of which is -K. The association constant of Hp and apoHb is $4.6 \pm 1.0 \times 10^6$. The observed linearity implies that the binding sites for apoHb are equivalent and non-interacting.

The observed association constant may be compared to that of haptoglobin to isolated α chains, which is also a reversible interaction. Chiancone et.al. (13) have carried out static fluorescence quenching experiments at 20-25°C. and estimated the association constant of Hp and α chains 5×10^6. Tsapis et.al. (14) working on agarose bound Hp, at 4°C, report an average association constant of 3.6×10^4. The results of this paper demonstrate that at moderate ionic strength the association constant of Hp and apoHb at 5°C is comparable to the constant for binding of α chain.

The most interesting and worthwhile comparison, however, would be with the Hp-Hb interaction, but the essential irreversibility of the latter precludes detailed comparison. The overall weakening of the interaction is probably due in part to diminished complementarity resulting from the modified $\alpha_1\beta_2$ surface in apoHb - a modification due to collapse of the empty heme pocket. A complete understanding of the haptoglobin-hemoglobin interaction will require elucidation of the difference in energy of interaction caused by this conformational alteration. In the immediate future, studies of haptoglobin binding to mutant globins with substitutions in the $\alpha_1\beta_2$ region may shed light on the specific residues in this surface required for binding to haptoglobin.

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