KINETIC PROPERTIES OF INTERMEDIATES IN HEMOGLOBIN FROM TROUT *SALMO IRIDEUS*

Maurizio BRUNORI, Bruno GIARDINA and Ernesto E. DI IORIO*

CNR Center of Molecular Biology, Institutes of Biochemistry and Chemistry, Faculty of Medicine, University of Rome, and Laboratory of Molecular Biology, Institute of Biochemistry, University of Camerino, Camerino, Italy

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1. Introduction

Understanding of the reaction mechanism in the binding of ligand to ferrous hemoglobin has greatly profited from successful attempts to prepare and characterize partially ligated intermediates. The availability of the isolated chains of human hemoglobin has made it possible to prepare frozen intermediates in which one type of chain is in the ligand-bound (ferric) state, and the other is free to react with oxygen or carbon monoxide [1–4]. However, it has not been possible to isolate intermediates in which either one or three sites in the tetramer are frozen in the ligand-bound state. With human hemoglobin the inability to isolate such intermediates has been interpreted on the basis of the fast dimer-exchange process occurring in the ligand bound form [5].

It has recently been reported that one of the hemoglobin components from trout's blood, the so-called Hb trout I, is a very stable tetramer, the value of the dissociation equilibrium constant being 10–100 times smaller than that of human hemoglobin [6]. Therefore, it seemed feasible in the case of Hb trout I to attempt the preparation and isolation of all the species which should exist upon partial oxidation of Hb CO. As reported below, it was indeed possible to isolate the five species expected on theoretical grounds, i.e.: $(\alpha_2\beta_2)(CO)_4$, $(\alpha_2\beta_2)^{+}(CO)_3$, $(\alpha_2\beta_2)^{+2}(CO)_2$, $(\alpha_2\beta_2)^{+3}(CO)$, and $(\alpha_2\beta_2)^{+4}$.

This note reports some of the kinetic properties of the isolated intermediates from Hb trout I, as compared to the corresponding intermediates obtained by partial photolysis of Hb CO. Hb trout I is particularly suitable also in this respect because, due to the high stability of the tetramer, no quickly reacting component due to free dimers is observed at protein concentrations equal to or higher than micromolar [7].

2. Materials and methods

Preparation of trout hemoglobin and purification of the various components was carried out as previously described [8].

The oxidation intermediates were purified by ion-focussing performed on a Hb solution in 0.1 M phosphate buffer pH 7.5 saturated with carbon-monoxide and treated with one half the stoichiometric amount (as referred to heme concentration) of K_3 [Fe $(CN)_6$]. The column used was the LKB 110 ml and the pH range of the ampholine was from 7–9. The electrophoresis was carried out at a constant voltage of 500 V for 24 hr.

Five distinct bands were obtained and, after elution, their spectra were recorded. The amount of Met-Hb in each solution was determined by the ratio between the absorption at 405 nm and that at 419 nm. In addition, an estimate of the % oxidation in each sample was obtained from the flash experiments by comparing the flood absorption change before and after addition of dithionite to each individual sample.

For the flash experiments the solutions were diluted with 0.1 M Tris–HCl buffer pH 7.4 to give a final concentration from 4 to 5.5 μM and then saturated with CO.

Flash photolysis measurements were performed using

* Permanent address: Friedrich Miescher-Institut, CH-4002 Basel, Switzerland.

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an apparatus similar to that previously described [9].

Spectrophotometric measurements were performed with a Beckman DBGT spectrophotometer.

Carbon monoxide was obtained from S.I.O. (Rome). All other reagents were analytical grade products.

3. Results and discussion

Flash photolysis studies have shown that, on partial photodissociation, a kinetic component characterized by a recombination rate constant with O_2 and CO higher than that obtained by mixing experiments is observed [1, 10]. The presence of this quickly reacting form (QRF) of hemoglobin has been correlated with the presence of ligand-linked conformational changes,

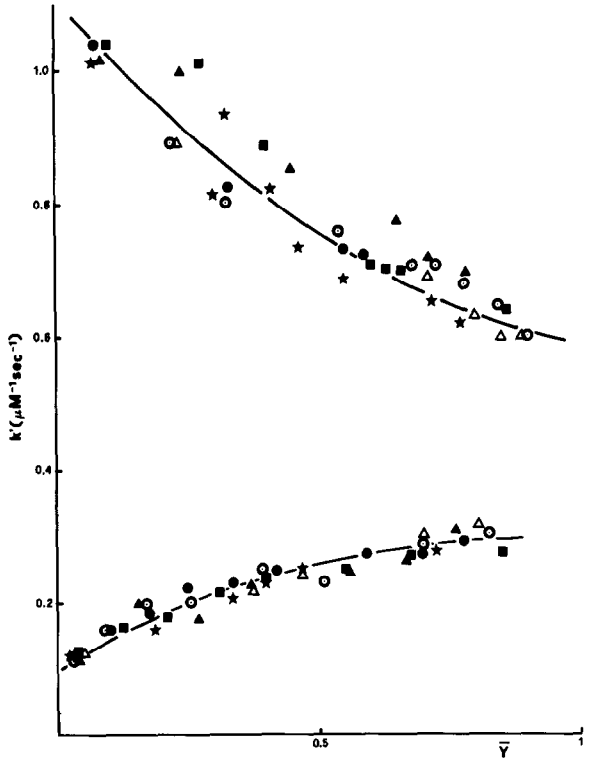

Fig. 1. Value of the apparent second-order velocity constant (k^1) vs. y for the combination with CO of Hb trout I fully and partially (∼19%) photodissociated at different ligand concentrations. Hemoglobin concentration: 1.4 μM, in phosphate buffer, 0.1 M, pH 7.5. Carbon monoxide concentration: (○) 10^{-3}M; (△) 1 × 10^{-4}M; (▲) 1.6 × 10^{-4}M, (■) 6.4 × 10^{-5}M; (●) 7.5 × 10^{-6}M; (★) 10^{-6}M.

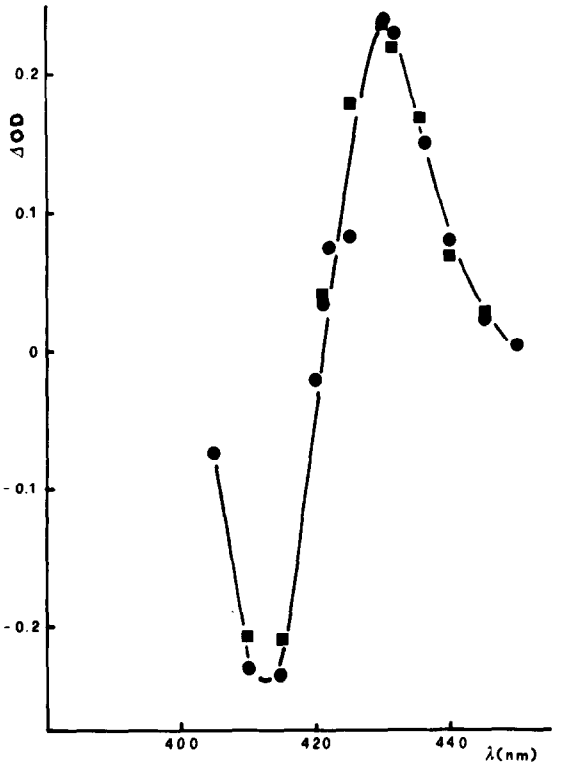

Fig. 2. The deoxyhemoglobin versus carbon-monoxide hemoglobin Soret difference spectra of the full (●) and partial (■) (∼19%) photodissociated Hb trout I. Experiments carried out in 0.1 M phosphate buffer, pH = 7.5; t = 20°C; [CO] = 4 × 10^{-4} M; Hb trout I = 1.4 μM. The difference spectrum of the partially photodissociated species was normalized to that of the fully dissociated species at 430 nm.

and therefore has been taken to represent a kinetic evidence of cooperative effects [1, 11, 12].

A QRF in the reaction with O_2 and CO has been observed in Hb trout I when the photolytic breakdown is progressively reduced from 100% downwards [7]. Thus, upon complete photodissociation, the CO recombination in the dark is typically autocatalytic, as shown in fig. 1 where the apparent second order rate constant is reported as a function of the fractional saturation with the ligand. When the photolysis is reduced (e.g. ∼19%), the recombination becomes faster and the time course becomes somewhat heterogeneous (see also fig. 1). The data summarized in fig. 1 also show that the observed time course, both upon full and partial photolysis, is independent of CO concentration over the range from 10^{-5} to 10^{-3} M. This implies that mono-

molecular decay processes should be considerably faster than the observed rates, as already known for human hemoglobin [1, 10, 12].

Fig. 2 reports the kinetic difference spectrum between CO–and deoxy hemoglobin as obtained on complete and partial photolysis. Under conditions in which the QRF is observed the difference spectrum is identical, after proper normalization, to that obtained on complete photolysis. Therefore, it seems that for Hb trout I the QRF by partial photolysis does not present those special spectral features which, on the other hand, are characteristic of the QRF of human hemoglobin [10].

A number of flash photolysis experiments have been performed with the oxidation intermediates of Hb trout I, separated as described above. It may be noted that the relevant species were obtained by two different procedures, i.e. by partially oxidizing a solution of CO hemoglobin (see Materials and methods) or by mixing equal amounts of CO– and ferric hemoglobin. The physico-chemical properties of the intermediates, as well as their distribution, are currently under investigation and will be published shortly.

Fig. 3 shows flash photolysis experiments performed on materials separated by electrofocussing and containing 0 to 3% Met-Hb, 23% Met-Hb, 49% Met-Hb and

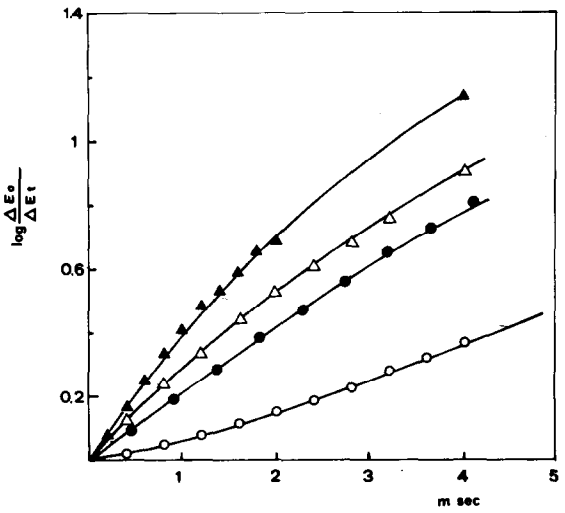

Fig. 3. Kinetics of CO combination of the oxidation intermediates of Hb trout I. Experiments carried out in 0.1 M Tris–HCl, pH 7.4; t = 20°C; λ = 431 nm, [CO] = 10^{-3} M. Other symbols as follows: (○) 0% Met-Hb, Hb = 4.0 μM; (●) ∿23%. Met-Hb, Hb = 3.9 μM; (△) ∿49% Met-Hb; Hb = 4.1 μM; (▲) ∿70% Met-Hb, Hb = 5.4 μM.

Table 1
Values of the initial combination velocity constants in the binding of CO by Hb trouth I obtained, under various conditions, by flash photolysis experiments

A)	% Photolysis	100	70	50	25
	k'_{on} (μM^{-1} sec^{-1})	0.1	0.3	0.4	1.1
B)	% Met-Hb	0	23	49	70
	k_{on} (μM^{-1} sec^{-1})	0.1	0.5	0.6	1.0
C)	% CN-Met-Hb	0	23	49	70
	k_{on} (μM^{-1} sec^{-1})	0.1	0.7	1.0	1.3

The initial combination rate constants were obtained by extrapolation of the apparent second order rate constant to $\bar{y}$ = 0 (as shown in fig. 1). Conditions: pH 7.4; 0.1 M Tris–HCl buffer; temperature = ~ 20°C. CO concentration = 10^{-3} M (in most experiments); [Hb] between 3.9 and 5.4 μM (heme).

70% Met-Hb. Within the errors of the measurements, the oxidation states of these hemoglobins correspond to those expected for molecules containing respectively: 0, 1/4, 1/2 and 3/4 of the hemes in the oxidized form. The recombination time course of partially oxidized molecules resembles, to a first approximation, that recorded in partial photolysis experiments. The overall reaction rate becomes faster as the number of sites in the ferrous state, i.e. capable of reversible reaction with CO, becomes smaller. It may also be noticed that the time course of CO combination appears progessively more heterogeneous as the number of oxidized sites is increased in the molecule. Table 1 reports the value of the initial second order rate constants, k_{on}, obtained for photolytic and oxidation intermediates, as compared to the initial value obtained on complete photodissociation for fully reduced Hb trout I.

Parallel experiments were performed with the oxidation intermediates in the absence, as well as in the presence, of cyanide (about 5 to 10-fold excess over the hemes). As shown in fig. 4, binding of CN^- to the ferric hemes is associated with a change in the time course of reaction; at constant CO concentration, ligand binding becomes faster in the presence of cyanide*. The values of the initial second order rate constant, recorded under these conditions, are also given in table 1.

These results, although preliminary, allow some con-

* It should be recalled that the kinetic and equilibrium properties of Hb trout I are unaffected by pH and organic phosphates [6–8].

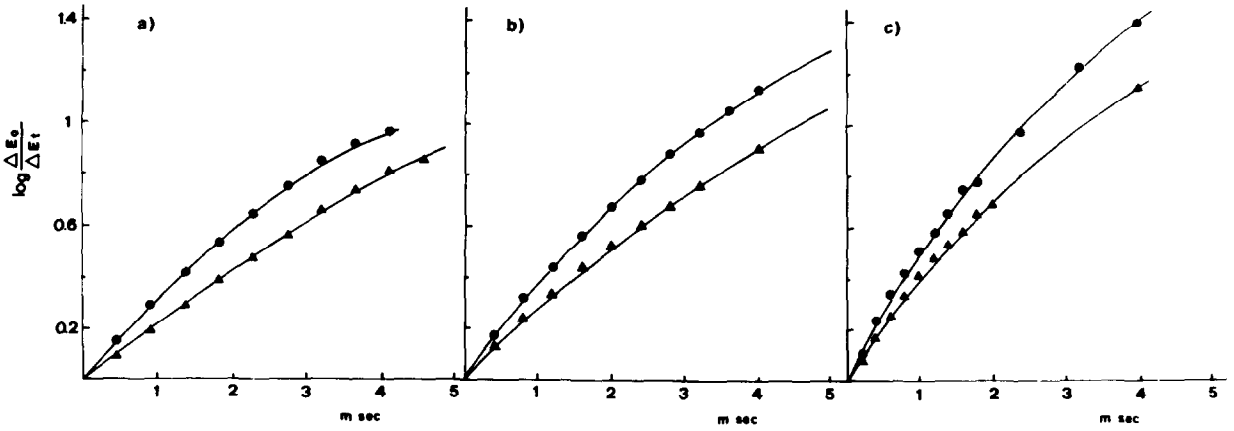

Fig. 4. Kinetics of CO combination of the oxidation intermediates of Hb trout I in the absence and in the presence of KCN. Experiments carried out in 0.1 M Tris–HCl, pH 7.4, $t = 20°C$; $\lambda = 431$ nm, [CO] = 10^{-3} M. (▲) without KCN; (●) in the presence of $\sim 10^{-5}$ M KCN; a) ∼23% oxidation, b) ∼49% oxidation, c) ∼70% oxidation.

clusions to be drawn concerning the kinetic properties of intermediates in the reaction of Hb trout I with ligands.

a) In agreement with previous experiments, it is shown that Hb trout I displays a quickly reacting component, as expected from the fact that ligand binding by this component is cooperative. At low photolysis levels, where $(\alpha_2\beta_2)(CO)_3$ is the dominant intermediate, the time course of CO combination is fast, somewhat heterogeneous and the shape of the reaction is ligand concentration independent. The lack of a spectrum typical of the QRF is at variance with the findings reported for mammalian hemoglobins. In view of the great significance attributed to the spectral features characteristic of the deoxygenated derivative of the QRF and of the isolated hemoglobin chains [10, 13, 14], this finding appears of particular interest and will be investigated in greater detail.

b) An effect similar to that obtained in partial photolysis experiments is observed with the oxidation intermediates. This suggests, once more, that to a first approximation ferric-hemes and CO-ferrous heme can be considered both liganded species.

c) It may be appreciated (fig. 3 and table 1) that the increase in rate constant is not linearly proportional to the % oxidation, suggesting that some of the intermediates (e.g. 25% and 50% oxidation) are closer than others to switch-over point from T to R.

d) The oxidation intermediates, which at pH values from 7 to 7.5, are largely in the high-spin configuration, change in the presence of cyanide to the low-spin configuration. In parallel, a definite change in the time course of the reaction with CO is observed, as shown in fig. 4. Thus, the transition of the heme iron from high-spin to low-spin is associated with an increase of the population in the highly reactive state. This may be brought about by a shift of the conformational equilibrium of partially ligated species towards the R-state, as has been previously suggested for the oxidation intermediates of human hemoglobin [3, 4]. These findings are in agreement with current ideas on the relationships between quaternary conformational state of the hemoglobin molecule and structural changes within the subunits (see [15] for a review).

Equilibrium and more complete kinetic studies on this type of intermediates of Hb trout I, and extention of this approach to Hb trout IV, should provide useful information to understand the molecular mechanism operative in the regulation of hemoglobin function.

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References

[1] Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, North Holland, Amsterdam.
[2] Brunori, M., Amiconi, G., Antonini, E., Wyman, J. and Winterhalter, K. H. (1970) J. Mol. Biol. 49, 461.
[3] Banerjee, R. and Cassoly, R. (1969) J. Mol. Biol. 42, 251.
[4] Ogawa, S. and Shulman, R. G. (1972) J.Mol. Biol. 70, 315.
[5] Guidotti, G., Konigsberg, W. and Craig, L. C. (1963) Proc. Natl. Acad. Sci. U.S. 50, 174.
[6] Brunori, M., Giardina, B., Chiancone, E., Spagnuolo, C., Binotti, I. and Antonini, E. (1973) Eur.J. Biochem. 39, 563.
[7] Giardina, B., Brunori, M., Binotti, I., Giovenco, S. and Antonini, E. (1973) Eur.J.Biochem. 39, 571.
[8] Binotti, L., Giovenco, S., Giardina, B., Antonini, E., Brunori, M. and Wyman, J. (1971) Arch.Biochem.Biophys. 142, 274.
[9] Antonini, E., Chiancone, E. and Brunori, M. (1967) J. Biol.Chem. 242, 4360.
[10] Gibson, Q. H. (1959) Biochem.J. 71, 293.
[11] Gibson, Q. H. (1959) Progr.Biophys.Chem. 9, 1.
[12] Hopfield, J. J., Shulman, R. G. and Ogawa, S. (1971) J.Mol.Biol. 61, 425.
[13] Perutz, M. F., Ladner, J. E., Simon, S. R. and Chien, Ho (1974) J.Mol.Biol., in press.
[14] Brunori, M., Antonini, E., Wyman, J. and Anderson, S. R. (1968) J.Mol.Biol. 34, 357.
[15] Perutz, M. F. (1970) Nature 228, 726.