Kinetic properties of cobalt–iron hybrid hemoglobins

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Received 16 December 1982; revision received 14 January 1983

The replacement of O_2 with CO was studied on cobalt–iron hemoglobin hybrids. Both proto- and meso-cobalt hemes were used for the reconstitution. In the oxy quaternary conformation no difference is observed between α- and β-subunits when only proto hemes are present in the hybrid ($k_4 = 30\ s^{-1}$, $k'_4/l'_4 = 2.5$). If Co-meso heme is present on the β-chains the binding properties of the partner subunit are modified ($k'_4/l'_4 = 4$).

Hemoglobin *Cobalt* *Unnatural heme* *Hybrid* *Kinetics*

1. INTRODUCTION

The Fe–Co hybrid hemoglobins provide a unique opportunity for the investigation of individual specific kinetic properties of the α and β subunits in the Hb tetramer [1]. CoHb's can be reconstituted, from apoprotein and Co-hemes, to yield a protein capable of cooperative oxygen binding [2]. Subunits can be obtained from the reconstituted material and, upon reassociation, they give a product functionally indistinguishable from the untreated protein [3].

Taking advantage of these features we have reconstituted Co–Fe hybrids, in which the Co heme is either the proto or the meso derivative, and used them for studies of replacement of O_2 by CO. The fact that the Co-substituted sites do not bind carbon monoxide, gave us the opportunity to study the replacement just on the iron subunits.

Ligand replacement reactions in Hb have been intensively studied by Gibson and Roughton [4]. On the basis of the Adair scheme they have shown that, if the protein is constantly kept in the fully ligated form, the velocity of the ligand replacement is given by:

$$r = k_4/(4l + [k'_4\ (O_2)]/[l'_4\ (CO)]) \qquad (1)$$

where:

r = the overall replacement rate;
k_4 = the dissociation rate for the fourth O_2 molecule;
k'_4 and l'_4 = the association rates for the fourth O_2 and the fourth CO molecule, respectively.

Thus a plot of $1/r$ *vs* $(O_2)/(CO)$ gives a straight line with slope equal to $4k'_4/k_4l'_4$ and the intercept on the Y axis is equal to $4/k_4$.

Using Co–Fe hybrid molecules this procedure allows a study of the kinetic differences between the subunits in a fully ligated tetramer. The effects of tertiary structure changes in the vicinity of the heme of one type of subunit on the binding properties of the other type can also be studied if unnatural porphyrins are present on the Co-containing chain.

2. MATERIALS AND METHODS

All operations were performed at 4°C unless otherwise stated. Protoporphyrin IX was purchased from Calbiochem [Calbiochem-Behring Corp. (La Jolla CA) cat. no. 5395]. Mesoporphyrin was prepared according to [5] starting from hemin obtained as in [6]. Co-hemes were obtained according to [2]. Co-Hbs were reconstituted as in [2], using apoprotein prepared by acid–acetone precipitation

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Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

[7]. Chain separation and SH group regeneration were achieved as in [8]. The hybrid tetramers were reconstituted from freshly prepared chains [9] and kept in liquid nitrogen [10] until immediately before use. The replacement of O_2 by CO was followed at 20°C on a Gibson type [11] stopped-flow apparatus, equipped with a 2 cm observation tube and mounted on a Durrum optical unit mod. D-120 [Durrum Instr. Corp. (Palo Alto CA)]. The signal from the amplifier was sent to a MINC-11 computer [Digital Equipment Corp. (Maynard MA)], equipped with a 12-bit resolution analog-to-digital converter and a programmable clock. Absorption changes were followed at 419 nm and, for each experiment, several traces were accumulated and subsequently averaged. All experiments were performed using 8 μM protein (on heme basis, after mixing) in 0.2 M Tris–HCl buffer (pH 7.4). The desired concentrations of ligands were obtained by mixing, in the appropriate amounts, buffers saturated at 20°C with Ar CO or O_2. Care was taken to have the same $[O_2]$ in both reactants.

3. RESULTS AND DISCUSSION

Fig.1 shows the plot of the reciprocal velocity for the replacement of O_2 with CO *vs* the ratio between the concentrations of the two ligands. The data refer to the two Co-proto–Fe-proto as well as to the two Co-meso–Fe-proto hybrids. It is evident that, while the proto hybrids and the α-Co-meso–β-Fe-proto one behave identically, the β-Co-meso–α-Fe-proto derivatives display the same dissociation rate for O_2, but differ in the binding rate(s) for O_2 and/or CO. The values derived from the plot are 30 s^{-1} for k_4 for all hybrids and 4.4 and 2.5 for the ratio between k'_4 and l'_4, respectively, for the β-Co-meso–α-Fe-proto and all other

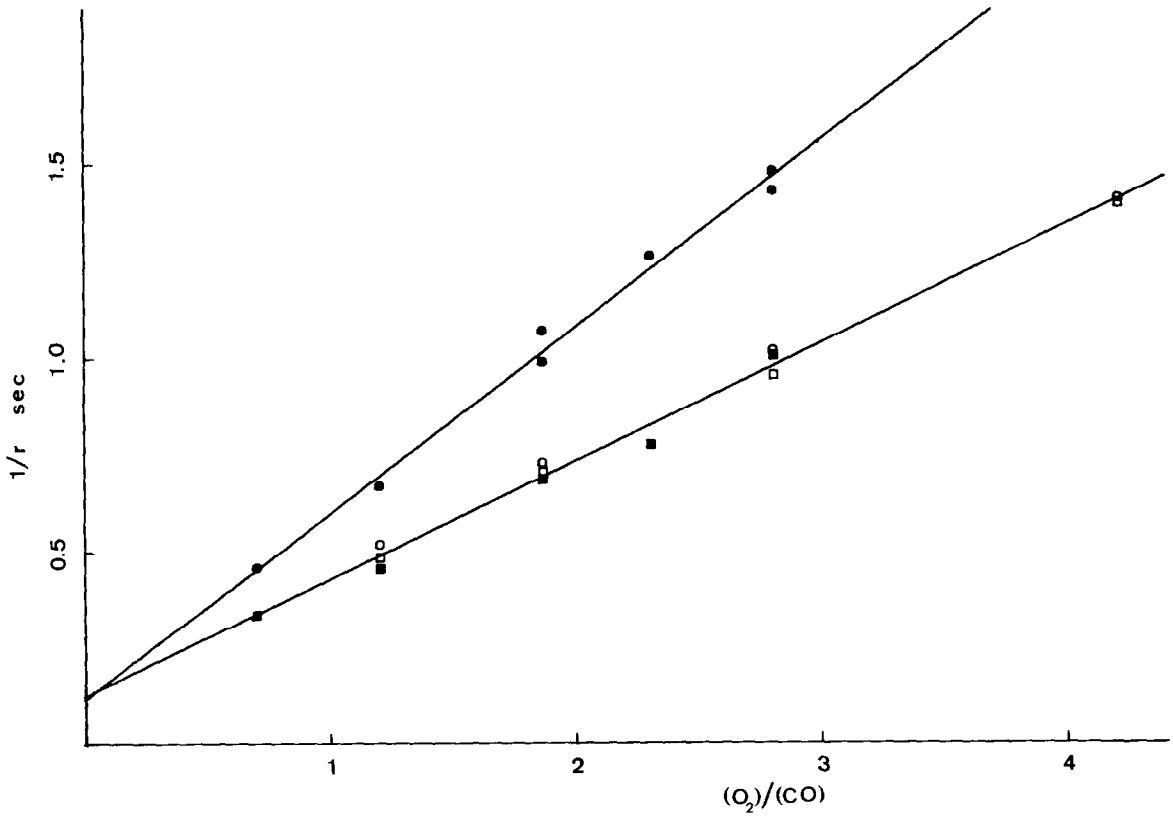

Fig.1. Plots of the reciprocal velocities of replacement of O_2 with CO *vs* the $[O_2]/[CO]$: (●) α-Fe-proto–β-Co-meso; (○) α-Fe-proto–β-Co-proto; (■) α-Co-meso–β-Fe-proto; (□) α-Co-proto–β-Fe-proto.

derivatives. For native human Hb, at pH 7.1, values of 26 s^{-1} for k_4 and of 3 for k'_4/l'_4 have been reported [12].

Due to the low affinity of CoHb for O_2 [2] it is possible that, in our experiments the Co sites were not fully saturated with the ligand (particularly the hybrids containing Co-proto hemes). This however did not seem to represent a major complication, probably also in view of the fact that the $[O_2]$ was the same before and after mixing. In fact in all experiments the replacement reaction followed a simple exponential time course, as shown in fig.2, where the results of a set of 7 expt with the α-Co-proto–β-Fe-proto hybrid, at the lowest $[O_2]$ used, are depicted. This implies that the requirements for the application of eq. (1) to this experimental system were fulfilled.

The data in fig.1 refer to different preparations of both Co-substituted and native subunits. An electrophoretic control of the hybrids showed a complete reconstitution. Furthermore light absorption spectra of both the isolated subunits and the reconstituted hybrids were always normal. This indicates that no appreciable denaturation of the protein had occurred, neither during the reconstitution of the apoprotein with the Co hemes, nor during the chain separation and the successive reconstitution to hybrid tetramers.

The results reported here indicate no difference between the kinetic properties of the α and β subunits (containing proto-heme) in the Hb tetramer, when this is in the liganded quaternary structure. In contrast the deoxy quaternary conformations exhibit a marked difference between the association rates of the α and β chains in Fe–Co hybrids [1].

The fact that the presence of a Co-meso heme on the β-chains alters the ligand association properties of the partner subunit may be explained on the basis of a tertiary structure effect. Such an effect

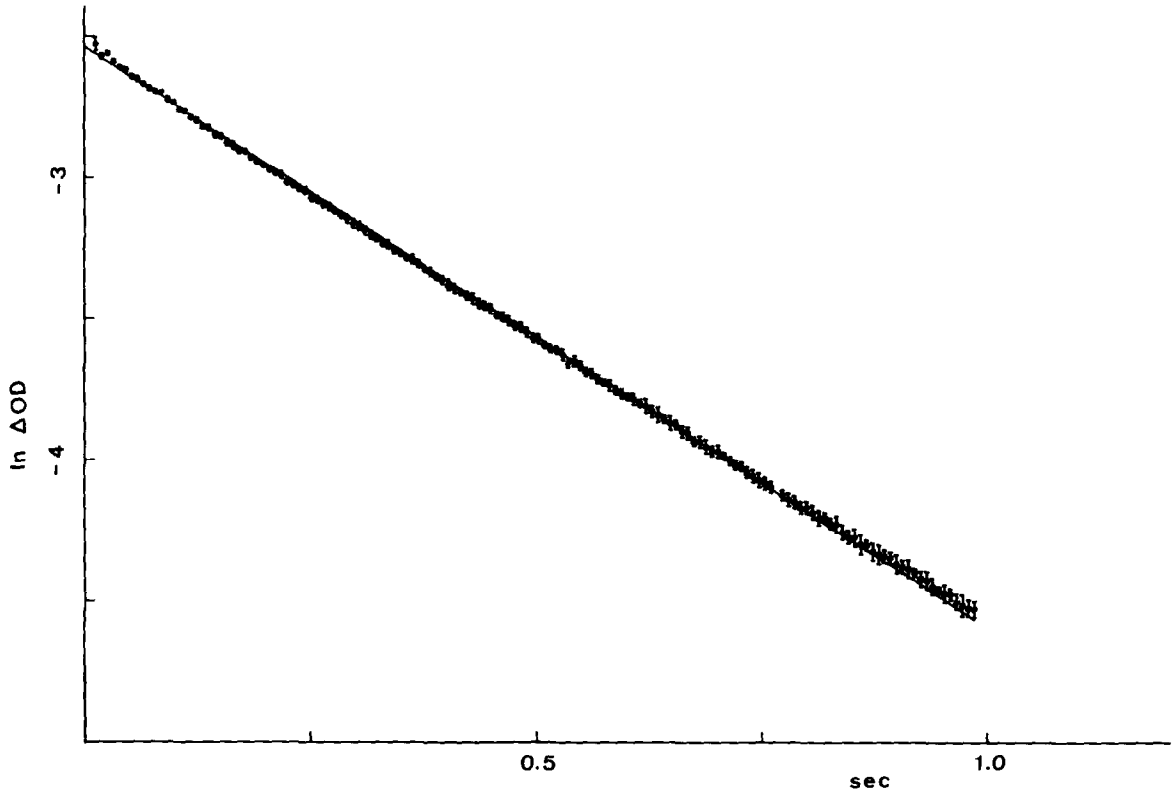

Fig.2. Semi-ln plot of the replacement reaction of O_2 with CO for the α-Fe-proto–β-Co-meso hybrid: 0.42 mM O_2 and 0.35 mM CO after mixing.

is likely to be due to an interaction of the π electrons of the porphyrin with the protein moiety. A good candidate for such an interaction is Phe CD1 which is located on the distal side of the heme and almost parallel to it. In the α subunits this residue is closer to the heme than on the β chains [13]. In addition the replacement of the vinyl side chains by ethyl groups on the meso hemes is reflected in a reduced density of π electrons. It thus appears that the interaction between the π electrons of the heme and the ones of Phe CD1 plays an important role in the regulation of the ligand binding in the oxy quaternary structure. Such interaction seems to be particularly favourable in the α chains in which the substitution of proto with meso heme does not bring any alteration in the 'mode of binding' of the partner subunit. On the contrary in the β subunits the larger distance between the heme plane and the π system of Phe CD1 makes the interaction looser and the substitution of the proto for a meso heme abolishes it completely. One could also speculate that the reduced cooperativity found in hemoglobins reconstituted with meso hemes on all sites is related to the same phenomenon [1,9].

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