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Kinetics of Oxygen and Carbon Monoxide Binding to Liver Fluke (*Dicrocoelium dendriticum*) Hemoglobin

AN EXTREME CASE?*

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The kinetics of oxygen and carbon monoxide binding to the monomeric liver fluke (*Dicrocoelium dendriticum*) hemoglobin have been studied. The ligand association rates are ~1 × 10^8 and ~3 × 10^8 M^{-1} s^{-1}, respectively, for CO and O_2 and show no pH dependence. On the contrary the ligand dissociation rates decrease by lowering the pH below 7, the pK of the transition being around 5.5. These findings, together with spectroscopic properties of the protein, are discussed in relation to the fact that, in this hemoglobin, the distal histidine is replaced by a glycine.

Comparative studies on the functional properties of hemoglobins, differing in their primary structure, have provided valuable information on the "mode of action" of heme proteins. Studies on hemoglobins with structural differences in the heme pocket in general and, more specifically, with the distal histidine E7 replaced by another amino acid are particularly interesting (1, 2). Heme model compounds have also been successfully used for this purpose, leading to the formulation of very interesting mechanisms for the modulation of the heme-iron reactivity (3).

In the present work we report on the kinetic and some spectroscopic properties of the liver fluke Dd-Hb,[1] a monomeric protein with an extremely high oxygen affinity (4) and a large acid Bohr effect (5). The available structural data (6, 7),[2] show that, in this protein, the distal histidine is replaced by a glycine and that it represents a very old hemoglobin folding in the evolutionary scale.

MATERIALS AND METHODS

All operations were carried out at 4 °C unless otherwise stated. The reagents were of the highest commercially available grade and used without further purification. Sperm whale myoglobin type II was purchased from Sigma and converted to the oxygenated form as described elsewhere (8).

Purification of the Dd-Hb—The procedure used for the purification of the Dd-Hb is a modification of the one used by Ascenzi *et al.* (9). The bile ducts of infected sheep livers were perfused, at room temperature and under 1 atm pressure, with 0.9% NaCl. The flukes were collected, separated from the larger blood clots and pieces of liver tissue, and washed several times with isotonic saline. If not used immediately the parasites were quickly frozen in liquid nitrogen.

Typically, 10 ml of flukes were mixed with an equal volume of 15 mM sodium phosphate buffer, containing 50 μM phenylmethanesulfonyl chloride, pH 7.4, and homogenized at 0 °C. After centrifugation for 100 min at 100,000 × *g* the clear supernatant was carefully collected through the floating lipid layer, filtered through a microfilter with a pore size of 0.2 μm, and concentrated by ultrafiltration. The resulting solution was passed through a Sephadex G-100 (Pharmacia Fine Chemicals, S-75104, Uppsala, Sweden) column (3.5 × 40 cm) equilibrated with the above phosphate buffer. The fractions containing Dd-Hb were pooled, concentrated, and dialyzed against 50 mM Bistris buffer containing 40 μM sodium chloride, pH 6. After centrifugation for 10 min at 10,000 × *g* the material was applied on a DEAE-Sephadex A-50 (also from Pharmacia Fine Chemicals) column (3 × 33 cm) equilibrated and eluted first with the buffer used for dialysis, followed by one containing 100 μM NaCl. The fractions corresponding to the two major peaks obtained (Fig. 1) were separately pooled, concentrated by ultrafiltration, and checked for purity by polyacrylamide gel electrophoresis, both in the presence and absence of sodium dodecyl sulfate (10-12). The samples were frozen and kept in liquid nitrogen until immediately before use.

Spectrophotometric Measurements—The derivatives used for the spectrophotometric studies were prepared as reported previously (13). Electronic absorption spectra were recorded either on a Cary 219 or on a Varian model 635 spectrophotometer while circular dichroism studies were performed using a Jasco 740 AS dichrograph.

The extinction coefficients of Dd-Hb were calculated from the iron content in different solutions of the protein. For this purpose all glassware was washed with *o*-phenanthroline in order to remove eventual iron contaminations. The content of this metal in the Dd-Hb solutions was determined by atomic absorption using the dilution buffers as blanks. For each sample the determination was done before and after a 10-fold dilution and repeated twice.

Analytical Ultracentrifugation—The measurements were performed on a Beckman model E analytical ultracentrifuge. Sedimentation velocity experiments were done at 20 °C and 60,000 rpm while equilibrium experiments were done at 13.5 °C and 30,000 rpm. The samples used had protein concentrations between 4 and 6 μM, and the buffers were either 0.2 M citrate, pH 5, or 0.2 M Bistris, pH 7. Monitoring was done at 410 nm.

Rapid Kinetics—O_2 and CO association as well as O_2 dissociation rates were measured by flash photolysis. The features of the apparatus used are essentially the ones already described (14). A flash lamp pumped dye laser (model 34 from Electro Photonics Ltd., Belfast, Northern Ireland, United Kingdom), equipped with a 1.4-microfarad capacitor (Maxwell Laboratories, Inc., San Diego, CA) was used. In place of the standard output coupler (a dielectric mirror) a broad band resonator plate (Phase-R Co., New Durham, NH) (14) was mounted on the laser. Rhodamine 6G (Lambda Physik, D-3400, Goettingen) was used as dye, giving a light pulse centered around 585

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This work is dedicated to the outstanding scientist and unforgettable friend, Eraldo Antonini.

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[1] The abbreviations used are: Dd-Hb, *Dicrocoelium dendriticum* hemoglobin; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Mb, myoglobin.

[2] K. J. Wilson, personal communication.

nm. Its duration was of ~4 μs. Monitoring of the reaction was done by recording the per cent of transmission changes on a transient recorder (TM-109, R. Maurer, Lucerne, Switzerland) with a time resolution of 1 μs/point. Synchronization of the data acquisition system with the laser was performed by means of a fast photodyode which was triggering the transient memory when illuminated by the laser pulse. The whole system was controlled by a MINC-11/23 computer (Digital Equipment Corporation, Maynard, MA) by means of an IEEE-488 interface and a digital to analog converter module. This allowed direct recording and averaging of several traces with considerable improvement of the signal to noise ratio.

Oxygen dissociation rates were measured by photodissociating the CO derivative of the protein, in the presence of variable concentrations of CO and O_2, and monitoring the formation of the carbonyl derivative from the oxygenated one formed immediately after the flash. Ligand concentrations were chosen so that the product $(O_2)\ k_{on}\ l_{off}$ could be neglected as compared to $(CO)\ l_{on}\ k_{off}$. Thus plots of the rate of displacement of O_2 by CO against the ratio of the O_2 to the CO concentration could be used to calculate the O_2 dissociation rates (15). Measurements of the oxygen and CO binding rates were done by direct photodissociation of the corresponding derivatives. The experiments were carried out at different ligand concentrations, and the second order rate constants obtained from the slope of the line result by plotting the experimental pseudo first order rates *versus* the free ligand concentration at time zero. The independently measured values of the dissociation rates were also taken into account.

The quantum yield of the Dd-Hb·CO complex was determined by the pulse method (16), using sperm whale Mb as reference.

CO dissociation rates were measured by displacement with NO (17) using the stopped flow apparatus described elsewhere (18).

RESULTS

Fig. 1 shows the elution pattern of the Dd-Hbs from the DEAE-Sephadex A-50 column. As compared to the gradient elution (9), the stepwise procedure greatly reduces the time required for the purification, thus improving the quality of the material. Despite their difference in charge, no functional difference has been found between the two main Dd-Hb fractions. However, also in view of the presence of residual sheep Hb (*PI*) and of a third minor Dd-Hb component (*PIV*) in the G-100 eluate, the DEAE-chromatography step was always performed. Most of the results hereafter reported refer to the Dd-Hb component eluted as *PIII*.

In Fig. 2 the association and dissociation rates for the reaction between Dd-Hb and both CO and O_2, at different pH values, are reported. While the dissociation constants (l_{off} and k_{off}) are markedly influenced by pH, no significant pH effect is observed on the association constants (l_{on} and k_{on}). At pH 4, in very diluted solutions and in the presence of oxygen, the protein is not very stable, thus not allowing kinetic investigations on its reaction with O_2.

From the changes in l_{on} on varying the temperature between 5 and 25 °C (Fig. 3) an activation energy value of ~8 kJ/mol is obtained, both at pH 7 and 5.

The photochemical efficiency for the dissociation of CO from Dd-Hb is illustrated in Fig. 4 together with parallel measurements performed on sperm whale Mb. From these data on apparent quantum yield of 0.35 is obtained, according to Brunori *et al.* (16).

An attempt in characterizing Dd-Hb from the spectrophotometric point of view has also been made. The mM absorption coefficients for the CO derivative of Dd-Hb at pH 7, obtained from the atomic absorption determination of the iron content in the protein, are given in Table I, and the CD spectra of the same derivative at pH 5 and 7 are reported in Fig. 5.

From the analytical ultracentrifugation experiments the same values of sedimentation coefficients are obtained, regardless of pH and state of ligation of the protein. The average molecular weight, calculated from these measurements, is 16,330 (±200) daltons.

DISCUSSION

Comparing the kinetic properties of Dd-Hb with the ones of other heme proteins, capable of reversibly binding ligands such as O_2 or CO, illustrates the uniqueness of Dd-Hb (Fig. 2 and Table II). It has the fastest CO binding and dissociation rate reported in the literature to date and its O_2 binding velocity equals that of *Chironomus thummi-thummi* erythrocruorin III (19). At the opposite extreme we find horseradish peroxidase with kinetic constants several orders of magnitude lower than the ones of Dd-Hb (20, 21). It is striking that proteins with very similar overall structural features, *i.e.* the same heme group inserted in a hydrophobic pocket and with a histidine as one of the axial ligands, differ so dramatically in their kinetic properties.

A relevant role in determining the kinetic properties of heme proteins is attributable to the geometry of the heme pocket at the distal side. Several hemoglobins, in which the distal histidine is replaced by another amino acid, have been reported (2, 22–24), and often they display increased rates of

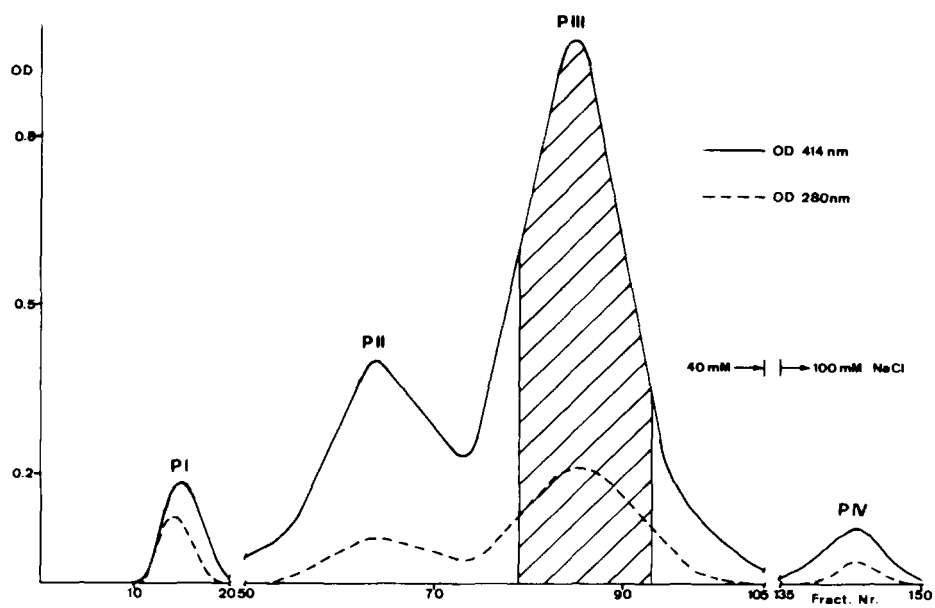

FIG. 1. **Elution pattern from the DEAE-A50 column of the liver fluke hemoglobin components.** A flow rate of ~35 ml/h was used, and 8.5-ml fractions were collected. Monitoring was done both at 280 nm (– – –) and 414 nm (——). For more details see text.

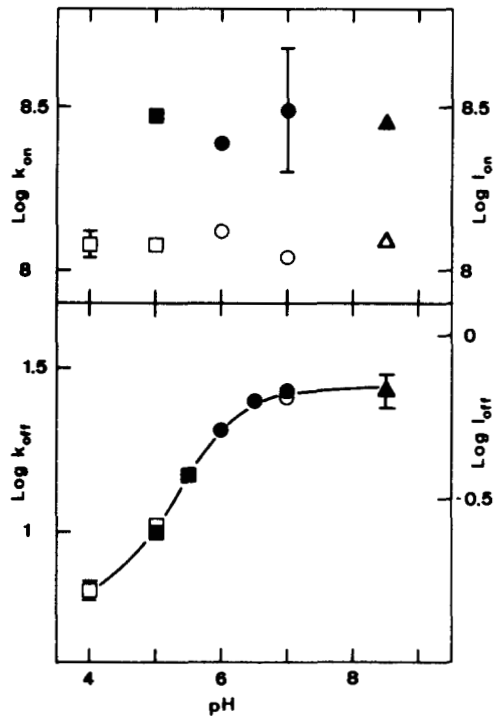

FIG. 2. **pH dependence, at 20 °C, of the combination (on) and dissociation (off) rate constants for the reaction of Dd-Hb with O_2 (*k*, ■, ●, ▲) and CO (*l*, □, ○, △).** Buffers used are 0.1M Citrate (squares), 0.1M Bistris-HCl (circles), and 0.1M Tris-HCl (triangles). Typical error bars for each type of measurements are also given.

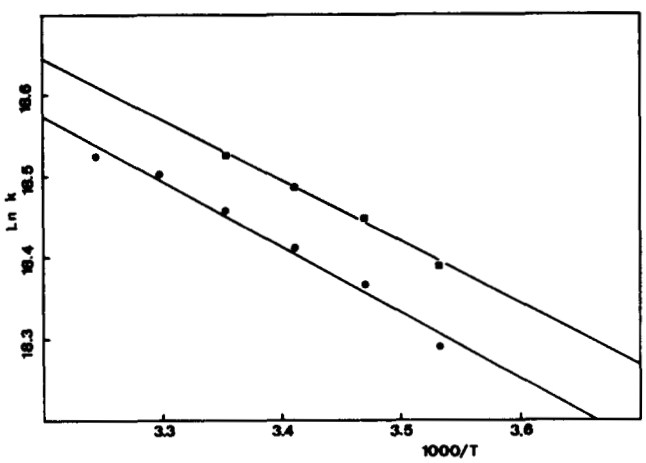

FIG. 3. **Effect of temperature on the rate of combination of Dd-Hb with CO.** The reaction was followed at 420 nm, using 3.5 μM protein in 0.2 M citrate buffer, pH 5 (●) or 0.2 M Bistris buffer, pH 7 (■). The *solid lines* were obtained by least squares fitting.

CO binding (2, 22, 25, 26). However, the replacement of the distal histidine is not always accompanied by increased CO binding rates. For example, *Aplysia* Mb has the same l_{on} as sperm whale Mb despite the presence of a valine at the distal side (1, 24). A feature common to many heme proteins with increased CO binding rates is the existence of a large cavity at the distal side (27, 28). In view of the replacement of the distal histidine by a glycine (6, 7),[2] such a situation is easily predictable also for Dd-Hb. This conclusion is further supported by the very low ratio between O_2 and CO binding rates, found to be around 3 for Dd-Hb (on purely statistical grounds, a value of 2 would be expected for such a ratio, since for the

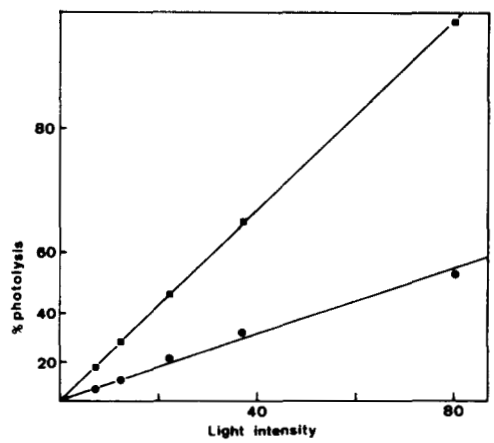

FIG. 4. **Dependence of the amount of dissociated CO from liver fluke hemoglobin (■) on changes of the photolysing light intensity.** Neutral filters were used to attenuate the intensity of the laser beam. Sperm whale myoglobin (■) was used as a reference. 3.5 μM protein and 20 μM CO concentrations were used. Monitoring was done at 420 nm.

TABLE I

Absorption coefficients of CO liver fluke hemoglobin in 50 μM Bistris buffer, pH 7

Values are given in mM^{-1} cm^{-1}.

420 nm	540 nm	570 nm
166.4 (±2.4)	12.2 (±0.1)	13.1 (±0.1)

O_2 molecule the probability for approaching the iron with the correct orientation is twice that for CO molecules). However, an increased size of the heme pocket alone is not sufficient to explain the extraordinarily high ligand binding rates reported in this work (2, 25, 26, 29). A factor which may significantly contribute to the increased velocity of ligand binding is the position of the iron atom relative to the heme plane. In this context *Chironomus* erythrocruorin III is interesting. This protein has fast ligand binding rates, but not a particularly wide cavity at the distal side of the heme (29). The high resolution crystallographic data available (29) show that, in the deoxy structure, the iron is only 0.17 Å out of plane, as compared to 0.58 Å found in deoxy human hemoglobin (30), but its electronic structure is as normally found in hemoglobins (29). This may explain its increased velocities in the binding of ligands. In the EPR spectra of the NO derivative of Dd-Hb above pH 7, the superhyperfine structure, related to the interaction of the iron with the proximal histidine, is lacking (31). The same observation has been made for Hb Zurich (32), and here again no alteration of the electronic structure of the iron is observed (33, 34). All these observations are consistent with an increased distance between the iron atom and its proximal ligand, possibly related to a more planar structure of the heme.

The very low absorption coefficient of carbonyl Dd-Hb (Table I) and its peculiar dichroic properties in the Soret region (Fig. 5) are difficult to interpret in the absence of more detailed structural data. However, some qualitative analysis of these results can be done. The splitting of the Soret CD spectrum cannot be attributed to transition-dipole interaction between two or more hemes since Dd-Hb is monomeric and does not form aggregates, as shown by the ultracentrifugation data. The strong pH dependence of the splitting, taken together with the EPR data (31), suggests the existence of a relationship between the dichroic properties of Dd-Hb in the Soret region and the position of the iron, as referred to the

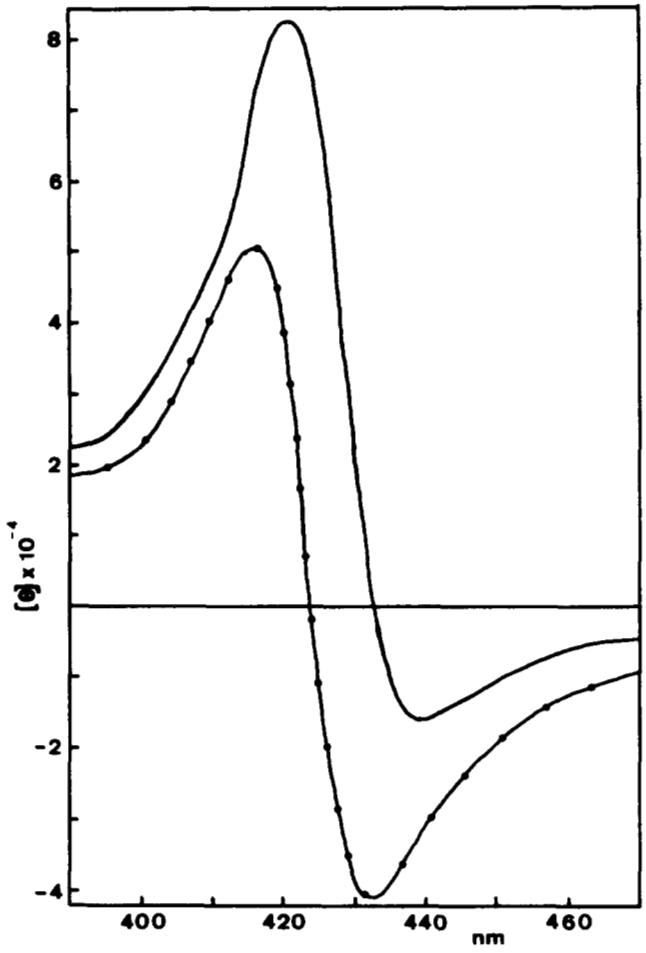

FIG. 5. **Circular dichroism spectra of liver fluke hemoglobin in the carbonmonoxy form in 0.5 mM citrate, pH 5 (——), and 0.5 mM Bistris, pH 7 (●——●).**

TABLE II

Comparison of the kinetic properties, at 20 °C, of Dd-Hb with other heme proteins

Protein	l_{on}	l_{off}	k_{on}	k_{off}
	$M^{-1}\,s^{-1} \times 10^{-7}$	s^{-1}	$M^{-1}\,s^{-1} \times 10^{-7}$	s^{-1}
Dd-Hb	11	0.65	30	30 pH 7
Dd-Hb	12	0.27	30	10 pH 5
SW-Mb	0.065	0.015	1.9	10[a]
α human	0.4	0.013	5.0	28[b]
β human	0.45	0.008	6.0	16[c]
β^{ZH}	2.5		7.0	34[d]
GD-Hb	2.2	0.055	19.0	2800[e]
CTT-Ery	2.7	0.095	30.0	218[f]
Lg-Hb	1.3	0.012	15.0	11[g]
HRP	0.00034	0.000016	0.0058	0.007[h]
Ascaris	0.017	0.018	0.15	0.004[i]
AL-Mb	0.05	0.02	1.5	70[j]

[a] Sperm whale Mb, pH 7, data from Ref. 1.
[b] pH 7.4, data from Ref. 1.
[c] pH 7.4, data from Ref. 1.
[d] pH 7.4, data from Ref. 2.
[e] *Glycera dibranchiata* Hb, pH 7, data from Ref. 22.
[f] *Chironomus T. T.* erythrocruorin, pH 7, data from Ref. 19.
[g] Leghemoglobin, 25 °C, data from Ref. 3.
[h] Horseradish peroxidase, data from Refs. 20 and 21.
[i] Perienteric fluid Hb, data from Ref. 3.
[j] *Aplysia* Mb, data from Ref. 1.

porphyrin ring, due to a change in its axial ligation state (35). The pH dependence of the Soret CD properties of Dd-Hb also correlates with the presence in this protein of an acid Bohr effect (5). The data of Fig. 2 show that this Bohr effect solely depends upon changes in the dissociation rates. The pK of the transition is around 5.5 suggesting a possible role of a histidine, interacting with the heme, in determining the changes in ligand dissociation rates. In the NO derivative of Dd-Hb, lowering of the pH below 7 leads to the rupture of the bond with the proximal histidine (31). An analogous situation, although less pronounced, has been described for *Aplysia* Mb (36). The rupture of the proximal bond of the iron has been correlated to an increase in the ligand binding rates (37, 38). However, the EPR data on Dd-Hb NO (31) and the kinetic results of Fig. 2 strongly indicate a proximal side regulation of the ligand dissociation velocities. It should be pointed out that Dd-Hb is probably the ideal system for the study of this type of regulatory processes since the association rates are already at the upper limit and practically regulated only by diffusion of the ligand through the protein matrix (39).

The high values of l_{off} found for Dd-Hb and the aforepostulated large size of its heme pocket are in apparent disagreement with the low quantum yield obtained from the data depicted in Fig. 4. The multiple barriers model proposed by Frauenfelder (40) offers a plausible explanation for this phenomenon. It has been demonstrated that to a fast binding velocity of the ligand from the solvent, as measured at room temperature, corresponds a very small energy barrier for the innermost process (*i.e.* direct binding of the ligand to the iron from inside the pocket) (25, 26). We can thus assume that the activation energy for the innermost process in Dd-Hb is not too different from the one reported for the β^{ZH} chains (~1 kJ/mol). Being the overall activation energy of CO binding to Dd-Hb of 8 kJ/mol (Fig. 3), geminate ligand recombination is favored over escaping from the pocket, resulting in a low apparent quantum efficiency.

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