ELSEVIER

Biophysical Chemistry 61 (1996) 169–176

Biophysical Chemistry

Functional heterogeneity of the α and β subunits in the association reaction between hemoglobin and carbon monoxide

Michele Perrella [a,*], Marilena Ripamonti [b], Louise Benazzi [b], Ilya Denisov [a]

[a] *Dipartimento di Scienze e Tecnologie Biomediche, Università di Milano, Italy*
[b] *Istituto di Tecnologie Biomediche Avanzate, C.N.R., Milano, Italy.*

Received 22 March 1996; revised 7 June 1996; accepted 10 June 1996

Abstract

A technique is described for the rapid inactivation and removal of excess ferricyanide used for the non-cryogenic oxidation of the unliganded subunits of the intermediates in the association reaction between hemoglobin and carbon monoxide. Under these conditions the asymmetric oxidized intermediates, which dissociate into non-identical dimers, disproportionate into their parent tetramers and four species, Hb^+, HbCO, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$, are isolated by non-cryogenic isoelectric focusing. The relative concentrations of species $\alpha_2^{CO}\beta_2^+$ and $\alpha_2^+\beta_2^{CO}$ measure the overall distribution of the ligand between the α and β subunits in the association reaction. At 20°C in 0.1 M KCl, pH 7, preferential CO binding to the β subunits was observed, in agreement with observations made by the cryogenic technique for the isolation of the intermediates [M. Perrella, N. Davids and L. Rossi-Bernardi, J. Biol. Chem. 267 (1992) 8744].

Keywords: Allostery; Hemoglobin ligation intermediates; Hemoglobin oxidation; Cryogenic focusing

1. Introduction

Fundamental aspects of the mechanisms of hemoglobin cooperativity have been discovered by the thermodynamic studies of ligand binding to hemoglobin by Ackers et al. [1]. The isolation of the carbon monoxide intermediates under kinetic and dynamic conditions has partly confirmed and extended such discoveries [2]. In particular, the isolation of the intermediates in the association reaction (Fig. 1) at neutral pH by cryogenic techniques has yielded two important pieces of information: 1) the rates of the reactions increase continuously with an acceleration after the binding of two molecules of ligand; 2) the rates of the reactions of the α and β subunits in the first ligation step differ slightly, but significantly, and are modulated by inositol-hexaphosphate (IHP) [3]. Thus, contrary to what has been previously predicted on the basis of kinetic stopped-flow experiments [4,5], no accumulation of intermediates occurs during the association process, particularly with regard to the double liganded species. Furthermore, the modulation of the reactivity of the α and β subunits by IHP indicates that IHP can influence the affinity for hemoglobin of modulators that bind to sites other than that of phosphate (the β subunit pocket), such as chloride and, partly, proton (α-amino group of the α subunit). Thus mechanisms of interaction between organic

* Corresponding author. Fax 0039-2-2663030.

PII S0301-4622(96)02196-5

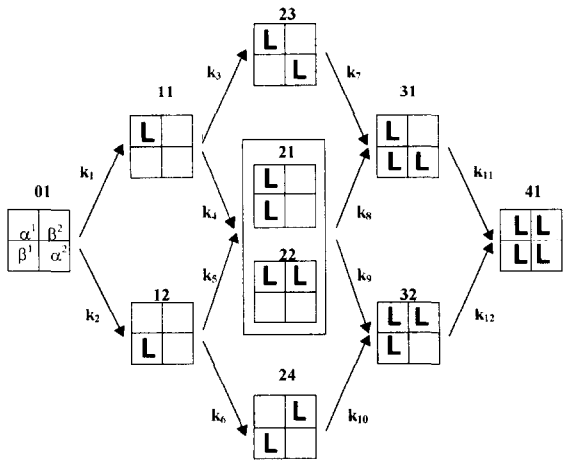

Fig. 1. Kinetic scheme of the association reactions between hemoglobin and CO as studied by the cryogenic technique [3]. L indicates the liganded subunit. Since the cryogenic technique does not resolve intermediates 21 and 22, this scheme involves 12 rate constants, four constants fewer than the general scheme [3]. The intersubunit contacts that break reversibly under physiological conditions are the $\alpha_1\ \beta_2$ and $\alpha_2\ \beta_1$ contacts and the weaker $\alpha_1\alpha_2$ contact.

phosphate and other modulators are envisaged, in agreement with similar observations on the effects of chloride and organic phosphates on the equilibrium distribution of oxygen between the α and β subunits made by the NMR technique [6]. Such mechanisms can be of relevance for the fine tuning of oxygen delivery to the tissues under various physiopathological conditions.

Observations on the functional heterogeneity of the α and β subunits contrasting with the results of the cryogenic technique were made by non-cryogenic techniques [7–9] and, in general, the confusion in the state of the art as far as the intermediates in CO binding to hemoglobin is concerned is apparent from a survey of the field given in a recent review [10].

A reinvestigation of the functional heterogeneity of the α and β subunits in the association reaction between hemoglobin and CO by means of a non-cryogenic approach can help to understand the reason of the contrast with the results of the cryogenic technique. Removal of this contrast would increase confidence in the cryogenic technique as a method suitable to study the mechanisms of linkage between the interactions of some modulators with hemoglobin.

2. Experimental approaches

2.1. Cryogenic and non-cryogenic techniques

Using the cryogenic technique the intermediates in the reaction between hemoglobin and CO are trapped by injecting the protein solution into an anaerobic cryosolvent at $-30°$ containing an excess of ferricyanide (FeCy). The unliganded subunits are oxidized to the ferric state and the liganded subunits are protected by the ligand, which dissociates slowly at this temperature. At subzero temperatures the dissociation reactions of all the tetrameric species yielded by the oxidation reaction are slow enough to make the dimer exchange reactions negligible (Fig. 2).

Under cryogenic conditions the excess oxidant is removed electrophoretically during the separation of the oxidized intermediates. In Section 3 we describe a technique for the inactivation and removal from the solution of the excess oxidant when the unliganded subunits are oxidized under non-cryogenic conditions.

Under non-cryogenic conditions only Hb^+, HbCO, $\alpha_2^+\beta_2^{CO}$, and $\alpha_2^{CO}\beta_2^+$, which dissociate into identical dimers, are isolated by isoelectric focusing (IEF) or chromatography. The oxidized intermediates $[11]_{ox}$, $[12]_{ox}$, $[21]_{ox}$, $[22]_{ox}$, $[31]_{ox}$, $[32]_{ox}$ made of dimers under different states of oxidation and ligation, or asymmetrical intermediates, disproportionate into the parental, symmetrical species, since the dissociation reaction of the tetrameric liganded and/or oxidized species is fast ($t_{1/2}$ = 1–2 s [11]). In the following, we shall indicate as $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ the partially oxidized species isolated by IEF, be they oxi-

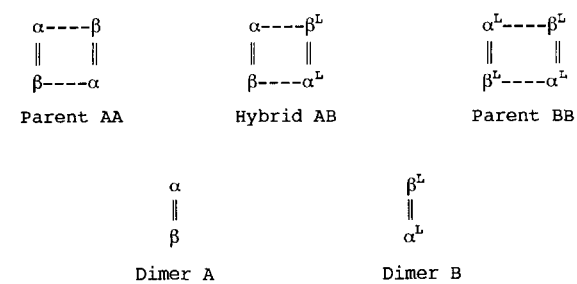

Fig. 2. Scheme of the dimer exchange reactions. Parents AA and BB equilibrate with hybrid AB by dimer exchange. Conversely hybrid AB disproportionates into the parent species.

dized intermediates or products of disproportionation of the oxidized intermediates. The concentrations of $\alpha_2^{CO}\beta_2^{+}$ and $\alpha_2^{+}\beta_2^{CO}$ measure the overall distribution of CO between the α and β subunits, but do not provide information on the type and amount of the intermediates.

3. Methods

3.1. Non-cryogenic oxidation of the intermediates.

Deoxyhemoglobin (Hb) was reacted with substoichiometric amounts of CO and then with an excess of FeCy to oxidize the unliganded subunits, using the multi-mixing quench-flow apparatus represented schematically in Fig. 3 (top). Syringe A (5 or 2.5 ml gas-tight Hamilton syringes) contained the solution of deoxygenated HbA_0 (1.5 or 2.5 mM in heme); syringe B (5 or 2.5 ml) contained a 1 mM solution of CO and syringe C (2.5 ml) a deoxygenated 10 mM solution of FeCy. The solvent was 10 mM phosphate in 0.1 M KCl, pH 7. The Hb and CO solutions also contained 0.5 mM dithionite. The flow rates were 1 and 0.5 ml s^{-1} using the 5 ml and 2.5 ml syringes respectively. The reactions were carried out in teflon tubes (1 mm i.d.) and the reactants were mixed by "ball-mixers" [12].

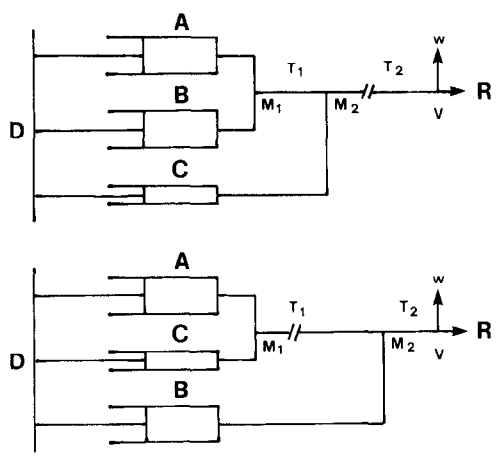

Fig. 3. Diagram of the double-mixing quench-flow apparatus. D, driving ram. A, B and C, gas-tight Hamilton syringes. M_1 and M_2, "ball-mixers" [12]. T_1 and T_2 teflon reaction tubes (1 mm i.d.). V, sampling valve. W, waste. R, reactor cooled at 1°C containing a stirred suspension of anion exchanger. Top diagram (experiment): A (5 or 2.5 ml), Hb; B (5 or 2.5 ml), CO; C (2.5 ml), FeCy. Bottom diagram (control): A, Hb; B, HbCO; C, FeCy.

The reaction time calculated for the complete binding of CO to Hb in Tube 1 under the conditions of the experiments varied from 2 to 15 ms [13]. The length of Tube 1 allowed for a reaction time of 70–100 ms depending on the flow-rate in M_1. This time was not sufficient for a significant dissociation of the bound ligand [14]. The reaction time for 99.9% oxidation of Hb by FeCy, using a ratio of FeCy to the Hb fraction of about 2:1, was calculated, from published values of the rate constants under the same conditions, to be in the range 190–300 ms depending on the flow-rates [15]. As a precaution, at the flow-rates of the experiments, the length of Tube 2 was chosen to allow for reaction times of 300–370 ms.

Fig. 3 (bottom) shows a scheme for a control reaction used to obtain the baseline of the IEF separation experiment (see below). Syringe A contained the solution of Hb, which was mixed in M_1 with the FeCy solution contained in Syringe C. The reactants were then mixed in M_2 with a solution of HbCO contained in Syringe B. The time of the oxidation reaction of Hb in Tube 1 and Tube 2 was similar to the time of the experiment carried out using similar concentrations of the reactants. The time of exposure of HbCO to the oxidant in Tube 2 was about 150 ms.

3.2. Removal and inactivation of the excess FeCy

FeCy was removed efficiently from the solution by an anion exchanger such as Bio-Rad Ag1X8, 300–400 mesh, in Cl^- form. Aliquots (0.5–2 ml) of 10 mM FeCy were added to the suspension (6 ml) of the resin in water (50% w/w) in a reactor provided with a Radiometer TTA-80 stirrer (Radiometer, Copenhagen) and cooled to 1°C. Samples of solution (2–3 ml) were drawn after 10 s into a syringe through a tube filled with a nylon net to filter the resin. Fig. 4 shows the absorbance at 420 nm of the filtrate. The absorbance after the addition of the first aliquot (0.5 ml), corresponding to the amount added in the experiments aimed at trapping the intermediates, was $< 1\%$ of the value measured in the absence of the resin.

The following test proved that FeCy bound to the

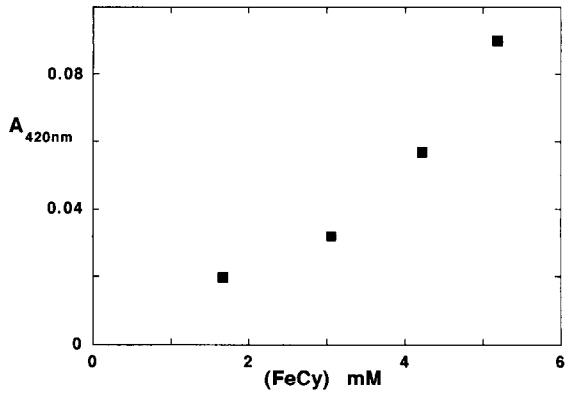

Fig. 4. Absorbance at 420 nm of the filtrate from the resin suspension in water (6 ml, 50%w/w) after the addition of successive aliquots (0.5 ml) of 10 mM FeCy.

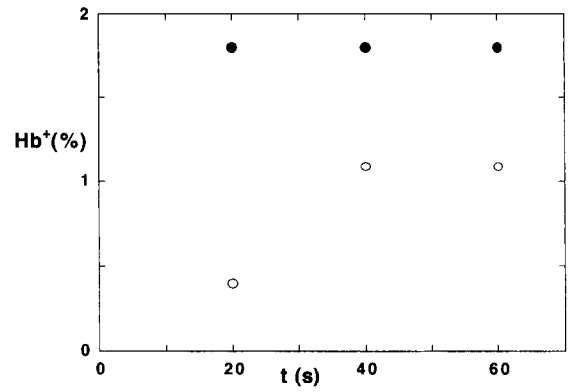

Fig. 5. Concentration of Hb^+ (%) in the filtrate from the resin suspension in water (6 ml, 50% w/w) containing 0.5 ml of 10 mM FeCy at various times after the addition of 2 ml of 1 mM (heme concentration) Hb (∘) or HbCO (·).

resin does not oxidize hemoglobin. A solution of HbCO, under aerobic conditions, or a solution of Hb, under anaerobic conditions, was injected into the reactor containing FeCy bound to the resin and the methemoglobin content was determined in the filtrate sampled at various times after the injection. In both cases the Hb^+ concentration, plotted in Fig. 5, was within error equal to the methemoglobin content of the solutions. To check that only the unliganded subunits were quantitatively oxidized by the procedure, a sample of the products of the oxidation of the intermediates filtered from the resin was diluted in phosphate buffer, pH 7, containing CN^- and CO and the fraction of CO liganded subunits, $(S_{CO})_{abs}^{Hb^+CN^-}$ was calculated from the values of the absorbance at 540 and 568 nm. Such a fraction was the same, within error, as: a) the CO saturation in M_1, $(S_{CO})_{Th}$, (Fig. 3, top) calculated from the concentrations of the solutions of Hb and CO and their mixing ratio, b) the value of the CO saturation, $(S_{CO})_{Abs}$, determined spectrophotometrically by delivering the reactants from M_2 into a Thurnberg cuvette containing 40 mM borate and some dithionite to exclude oxygen. These data, listed in Table 1, also indicate that the removal and inactivation of FeCy by the procedure were faster than CO dissociation and oxidation of the unprotected subunits.

3.3. Non-cryogenic IEF of the oxidized intermediates

The products of the oxidation reaction were separated by IEF on 1 mm thick gel plates containing 3% ampholines pH range 6.5–7.5 (Sigma Aldrich, Milano). Samples (130 μl) were layered in troughs (4 or 5) in the gel and focused for 3 h at 7°C and 800 V. The zones containing the four components, Hb^+, HbCO, $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$, were excised and the hemeprotein eluted in 2 ml of 20 mM phosphate, pH

Table 1
Non-cryogenic oxidation and IEF separation of the intermediates in the reaction between Hb and CO at 20°C in 0.1M KCl, 10 mM Pi, pH 7.

Conditions	$[Hb]_0$ (mM)	$[CO]_0$ (mM)	$(S_{CO})_{Th}$ (%)	$(S_{CO})_{Abs}$ (%)	$(S_{CO})_{Abs}^{Hb^+CN^-}$ (%)	$(S_{CO})_{IEF}$ (%)	$[Hb^+]$ (%)	$[\alpha_2^+\beta_2^{CO}]$ (%)	$[\alpha_2^{CO}\beta_2^+]$ (%)	[HbCO] (%)
(A)	1.94	1	51.5	48.6	53.8	51.9	36.2	14.9	8.9	40.0
				±0.93	±1.0	±1.45	±1.4	±0.07	±0.20	±1.5
(B)			60		56.08	61.0	37.1	1.87	1.97	59.1
					±0.26	±1.19	±1.55	±0.51	±0.3	±0.91
(C)			60		56.8	63.3	36.0	2.16	1.19	61.4
					±0.26	±0.70	±0.83	±0.36	±0.40	±1.0

7, 10 mM NaCl for 36 h and assayed by the pyridine hemochromogen method [16].

4. Results

4.1. Distribution of CO between the α and β subunits

Fig. 6 shows the data on the concentrations of Hb^+, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$ and HbCO obtained by the non-cryogenic oxidation and IEF of the intermediates in the association reaction between Hb and CO at 20°C, in 0.1 M KCl, 10 mM phosphate, pH 7, in a CO saturation range from 25 to 75%. Detailed data with the relative errors regarding several such non-cryogenic determinations of the intermediates at a constant $[CO]_0/[Hb]_0$ ratio are listed in Table 1. The CO saturation calculated from the concentrations of

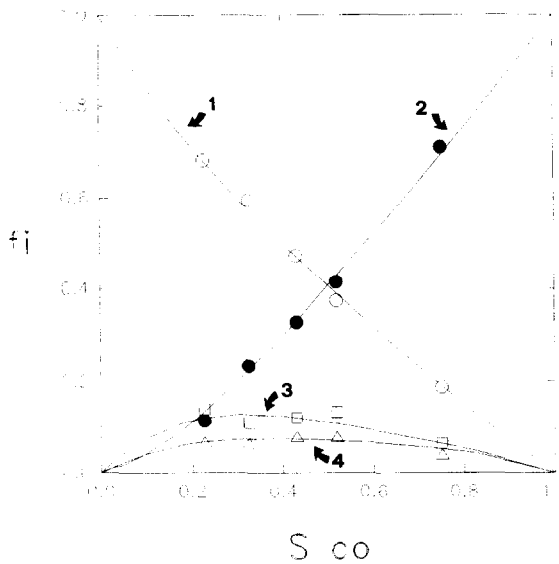

Fig. 6. Association reaction between Hb and CO in 0.1 M KCl, pH 7, 20°C. Experimental values of the fractional concentrations, f_i, of the products of non-cryogenic oxidation of the intermediates isolated by IEF at 7°C against fractional CO saturation S_{CO}. (○), Hb^+; (·), HbCO; (□), $\alpha_2^+\beta_2^{CO}$; (Δ), $\alpha_2^{CO}\beta_2^+$. The lines (1–4) represent the theoretical values of the concentrations of Hb^+, HbCO, $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ respectively, calculated assuming that the oxidized intermediates $[11]_{ox}$, $[12]_{ox}$, $[21]_{ox}$, $[22]_{ox}$, $[31]_{ox}$, $[32]_{ox}$ disproportionate completely into their parent species and that intermediates $[21]_{ox}$ and $[22]_{ox}$ in the component unresolved by cryofocusing are present in a 3:2 ratio (see text).

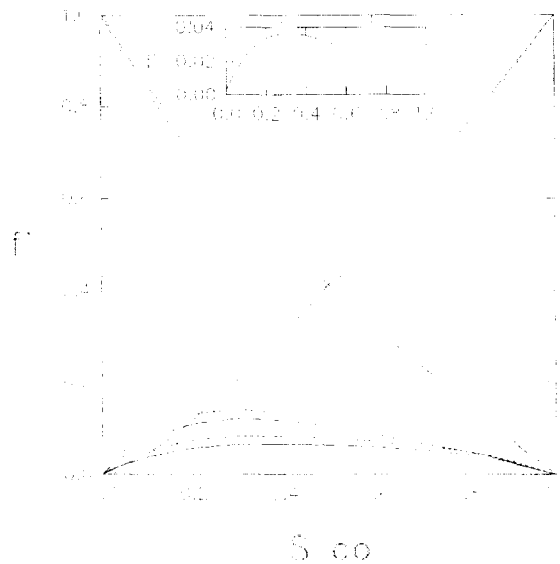

Fig. 7. Theoretical lines calculated as described in the legend of Fig. 6, assuming that the component unresolved by cryofocusing is made up of species $[21]_{ox}$, continuous lines, or species $[22]_{ox}$, dashed line, exclusively. Identity of the lines as in Fig. 6. Insert: concentration, f_i, of the component unresolved by cryofocusing, $[21]_{ox}+[22]_{ox}$, versus CO concentration, S_{CO}, data from Ref. [3].

the species separated by IEF, $(S_{CO})_{IEF}$ in Table 1, agrees within the experimental error with the values of the CO saturation determined in the preceding steps of the procedure. Also listed in Table 1 are the data relative to the controls carried out according to the multimixing scheme shown in Fig. 3 (bottom diagram), which provide the baseline and were used to correct the experimental data.

The data on the intermediates in the association reaction between hemoglobin and CO obtained by the cryogenic technique can be fitted using the kinetic model shown in Fig. 1, which makes no assumption with regard to the concentrations of the component, $[21]_{ox}$ plus $[22]_{ox}$, unresolved by cryofocusing. To calculate the concentrations of Hb^+, $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ yielded by the oxidation of the symmetrical species Hb, 23 and 24 plus the concentrations of Hb^+, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$ and HbCO yielded by the oxidation and complete disproportionation of the asymmetrical intermediates 11, 12, 21, 22, 31 and 32 one has to know the concentrations of species $[21]_{ox}$, and $[22]_{ox}$, or must make some assumption with regard to these species. Preliminary non-cryo-

genic experiments carried out to analyse the component unresolved by cryogenic IEF indicate that in the 40–60% CO saturation range of the association reaction, in 0.1 M Cl^- at neutral pH, this component contains species $[21]_{ox}$ and $[22]_{ox}$ in an approximately 3:2 ratio. [17]. We have used this preliminary information to calculate the curves in Fig. 6 making use of the kinetic scheme in Fig. 1.

In Fig. 7 the curves were recalculated assuming that the unresolved component is made either of species $[21]_{ox}$ or $[22]_{ox}$ exclusively. These curves and the curves in Fig. 6 indicate that, whatever the assumption made, the calculated difference in the concentrations of species $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ is the same as the difference obtained by the non-cryogenic experiments. This is expected because species $[21]_{ox}$, $[22]_{ox}$ and their products of disproportionation contain the same amounts of β and α CO − liganded chains. Furthermore, the curves in Figs. 6 and 7 are all very close to the experimental non-cryogenic data because of the paucity of species $[21]_{ox}$ and $[22]_{ox}$, which constitute the unresolved component, as shown in the insert of Fig. 7.

5. Discussion

5.1. Validity of the non-cryogenic procedure for the oxidation of the intermediates

The non-cryogenic oxidation of the unliganded subunits of the CO intermediates can provide correct information on the relative distribution of the ligand between the α and β subunits only if an efficient technique to remove and inactivate the excess oxidant is available. We have shown that the anion exchanger removes $> 99\%$ of FeCy from solution (Fig. 4) and FeCy bound to the resin does not oxidize Hb or HbCO to any significant extent (Fig. 5).

Furthermore, the experiment was carefully designed to meet with the following requirements. A large excess of FeCy was avoided because of the possibility of side-reactions such as the oxidation of the protein thiol groups [18]. Using a low FeCy to Hb ratio the reaction time of the oxidation reaction was carefully estimated. An overestimation would allow a significant dissociation of the ligand and oxidation of the exposed subunits. Since the β subunits are oxidized faster than the α subunits [15], the incomplete oxidation due to an underestimation of the reaction time would yield an apparent increase in the α relative to the β liganded subunits in the IEF separation step. This would lead to an incorrect assessment of the relative distribution of the ligand between the subunits.

In the design of the experiment we have taken advantage of our detailed knowledge of the mechanism of the oxidation of deoxyhemoglobin by FeCy [15] and validated the approach by experimental controls. The theoretical fractional value of the CO liganded subunits calculated from the mixing ratio of the reactants was the same as that measured throughout the various steps of the procedure (row A in Table 1). This indicates that: a) within the experimental error of 2–3%, only the unliganded subunits are oxidized by FeCy, in agreement with approximate estimates, based on available kinetic data [14], suggesting no more than 5% of possible oxidation of CO liganded subunits; b) the four symmetrical species Hb^+, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$ and HbCO, products of the non-cryogenic oxidation and disproportionation of the intermediates, were correctly and quantitatively estimated.

Methemoglobin is present in solutions of oxy- or carboxyhemoglobin mainly as species $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$. A total concentration of partially oxidized species, $\alpha_2^+\beta_2^{CO}$ plus $\alpha_2^{CO}\beta_2^+$, equal to $3.6 \pm 0.2\%$ in the control experiment (rows B and C in Table 1) would correspond to a methemoglobin content of the hemoglobin solution of about $1.8 \pm 0.1\%$, which compares well with the assayed value of about 2%. Ferrocyanide added to mixtures of Hb^+ and HbCO catalyses a rapid formation of partially oxidized species yielding large amounts of $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ in IEF. The small amount of such species observed in the control experiments also indicates that charge transfer reactions catalysed by traces of ferrocyanide during the procedure were negligible.

5.2. Functional heterogeneity of the subunits

Fig. 6 indicates a slight preferential binding of CO to the β subunits in the presence of Cl^- and in the absence of organic phosphate. The difference in the concentrations of species $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$

yielded by the non-cryogenic experiments is comparable with the difference in the concentrations of these species calculated from the distributions of intermediates previously obtained by the cryogenic technique [3], on the assumption of a complete disproportionation of species $[11]_{ox}$, $[12]_{ox}$, $[21]_{ox}$, $[22]_{ox}$, $[31]_{ox}$, $[32]_{ox}$ into their parental symmetrical species. Such a difference is not influenced by the assumptions made on the nature of the unresolved component in the cryogenic experiments because species $[21]_{ox}$, $[22]_{ox}$ and their products of disproportionation contain the same proportions of liganded α and β chains. Although there is a vast body of evidence from thermodynamic studies in various models of ligation supporting the functional difference of intermediates 21 and 22 [1], it is not possible to infer from such studies the functional behaviour of these species in the association reaction between hemoglobin and CO. Clearly, the calculated values of the concentrations of Hb^+, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$ and HbCO depend on the relative amounts of species $[21]_{ox}$ and $[22]_{ox}$ assumed to be present in the component unresolved by cryofocusing. Thus the various calculated curves (Figs. 6 and 7) do not fit the data of the non-cryogenic experiments equally well. However, we do not believe that this kind of analysis is a satisfactory approach to the clarification of the nature of the unresolved component. The concentration of this component, $\leq 4\%$ of the total as shown in the insert of Fig. 7, is low in comparison with the concentration of the monoliganded species, $\leq 30\%$ of the total [3], which contribute to most of the $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ species isolated by non-cryogenic IEF. A more reliable approach is the direct analysis by non-cryogenic methods of the mixture of species $[21]_{ox}$ and $[22]_{ox}$ isolated by the cryogenic technique of which we have provided some preliminary results.

The data in Fig. 6 are consistent with the kinetic analysis of the cryogenic data of the intermediates in the association reaction between hemoglobin and CO, indicating that, at neutral pH in the absence of organic phosphate, the β chains react with CO slightly faster than the α chains in the first binding step. A similar conclusion has been reached by Mathews et al. [19,20] in their studies of the kinetics of CO binding to native and mutant hemoglobins using the stopped-flow method. Mathews and Olson [21] have discussed the possible structural origin of such a difference.

This work supports the sensitivity and reliability of the cryogenic technique in detecting a slight functional heterogeneity of the subunits of hemoglobin. The difference in reaction rates between the α and β subunits in the association reaction with CO is interesting for the understanding of the stereochemistry of CO binding to the heme pocket, and could have important physiological implications. The observation by the cryogenic technique that the rates of the reactions of CO association to the α and β subunits are modulated by IHP [3], which has been confirmed by Mathews et al. [19,20], suggests an interaction between Cl^- and organic phosphate, mediated by different changes in the tertiary structures induced in the α and β subunits by the binding of these modulators. Such an interaction, which could be of physiological importance for the fine tuning of the oxygen delivery to the tissues, was first suggested by NMR studies of the equilibrium between Hb and O_2 [6].

Acknowledgements

This work was supported by grants from the CNR and MURST (60%), Rome, Italy.

References

[1] G.K. Ackers, M.L. Doyle, D. Myers and M.A. Daugherty, Science, 255 (1992) 54.
[2] M. Perrella and I. Denisov, Methods Enzymol., 259 (1995) 468.
[3] M. Perrella, N. Davids and L. Rossi-Bernardi, J. Biol. Chem., 267 (1992) 8744.
[4] Q.H. Gibson and F.J.W. Roughton, Proc. R. Soc. London, Ser. B. 146 (1957) 206
[5] Q.H. Gibson, J. Biol. Chem., 248 (1973) 1281.
[6] G. Viggiano and C. Ho, Proc. Natl. Acad. Sci. U.S.A., (1979) 3673.
[7] V.S. Sharma, J. Biol. Chem., 264 (1989) 10582.
[8] M. Berjis, D. Bandyopadhyay and V.S. Sharma, Biochemistry, 29 (1990) 10106.
[9] V.S. Sharma, D. Bandyopadhyay, M. Berjis, J. Rifkind and G.R. Boss, J. Biol. Chem., 266 (1991) 24492.
[10] C. Ho, Adv. Protein Chem., 43 (1992) 154.
[11] F.R. Smith and G.K. Ackers, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 5347.

[12] R.L. Berger, B. Balko and H. Chapman, Rev. Sci. Instrum., 39 (1968) 493.
[13] R.L. Berger, N. Davids and M. Perrella, Methods Enzymol., 76 (1993) 517.
[14] M. Samaja, E. Rovida, M. Niggeler, M. Perrella and L. Rossi-Bernardi, J. Biol. Chem., 262 (1987) 5428.
[15] M. Perrella, R.I. Shrager, M. Ripamonti, G. Manfredi, R.L. Berger and L. Rossi-Bernardi, Biochemistry, 32 (1994) 5223.
[16] M. Perrella and L. Rossi-Bernardi, Methods Enzymol., 232 (1993) 445.
[17] M. Perrella and M. Ripamonti, unpublished results, 1996.
[18] E. Antonini and M. Brunori, Hemoglobin and Myoglobin in Their Reactions with Ligands, Elsevier/North-Holland Biomedical Press, Amsterdam, 1971.
[19] A.J. Mathews, R.J. Rohlfs, J.S. Olson, J. Tame, J.-P. Renaud and K.Nagai, J. Biol. Chem., 264 (1989) 16573.
[20] A.J. Mathews, R.J. Rohlfs, J.S. Olson, J.-P. Renaud, J. Tame and K.Nagai, J. Biol Chem., 266 (1991) 21631.
[21] A.J. Mathews and J.S. Olson, Methods Enzymol., 232 (1994) 363.