Calorimetric Determination of the Enthalpy and Heat Capacity Changes for the Association of Haptoglobin with Hemoglobin. I. Demonstration of Two Interacting Systems†

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ABSTRACT: Calorimetric determinations of the enthalpy and heat capacity changes for the association of haptoglobin with hemoglobin have been performed in function of the molar ratios of the two components. Analysis of the thermodynamic quantities permits unambiguous characterization of two different reacting systems [Hp/Hb = 1] and [Hp/Hb > 1], which can be differenciated by their respective ΔC_p value. Taking into account previous physical and chemical data on the nature of the complexes formed in these two systems the difference in ΔC_p can be attributed to an heterogeneity in the reaction product when the molar ratio Hp/Hb is equal to 1. From ΔG, ΔH, ΔS, and ΔC_p values it can be concluded that the haptoglobin–hemoglobin association reaction is similar to the refolding reaction of proteins and that they include contributions from hydrophobic interactions, hydrogen bonding, and loss in conformational entropy.

A large number of structural studies of the haptoglobin (Hp)–hemoglobin (Hb) system has recently been reviewed by Pintera (1971). Most of the previous studies have been performed with the genetic type of haptoglobin named Hp1-1 which is an oligomer of 85,000 molecular weight and homogeneous by all usual physical criteria. The reaction between Hp1-1 and Hb depends greatly upon molar ratio of the two proteins in the reaction mixture.

At a molar ratio of Hp1-1/Hb = 1 the reaction product has been characterized by electrophoresis on polyacrylamide gel, spectrophotometry, spectrofluorimetry, and ultracentrifugation (Chiancone *et al.*, 1968). All these types of measurements are consistent with a 1 : 1 reaction stoichiometry (one tetramer of Hb mol wt 64,500/mol of Hp1-1 mol wt 85,000). But the ligand (CO and O_2) binding (Alfsen *et al.*, 1970; Chiancone *et al.*, 1973) and the redox titration (Brunori *et al.*, 1968) reflect heterogeneity in the system. According to these previously published results, a tentative scheme was proposed by one of us (Waks *et al.*, 1969); the functional heterogeneity was accounted for by the existence of a molecular asymmetry of Hb bound to Hp. However the titration data obtained by stopped flow on the functional behavior of Hb bound to Hp (Alfsen *et al.*, 1970) suggest the simultaneous formation of two complexes; indeed the identical results obtained when Hp/Hb = 1 either by adding Hb to Hp or Hp to Hb is not compatible with the formation of irreversible complexes (Cx and Cd) in one case and the formation of a single reversible complex in the other case.

In contrast, at a molar ratio of Hp1-1/Hb > 1 two different complexes which we call Cd and Cx have been identified and separately characterized (Waks *et al.*, 1969; Lavialle *et al.*, unpublished results). The Cd complex is the result of the association of one molecule of Hp1-1 per Hb dimer ($\alpha\beta$); in the complex Cx one tetramer of Hb($\alpha_2\beta_2$) is associated with a molecule of Hp1-1. In this case the combination with CO and O_2 and the redox titration correspond to those of a single reacting species, although two types of complexes exist (Nagel and Gibson, 1966; Brunori *et al.*, 1968; Alfsen *et al.*, 1970). This homogeneity is consistent with a single type of interaction for the Hb dimer in both the Cx and Cd complexes.

In agreement with this set of data and the present work, a tentative scheme for the various association reactions can be proposed:

when Hp1-1/Hb = 1

$$\text{Hp1-1} + \text{Hb} \longrightarrow \text{Hp1-1}\cdot\text{Hb} + \text{Cx}\left[\frac{\text{Hp1-1}}{\text{Hb}} = 1\right] \quad (1a)$$

when Hp1-1/Hb > 1

$$\text{Hp1-1} + \text{Hb} \longrightarrow \text{Cx} + \text{Cd} + \text{Hp1-1}\left[\frac{\text{Hp1-1}}{\text{Hb}} > 1\right] \quad (1b)$$

Besides the genetic type of Hp1-1 there are two other common genetic types of haptoglobin: Hp2-1 and Hp2-2 which are formed as a series of stable polymers (Smithies, 1955). In spite of the presence of these polymers, one tetramer of Hb is bound per unit of 85,000 daltons.

This work was an attempt to analyze the thermodynamic quantities for the association reaction of the [Hp/Hb = 1] and [Hp/Hb > 1] systems for the three genetic types and to obtain information relative to the association.

Materials and Methods

Horse hemoglobin (Hb) was prepared according to Cann (1964). Methemoglobin was obtained from fresh material by addition of ferricyanide in large excess. The excess of FeCN was removed by chromatography on a P_2 Bio-Gel column equilibrated with 0.01 M acetate buffer–0.08 M in KCl, pH 5.5. The concentration of the samples was measured as cyanmet Hb ($E^{1\%}_{540}$ 7.0) on a Gilford 2400 spectrophotometer.

Haptoglobin 1-1, 2-1, and 2-2 were prepared from ascitic fluids by chromatography on DEAE-cellulose (DE-23 or DE-52 Whatman) as previously described (Waks and Alfsen, 1966). The extinction coefficient used is $E^{1\%}_{280} = 12$ (Herman-Boussier, 1960) using a molecular weight of 85,000. The hemoglobin binding capacity of each haptoglobin sample was obtained by fluorescence quenching titration on a differential

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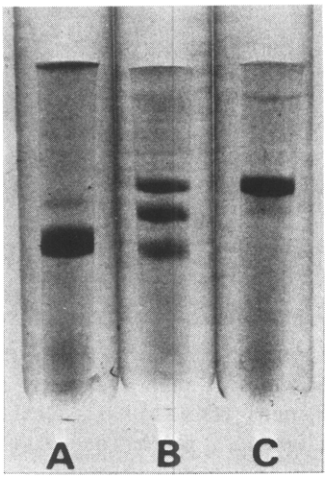

FIGURE 1: Electrophoretic pattern of Hp1-1 (A), Hpl-1/Hb = 2.2 (B), and Hp1-1/Hb = 1 (C).

spectrofluorimeter Fica 55. Haptoglobin shows fluorescence with an emission spectrum having a maximum at 329 nm, the maximum of excitation spectrum being at 281 nm. The binding of oxyhemoglobin leads to quenching. These titrations were carried out at 25° by adding to a constant amount of Hp (≃150 μg), small increments of a 0.2% Hb solution. (Both proteins were dissolved in the same buffer, 0.01 M in acetate, 0.08 M in KCl, pH 5.5.) The titration end point corresponds under these conditions to the binding of 1 mol of Hp/mol of Hb (Chiancone *et al.*, 1968).

The nature of the complex obtained at this end point was monitored by disc electrophoresis on polyacrylamide gel. The gels, at an acrylamide concentration of 7%, were prepared as described by Davis (1964) using a Tris–glycine continuous buffer at pH 8.9.

The calorimetric measurements were carried out using an LKB batch microcalorimeter. A Keithley 150B microvolt ammeter was used as amplifier and the signal recorded with a Servogor RE-512. The reaction cells were made from 18-carat gold. The integrated voltage–time curves were obtained with a read-out type Addo-X. The two compartments of the reaction cell were charged with 4 ml of metHb and 2 ml of Hp, respectively. Both protein samples have been dialyzed against common acetate buffer, overnight at 4°. The reference vessel was charged with 4 ml + 2 ml of acetate buffer, which is the same as that used for dialysis. The concentration of each reactant was adjusted such as to obtain a molar ratio Hp/Hb of approximately 1 and 2. The exact amounts of Hp and Hb for each experiment are listed in Table I.

It has been shown (Waks *et al.*, 1969) that Hb–Hp association is accompanied by a release of protons which is of course, pH dependent. This result suggests the involvement of ionic binding between the two proteins. The heat of protonation of acetic acid in the 0.01 M acetate buffer is less than 0.09 kcal/proton (Nelander, 1964) and hence is negligible. However, it must be noted that the heat associated with proton release by the proteins is still included in our reported heat values.

Results and Discussion

It should be remembered that the binding constant of the two proteins is so great that complete reaction is assured under experimental conditions. The ΔH values have been expressed in terms of kcal per Hb dimer. Indeed it is the only reference unit available in both molar ratio Hp/Hb = 1 and Hp/Hb > 1,

TABLE I: ΔH Values for Hp1-1–Hb, Hp2-1–Hb, and Hp2-2–Hb Association.

Genetic Type of Hp	$-\Delta H$ (kcal/Dimer)	Molar Ratio Hp/Hb	Amount (mg) in Initial Mixture		Temp (°C)
			Hp	Hb	
Hp1-1	10.6 ± 0.5[a]	1	3.8	2.88	
	11.6	1.07	3.66	2.74	
	16.9	2.26	3.8	1.26	
Hp2-1	10.9	1	3.44	2.52	19
	18.1 ● 0.55[a]	2.1	1.75	0.63	
	19.2	1.7	5.1	2.28	
Hp2-2	10.8	1	2.24	1.69	
	14.5 ± 0.47[a]	1.94	4.33	1.69	
	15.45	2	4.66	1.65	
Hp1-1	15.9	0.99	3.15	2.4	
	21.0	1.97	2.29	0.88	
Hp2-1	17.35 ± 0.55[a]	1	9.16	6.95	25
	17.43	1	10.6	8	
	20.2	1.88	3.25	1.31	
Hp2-2	17.34	0.97	3.07	2.4	
	20.02	2	1.26	0.48	
Hp1-1	21.9	1.01	2.98	2.23	
	21.5 ± 1.05[a]	2.15	1.46	0.52	
	23.6	1.94	7	2.74	
Hp2-1	22.9	1	2.33	1.77	30
	22.8	1.94	7	2.74	
Hp2-2	22.95	1.03	1.31	1.02	
	22.31 ± 0.05[a]	2.04	0.86	0.32	
	22.2	2.35	5.32	1.71	

[a] Standard deviation for ΔH.

since in both cases, the amount of Hb which has reacted is exactly the amount of Hb in the initial mixture. Moreover the total number of Hb dimer is very accurately and rapidly determined by spectrophotometry.

In the concentration range of the proteins used in these experiments (0.2 mg/ml < c < 2 mg/ml for Hb and 0.5 < c < 5 mg/ml for Hp), the measured heats of dilution are in the order of 5×10^{-2} mcal for experimental heats of reaction in between 0.7 and 1.5 mcal. Since these values of heats of dilution are in the limit of the measurement precision, they have been neglected for the reaction enthalpy.

Hp1-1–Hb Association

[Hp1-1/Hb = 1] System. As previously described the reaction product is homogeneous by electrophoresis (Figure 1c) as well as by other physical criteria. The ΔH values obtained for this reaction are reported in Table I. They are remarkably high but in agreement with the extremely large association constant of the two molecules (Chiancone *et al.*, 1968). The large temperature variation of ΔH is shown on Figure 2 from which ΔC_p can be calculated. The ΔC_p values were found to be negative and very large, −940 cal/Hb dimer per °C (Table II).

[Hp1-1/Hb > 1] System. According to previous data (Waks *et al.*, 1969) when Hp1-1 is in excess in the initial mixture two types of complexes are formed, as shown in the electrophoretic pattern in Figure 1B. The ΔH values measured for the simultaneous formation of Cx and Cd are of the same

TABLE II: ΔH and ΔC_p Values for Hp1-1/Hb = 1, Hp2-1/Hb = 1, Hp2-2/Hb = 1, and Hp1-1/Hb > 1, Hp2-1/Hb > 1, Hp2-2/Hb > 1 Systems.

	ΔH at 19° (kcal/Hb Dimer)	ΔC_p (cal/Hb Dimer per °C)
Hp1-1/Hb = 1 system Hp1-1·Hb + Cx	−11	−940
Hp1-1/Hb > 1 system Cx + Cd	−16.9	−400
Hp2-1/Hb = 1	−10.9	−1080
Hp2-1/Hb > 1	−18.5	−400
Hp2-2/Hb = 1	−10.8	−1080
Hp2-2/Hb > 1	−15	−580

order as those obtained for the Hp1-1/Hb = 1 reaction. The striking difference between the two reacting systems is the variation of ΔH as a function of temperature which gives rise to much lower ΔC_p for this system than for the [Hp1-1/Hb = 1] system (Table II).

After electrophoresis on polyacrylamide gel of the reaction products, spectrophotometric scanning was carried out in order to estimate the relative amount of Cx and Cd (N_1, N_1' and N_2, N_2') formed at different molar ratios. This was the only method available because of the small amounts (<10 mg) of proteins used for calorimetric experiments. Previous work was performed by chromatography on Bio-Gel DM-100 (Waks *et al.*, 1969) but in somewhat different conditions: the total amount of proteins was significantly higher ($\geq$100 mg) allowing a real separation of the components and a spectrophotometric measurement of the product amounts. Furthermore these results have never been reproduced since the column matrix was no more available and not replaced by the manufacturer. According to the equations

$$Q_1 = N_1\Delta H_{Cx} + N_1'\Delta H_{Cd}$$
$$Q_2 = N_2\Delta H_{Cx} + N_2'\Delta H_{Cd} \quad (2)$$

the value of ΔH for Cx formation (ΔH_{Cx}) and for Cd formation (ΔH_{Cd}) have to be estimated. Q_1 and Q_2 are the measured heats of reaction at two different molar ratio Hp1-1/Hb. The enthalpy of formation of each one of these two complexes (expressed in kcal per Hb dimer) is the same. By comparing eq 1a and 1b it can be seen that the difference between the reaction products of the two systems [Hp1-1/Hb = 1] and [Hp1-1/Hb > 1] is the presence of Hp1-1·Hb complex in the first one and of Cd in the second one. Since Cx and Cd have the same ΔH value of formation, the difference in ΔH values at 19° and in the ΔC_p values between [Hp1-1/Hb = 1] and [Hp1-1/Hb > 1] systems arises from the heterogeneity in the reaction product of the first system (eq 1). This agrees with heterogeneous functional behavior described for [Hp1-1/Hb = 1] system when ligand binding kinetics was studied by stopped flow or temperature-jump experiments (Alfsen *et al.*, 1970; Chiancone *et al.*, 1973), and confirms the existence of a Hp1-1·Hb complex in which the binding forces between Hp1-1 and Hb should be different from the Cx complex.

A striking feature of Table I is the equality of all ΔH's at 30°. According to Wyman (1948, 1964) the shape of the oxygen dissociation curve is independent of temperature which implies that the functional properties of Hb at 30° are not modified. Electrophoretic experiments have shown that at this

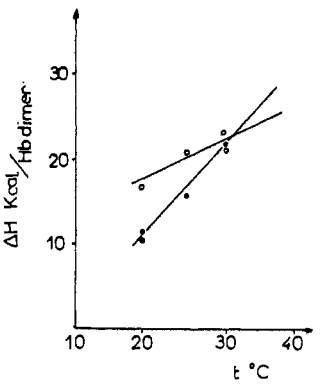

FIGURE 2: ΔH values in function of temperature: (●) [Hp1-1/Hb = 1] system; (○) [Hp1-1/Hb > 1] system.

temperature the same types of complexes are formed as at 20°: Hp·Hb + Cx when Hp/Hb = 1, Cx and Cd when Hp/Hb > 1. Furthermore Brandts (1969) pointed out that hydrophobic effects are extensively perturbed by small variations in temperature. The temperature dependence of ΔH in the [Hp/Hb = 1] system suggests therefore larger hydrophobic interactions in this system than in the [Hp/Hb > 1] system. The apparent equality of all ΔH values at 30° could be interpreted as a reinforcement of hydrophobic interactions with a higher enthalpy contribution to the global ΔH value in the 1 : 1 system.

Such an effect of the temperature leads to an equality of the global ΔH value for the two systems.

Hp2-2–Hb and Hp2-1–Hb Association. Hp2-1 and Hp2-2 behave like a series of stable polymers (Smithies, 1955). Fuller *et al.* (1973) have concluded from molecular weight estimations by sodium dodecyl sulfate acrylamide gel electrophoresis and ultracentrifugational analysis, that each polymer of Hp2-2 differs from the next number of the series by an average increment of 54,500. This approximates a subunit, consisting of one α- and one β-polypeptide chain. However few data on the binding of Hb with such polymers are actually available; but different types of studies (electrophoresis, chromatography) have shown that like for Hp1-1, two systems [Hp/Hb = 1] and [Hp/Hb > 1] can be described.

The ΔH values of the reaction with Hb obtained for these two genetic types are summarized in Table I. These values are in the same order of magnitude as those for Hp1-1–Hb association. In addition the existence of a large negative ΔC_p value for the [Hp2-1/Hb = 1] and [Hp2-2/Hb = 1] systems is apparent (Table II). The negative ΔC_p values are significantly lower for the [Hp2-2/Hb > 1] and [Hp2-1/Hb > 1] systems as it was the case for [Hp1-1/Hb > 1] system.

For these two genetic types it is not yet possible to describe a reaction model. The similarity between the ΔH and ΔC_p values for the three genetic types suggests, however, that the same types of interacting forces are involved in the binding with Hb dimer.

The first calorimetric study on Hp–Hb association was reported by Adams and Weiss (1969). The ΔH values obtained are of the same order of magnitude as those reported in this paper if expressed as kcal/Hb dimer. However it is difficult to make a more accurate comparison between the two studies since Adams *et al.* worked with a mixture of the three genetic types of unpurified Hp. Moreover they did not express all their results with the same reacting unit. In their interpretation "hydrogen bonding is the driving force reinforced by hydrophobic environment." On the other hand, Kalous and

TABLE III: ΔG, ΔH, ΔS, and ΔC_p Values for [Hp1-1/Hb = 1] System.

	ΔG (cal/Hb)[a]	ΔH (kcal/Hb)[a]	ΔS (cal/mol per °C)	ΔC_p (cal/Hb Dimer per °C)
Hp1-1/Hb = 1 system Hp·Hb, Cx	-9.15×10^3	−33.2	−80.6	−940

[a] H5 tetramer at 25°.

Pavlicek (1968) on the basis of chemical modifications of Hp and Hb molecules have indicated the participation of lysine and histidine in the association and suggested hydrogen-bond formation. But these two groups of workers did not differentiate between the [Hp/Hb = 1] and [Hp/Hb > 1] systems.

From the data presented in this work the importance of these two systems should be emphasized as well as the formation of the two complexes: Hp·Hb and Cx in [Hp1-1/Hb = 1] system.

The association constant for the reaction of haptoglobin with hemoglobin chains under equimolar conditions has been estimated to be 5×10^6 M^{-1} assuming the existence of four equivalent and independent binding sites (Chiancone *et al.*, 1968). Although this represents only a crude estimate of the intrinsic association constant, it does allow us to make an estimate of the free-energy and entropy changes for the reaction using our values for the enthalpy change. These data are summarized in Table III, from which it can be observed that the entropy change, the enthalpy, and heat capacity changes are all large and negative. These results allow us to eliminate the hypothesis that the association reaction is predominantly the result of an association of hydrophobic surfaces of the two proteins. This follows from the fact that the transfer of a nonpolar moeity from water to a nonaqueous environment is characterized by a positive entropy change, but a negative heat capacity change. While the sign of the heat capacity change for our association reaction is consistent with this hypothesis, the large negative entropy changes rules it out. However from the present data the hydrophobic interactions contribute in a larger part to the formation of the Hp·Hb complex than to Cx and Cd especially in view of the higher temperature dependence of the enthalpy values in the [Hp/Hb = 1] system.

We wish to point out that the sign and magnitude of the thermodynamic quantities for the haptoglobin–hemoglobin association reaction are similar to that which would be observed during a protein refolding reaction, *i.e.*, the reverse of protein denaturation. It thus appears that the Hp–Hb association reaction is similar to the refolding reaction of proteins, and the observed thermodynamic changes include contributions from hydrophobic interactions, hydrogen bonding, and loss in conformational entropy (Brandts, 1964). The temperature-jump measurements (Chiancone *et al.*, 1973) of the kinetic of O_2 binding on Hb bound to Hp support the loss of conformational entropy; the Hp molecule imposes contraints on the conformational flexibility of Hb. In order to obtain further informations on individual contributions, heat capacity determinations of the hemoglobin and haptoglobin molecules are currently underway.

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References

Adams, E. C., and Weiss, M. R. (1969), *Biochem. J. 115*, 441.
Alfsen, A., Chiancone, E., Antonini, E., Waks, M., and Wyman, J. (1970), *Biochim. Biophys. Acta 207*, 395.
Brandts, J. F. (1969), *in* Biological Macromolecules Series, Timasheff, S. N., and Fashman, G. D., Ed., New York, N. Y., Marcel Dekker, p 246.
Brunori, M., Alfsen, A., Saggesse, V., Antonini, E., and Wyman, J. (1968), *J. Biol. Chem. 243*, 2950.
Cann, J. R. (1964), *Biochemistry 3*, 74.
Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finazzi, Agro, A., Wyman, J., and Antonini, E. (1968), *J. Mol. Biol. 34*, 347.
Chiancone, E., Antonini, E., Brunori, M., Alfsen, A., and Lavialle, F. (1973), *Biochem. J. 133*, 205.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Fuller, G. M., Rasco, M. A., McCombs, M. L., Barnett, D. R., and Bowman, B. H. (1973), *Biochemistry 12*, 253.
Herman-Boussier, G. (1960), Thèse de Doctorat ès-Sciences, Paris.
Kalous, V., and Pavlicek, Z. (1965), *Biochim. Biophys. Acta 111*, 337.
Lavialle, F., Rogard, M., and Alfsen, A. (1974), unpublished results.
Nagel, R. L., and Gibson, Q. H. (1966), *J. Mol. Biol. 22*, 249.
Nelander, H. (1964), *Acta Chem. Scand. 18*, 973.
Pintera, J. (1971), *Ser. Haematol. IV*, 2.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Waks, M., and Alfsen, A. (1966), *Arch. Biochem. Biophys. 113*, 304.
Waks, M., Alfsen, A., Schwaiger, S., and Mayer, A. (1969), *Arch. Biochem. Biophys. 132*, 268.
Wyman, J. (1948), *Advan. Protein Chem. 4*, 407.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.