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The hemoglobin cyanomet ligation analogue and carbon monoxide induce similar allosteric mechanisms☆

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Abstract

Current thermodynamic models of protein cooperativity predicting sigmoidal ligand equilibrium curves differ in the assumptions regarding the structural/functional properties of the intermediate ligation states. Quantitative information on the intermediates cannot be extracted from the equilibrium curves, but must be obtained from direct studies of the intermediates. Since the intermediates are intrinsically unstable species, ligation analogues with reduced mobility are indispensable tools for cooperativity studies provided that the tertiary/quaternary changes triggered by the ligation analogue are similar to those observed using the physiological ligands. We demonstrate that the valency exchange reactions occurring in mixtures of deoxy and cyanomethemoglobin yield non-random distributions of deoxy/cyanomet intermediates that resemble those observed in the equilibrium with carbon monoxide. Previous and new data using the analogue, in agreement with the studies of the CO intermediates, indicate that the mechanism of hemoglobin cooperativity is neither purely concerted nor sequential nor combinatorial, but contains some elements of each of these models.

Keywords: Allostery; Cooperativity; Hemoglobin intermediates; Hemoglobin cyanomet ligation analogue; Hemoglobin valency exchange; Cryogenic focusing

1. Introduction

Allosteric proteins, either single polipeptide chains or assemblies of functional chains, play a key role in the regulation of fundamental biological processes. In general, the mechanisms by which they fulfill such a role exploit the flexibility of these molecules allowing them to take up different conformations upon binding specific ligands. In most cases of allosteric assemblies of protein functional units the ligand binds in a cooperative positive mode yielding equilibrium curves sigmoidal in shape [1]. Sigmoidal equilibrium curves are predicted by thermodynamic models of cooperativity, which differ in the assumptions regarding the structural/functional properties of the intermediates [2–4]. The nature and the concentrations of

Abbreviations: Hb, HbO_2, HbCO, HbCN, Hb^+, deoxy-, oxy-, carbon monoxy-, cyanomet- and methemoglobin, respectively; α^{CN} and β^{CN}, cyanide bound hemoglobin chains in the ferric state; O.P. optical path.

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$(\alpha^1\beta^1)(\alpha^2\beta^2)$

$(\alpha^L\beta)(\alpha\beta)$ $(\alpha\beta^L)(\alpha\beta)$

$(\alpha^L\beta^L)(\alpha\beta)$ $(\alpha^L\beta)(\alpha\beta^L)$ $(\alpha^L\beta)(\alpha^L\beta)$ $(\alpha\beta^L)(\alpha\beta^L)$

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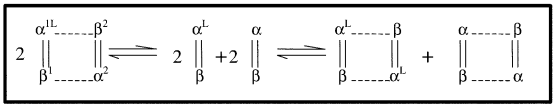

Fig. 1. The 10 hemoglobin ligation states. Superscript *L* indicates ligation. Superscripts 1 and 2 identify the four chains. The α^1–β^1, and α^2–β^2, contacts of the dimeric units, shown in parentheses, do not dissociate under physiological conditions. The weaker α^1–β^2 and α^2–β^1 inter-dimeric contacts allow a reversible tetramer–dimer dissociation. Symmetrical tetramers made up of dimers in the same ligation state are stable in pure form, since they re-associate into the original tetramer. Asymmetrical tetramers (insert) disproportionate into a ternary mixture of the asymmetrical and its parental symmetrical tetramers.

the molecules in a partial state of ligation, or intermediates, depend on the structure of the allosteric protein and the mechanisms of the cooperative interactions of the functional units. Therefore the determination of the properties of the intermediates is crucial for the choice of the model that is more adequate for the description of the cooperative mechanisms. Hemoglobin has a prototypic role in the study of cooperative proteins because of its physiological importance, the vast amount of data available on the structural/functional properties in the unliganded and fully liganded state, and, as an unique case among the cooperative proteins, because important thermodynamic properties of its intermediates are accessible to study. Ligand binding to hemoglobin yields the eight intermediates shown in Fig. 1. The study of the equilibrium curves cannot provide quantitative information on the type and properties of all these intermediates. Due to the dimer–tetramer equilibrium the intermediates that are accessible to study in a pure form are the symmetrical species, which dissociate into identical dimers. The asymmetrical intermediates can only be studied in mixture with the two symmetrical parental species, as illustrated in the insert of Fig. 1. The study of the intermediates in the reactions with gaseous heme ligands, such as O_2 and CO, is difficult because of the high mobility of the ligands, which dissociate from and re-associate to the four chain hemes. Nevertheless, the reduced CO mobility, as compared with that of O_2, has made it possible to trap and identify the CO intermediates under equilibrium and dynamic conditions using cryogenic analytical techniques [5,6]. The analyses of the equilibrium distributions have provided information on the thermodynamic properties of some CO intermediates [7,8]. To directly study the intermediates, either pure or in ternary mixtures, the ligand should have a low mobility compared with the time scale of the methodologies used for the study. To this end two types of ligation analogues have been used: non-native metal substituted hemoglobins, in which metals replacing the heme ferrous iron mimic the state of ligation or non-ligation, and cyanomet analogues, in which cyanide bound to ferric chains mimics the ligation state [9]. The cyanomet analogues, inexpensive and simple to prepare, have been intensively studied because of the assumed lack of mobility in comparison with the gaseous ligands. This assumption was incorrect, since these analogues undergo slow valency exchange reactions by which unliganded ferrous chains exchange electrons or the whole heme group with the cyanoferric chains [10]. The techniques that detect the valency exchange indicate that these reactions require several days to reach an apparent equilibrium, but do not provide information on the nature and distribution of the products of the exchange [10,11]. Such information is crucial for an assessment of the validity of the cyanomet analogue to represent cooperativity in ligation. Since the valency exchange reactions are slow, kinetic and thermodynamic properties of some intermediates can be obtained under condition of non-equilibrium, when the contamination by the products of the exchange is modest. However, if the valency exchange reactions yielded at equilibrium random distributions of intermediates,

the usefulness of this ligation analogue would be questionable. It would mean, in fact, that chains assuming this state of ligation are insensitive to the state of ligation of the other chains, which is in contrast to the present concept of allostery.

By the same cryogenic technique used for trapping the CO intermediates, we demonstrate that solutions of Hb[1] and HbCN reach reproducible non-random equilibrium distributions of deoxy/cyanomet intermediates in approximately 50–60 h. In these solutions the valency exchange reactions allow the attainment of a redox equilibrium and, in this respect, are equivalent to the dissociation and association reactions that allow the equilibration of the gaseous ligands with the protein. The distributions of deoxy/cyanomet intermediates were similar to those obtained with the highly cooperative CO, proving that the cyanomet analogue has the requisites of a true cooperative ligation analogue.

2. Materials and methods

2.1. Hemoglobin preparation

HbA_0 was purified by ion exchange chromatography, as previously described [12], and equilibrated with 50 mM KCl, pH 7.0. HbCN was prepared by HbO_2 oxidation at pH 6.8 with a 10–20% excess of ferricyanide and cyanide. HbCN was then exhaustively dialyzed against 0.1 M K-phosphate, 0.5 mM KCN, pH 6.8, to remove ferricyanide and any free and protein bound ferrocyanide, and equilibrated with 50 mM KCl, 0.5 mM KCN, pH 7.0. Samples of purified proteins at 5 mM heme concentration were stored in liquid nitrogen.

2.2. Hemoglobin deoxygenation

Deoxygenation of the HbO_2 solutions was carried out using a vibrating glass tonometer and a nitrogen or argon flow. Fig. 2a is the spectrum of a 2.1 mM deoxy HbA_0 sample measured after 40 min tonometry using a 0.2 mm O.P. flow cell fitted in a Beckman DU70 spectrophotometer. Residual amounts of HbO_2, 1–1.5%, detected by the spectral analysis were not removed by increasing the deoxygenation time, by replacing nitrogen with argon or purging the gases through a concentrated dithionite solution, since they were likely due to contamination by O_2 leaking into the unsealed cell. Fig. 2b is the spectrum of a similarly prepared Hb solution exposed to oxygen after 144 h incubation at 20° in a gas tight Hamilton syringe. After the addition of cyanide to this solution the spectral analysis indicated the presence of 2.1% HbCN. Deoxygenation of HbCN solutions brought about the rapid evaporation of free cyanide and some hemoglobin bound cyanide, as indicated by the appearance of 3–4% of the Hb^+ spectral component after 10 min tonometry. However, 2 min of tonometry were enough to remove dissolved oxygen from these solutions. The small cyanide loss under these conditions, which was measured by spectral analyses, was replenished by the addition of a calculated amount (1–5 μl) of a 90 mM cyanide solution. Free cyanide in HbCN solutions was determined spectrophotometrically by the addition of a known Hb^+ aliquot in excess with respect to the free cyanide. Anaerobic hemoglobin mixtures were prepared by filling gas tight syringes with each deoxygenated component and mixing by means of an argon bubble introduced after samples loading.

2.3. Anaerobic incubations

Anaerobic incubations were carried out in 0.5 ml gas tight Hamilton syringes. Methemoglobin formation, measured as HbCN, due to oxidation by contaminating oxygen, was $\leq 2\%$ of the total over long periods, 140–200 h (Fig. 2b). The free and bound cyanide concentrations were stable under these conditions. Table 1 lists the results of a typical stability test carried out as follows: a sample of 2.5 mM HbA_0 containing 98.7% HbCN and 1.4 Hb^+ was deoxygenated for 2 min. The Hb^+ concentration increased to 3.3%. After the addition of the calculated amount of 90 mM cyanide solution, samples (200 μl) were drawn into six syringes previously rinsed with argon and the same solution. A 2.5 mM HbA_0 solution, containing 100% HbO_2 according to the spectral analysis, was deoxygenated for 40 min and samples (200 μl) were drawn into the same syringes.

After mixing the contents the syringes were sunk in a bottle containing 0.1 M phosphate buffer and 2 g/l dithionite, pH 7, thermostatted at 20°. At the end of the incubation the syringe contents were exposed to air and the spectra of the undiluted solutions recorded.

2.4. Spectral analyses

Composite spectra were analyzed by least-squares fitting the mixture spectrum with a linear combination of the spectra of pure components using Matlab 6.5. As an example from Table 1, the analysis of the spectrum of the solution after 15 min incubation yielded: 50.7% HbO_2, 47.8% HbCN, 1.5% Hb^+, assuming three components ($\chi^2 = 7.6 \times 10^{-5}$), and 50.5% HbO_2, 49.5% HbCN, assuming two components ($\chi^2 = 1.1 \times 10^{-4}$).

2.5. Valency exchange determination by the cryogenic method

Samples of 100% HbCN solutions gel focused at −27° for 24 h migrated as a single homogeneous zone. Loss of protein bound cyanide during such a long electrophoretic run would show up as partially ferric zones. Samples of HbCN solutions that had lost a small amount of bound cyanide, e.g. during tonometry, did show additional components mainly corresponding to species $(\alpha^{+}\beta^{CN})(\alpha^{CN}\beta^{CN})$ and $(\alpha^{CN}\beta^{+})(\alpha^{CN}\beta^{CN})$. The amount of these components measured by the pyridine hemochromogen method, after elution

Table 1
Stability at 20° under unaerobic conditions of an equimolar mixture of 2.5 mM deoxy and cyanomet HbA_0 in 50 mM KCl, 20 mM K-phosphate, pH 7, in the absence of a detectable amount of free cyanide

t (h)	HbO_2 (%)	HbCN (%)	Hb^+ (%)
0.15	50.7	47.8	1.5
3	50.4	48.2	1.4
20	50.0	48.4	1.6
46	49.3	48.4	2.3
68	49.2	49.0	1.8
92	49.6	48.5	1.9

The spectral analyses were carried out at the end of the anaerobic incubations by exposing the mixtures to air.

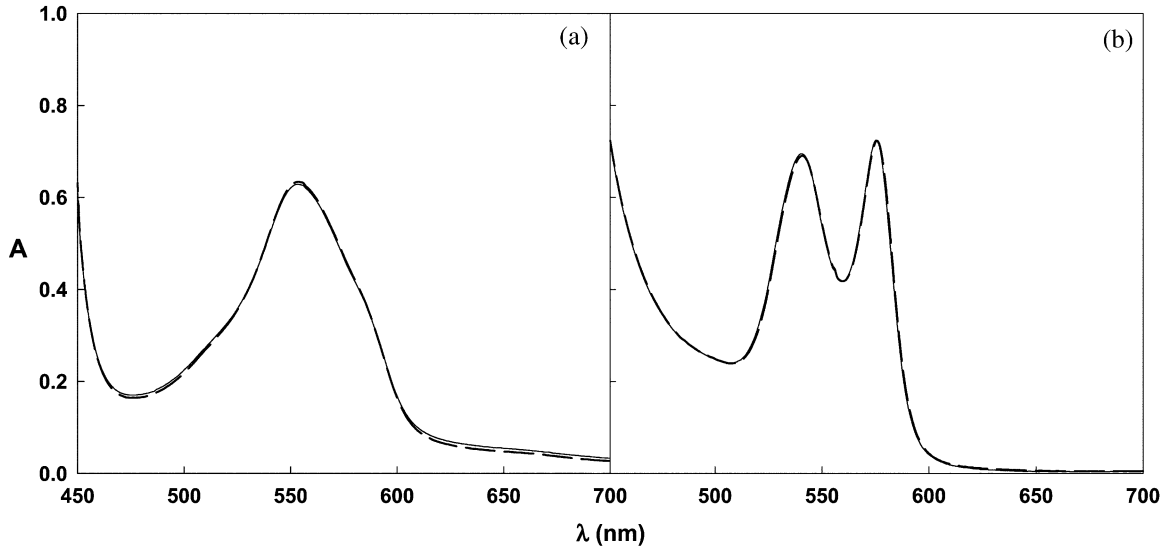

Fig. 2. Hemoglobin deoxygenation and stability during long anaerobic incubations. (a) Spectra of a sample of 2.1 mM HbO_2 in 20 mM phosphate, 50 mM KCl, pH 7, deoxygenated by 40 min argon tonometry at 20°. (—), Spectrum measured using a 0.2 mm O.P. flow cell immediately after deoxygenation; (----), calculated two component spectrum, 98.7% Hb and 1.3% HbO_2. (b) (—), Spectrum of a 2.5 mM Hb solution, prepared as in (a), after the addition of oxygen and cyanide at the end of 144 h incubation at 20° in a gas tight syringe; (----), calculated two component spectrum, 97.8% HbO_2 and 2.1% HbCN.

Table 2
Comparison of the fractions (%) of total Hb^+, [Hb^+], determined spectrophotometrically in 2.5 mM HbCN solutions in 50 mM KCl, 20 mM K-phosphate, 0.5 mM KCN, pH 7, after 2–3 min argon tonometry at 20°, and the fractions of the partially ferric species, $[(\alpha^+\beta^{CN})(\alpha^{CN}\beta^{CN})+(\alpha^{CN}\beta^+)(\alpha^{CN}\beta^{CN})]$, isolated by cryofocusing of the same solutions, eluted from the gel and determined by the pyridine hemochromogen method [5]

[Hb^+]	$[(\alpha^+\beta^{CN})(\alpha^{CN}\beta^{CN})+(\alpha^{CN}\beta^+)(\alpha^{CN}\beta^{CN})]$
2.9	2.5
4.6	3.1
	4.6*
4.4	3.2
	2.8*
3.2	2.2
2.6	3.0

*Duplicate determinations.

from the gels [5], was correlated to the Hb^+ amount determined spectrophotometrically in the HbCN solutions before cryofocusing. Some data are listed in Table 2. Since the complex between cyanide and the ferric heme at $-27/-30°$ was at least as stable as the CO ferrous heme bond at the same temperature, the method for trapping the CO intermediates could also be applied to the isolation of the deoxy/cyanomet intermediates [5]. Briefly, the procedure was as follows: a sample of aqueous deoxy/cyanomet hemoglobin solution at 20° was injected into an anaerobic mixture of ethylene glycol and 20 mM Na-phosphate buffer, pH 8.0, containing a 30-fold excess of ferricyanide, stirred and cooled at −30°. Under these conditions while cyanide was firmly bound to the heme, the unliganded hemes were rapidly oxidized. The dimer exchange reactions are extremely slow under these conditions. A sample of the quenched solution was transferred onto a gel for isoelectric focusing at −27°. The equilibrium was reached in 20–24 h. The protein sample was resolved into nine out of the ten oxidation products of the species depicted in Fig. 1, all of which have been identified [5]. Species $(\alpha^+\beta^+)(\alpha^{CN}\beta^{CN})$ and $(\alpha^+\beta^{CN})(\alpha^{CN}\beta^+)$ co-focus and therefore the corresponding intermediates, $(\alpha\beta)(\alpha^{CN}\beta^{CN})$ and $(\alpha\beta^{CN})(\alpha^{CN}\beta)$, were not resolved by the procedure, as in the case of the CO intermediates. By injecting samples of deoxy and cyanomet HbA_0 solutions separately into the quenching medium controls of the procedure and blanks for baseline correction were obtained.

2.6. Equilibrium data analysis

The analyses were carried out using an Adair scheme describing the ligand equilibria by means of 16 microscopic stepwise binding constants [13], and a model independent site-specific approach [14]. Assuming an arbitrary unit for the ligand concentration, [*L*], apparent values of the microscopic Adair constants were obtained. Using the apparent constants one can calculate an apparent equilibrium curve and the Hill's coefficient *n*, to provide a measure of the cooperativity of the system. The apparent constants can also be used to measure the different stability of the hemoglobin α and β chains in the various cyanomet liganded states. Due to a limitation of the cryogenic technique, which cannot resolve the oxidation products of intermediates $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ and $(\alpha\beta^{CN})(\alpha^{CN}\beta)$, the analysis was simplified further by using the eight constants scheme illustrated in Fig. 3. In addition, the experimental evidence indicated that, within the error, the total concentration of asymmetrical diliganded intermediates $[(\alpha\beta)(\alpha^{CN}\beta^{CN})+(\alpha\beta^{CN})(\alpha^{CN}\beta)]$ was equal to the total concentration of the symmetrical diliganded intermediates $[(\alpha^{CN}\beta)(\alpha^{CN}\beta)+(\alpha\beta^{CN})(\alpha\beta^{CN})]$. This observation suggested, as a further simplification, that $k_{2\alpha}=k_{2\beta}=k_2$. The concentrations of the intermediates, expressed as a function of seven apparent microscopic constants and the ligand concentration [*L*], form a set of eight non-linear equations:

$$\left[(\alpha^L\beta)(\alpha\beta)\right]=k_{1\alpha}\left[(\alpha\beta)(\alpha\beta)\right][L] \quad (1)$$

$$\left[(\alpha\beta^L)(\alpha\beta)\right]=k_{1\beta}\left[(\alpha\beta)(\alpha\beta)\right][L]$$

$$\left[(\alpha^L\beta)(\alpha^L\beta)\right]=k_{1\alpha}k_2\left[(\alpha\beta)(\alpha\beta)\right][L]^2$$

$$\left[(\alpha\beta^L)(\alpha\beta^L)\right]=k_{1\beta}k_2\left[(\alpha\beta)(\alpha\beta)\right][L]^2$$

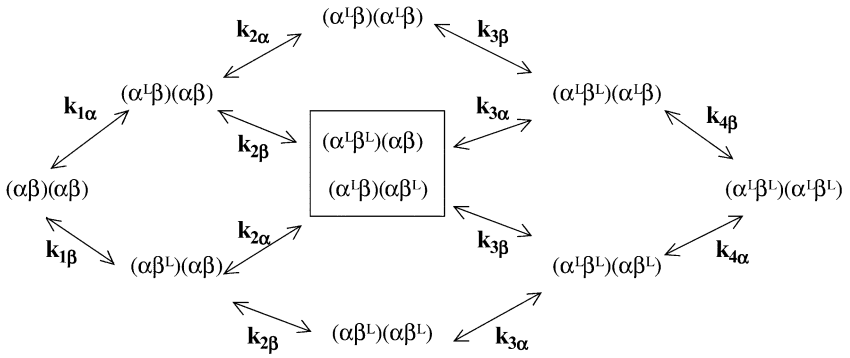

Fig. 3. Scheme of the equilibrium reactions between the 10 hemoglobin species in Fig. 1 and ligand L. The two asymmetrical intermediates $(\alpha^L\beta^L)(\alpha\beta)$ and $(\alpha^L\beta)(\alpha\beta^L)$ are enclosed in a box to indicate that they are not distinguishable because unresolved by the cryogenic technique. The scheme involves eight microscopic stepwise associative constants, but was simplified further by assuming $k_{2\alpha}=k_{2\beta}=k_2$. Such an assumption was based on the experimental evidence that the total concentration of the asymmetrical diliganded intermediates was equal to the total concentration of the symmetrical diliganded intermediates within the error of the analytical technique.

$$\left[(\alpha^L\beta^L)(\alpha\beta)\right] = k_2(k_{1\beta}+k_{1\alpha})\left[(\alpha\beta)(\alpha\beta)\right][L]^2$$

$$\left[(\alpha^L\beta^L)(\alpha^L\beta)\right] = (k_{3\alpha}k_{1\beta}k_2+k_{3\alpha}k_{1\alpha}k_2+k_{3\beta}k_{1\alpha}k_2)\times\left[(\alpha\beta)(\alpha\beta)\right][L]^3$$

$$\left[(\alpha^L\beta^L)(\alpha\beta^L)\right] = (k_{3\alpha}k_{1\beta}k_2+k_{3\beta}k_{1\beta}k_2+k_{3\beta}k_{1\alpha}k_2)\times\left[(\alpha\beta)(\alpha\beta)\right][L]^3$$

$$\left[(\alpha^L\beta^L)(\alpha^L\beta^L)\right] = \left[k_{4\alpha}(k_{3\alpha}k_{1\beta}k_2+k_{3\beta}k_{1\beta}k_2+k_{3\beta}k_{1\alpha}k_2)+k_{4\beta}(k_{3\alpha}k_{1\beta}k_2+k_{3\alpha}k_{1\alpha}k_2+k_{3\beta}k_{1\alpha}k_2)\right]\times\left[(\alpha\beta)(\alpha\beta)\right][L]^4$$

The fractions of the intermediates, i.e. the ratio between each intermediate concentration and the total concentration of the intermediates, were the experimentally accessible quantities. Such fractions, calculated from Eq. (1), form a set of seven independent equations, which can be solved by a non-linear least-squares procedure using Matlab 6.5. The apparent saturation Y vs. $[L]$ is then calculated from the fraction values:

$$Y=\frac{1}{4}(f_\alpha+f_\beta+2(f_{\alpha a}+f_{\beta\beta}+f_{\alpha\beta})+3(f_{\alpha\alpha\beta}+f_{\alpha\beta\beta})+4f_{\alpha\alpha\beta\beta}) \quad (2)$$

where the subscripts indicate the liganded chains of the intermediates. The model independent site-specific analysis describes the system using the partition function, Ψ,

$$\Psi = 1+2(K_\alpha+K_\beta)x+\left[c_{\alpha\alpha}K_\alpha^2+2(c_{\alpha\beta}+c'_{\alpha\beta})K_\alpha K_\beta+c_{\beta\beta}K_\beta^2\right]x^2+2(c_{\alpha\alpha\beta}K_\alpha+c_{\alpha\beta\beta}K_\beta)K_\alpha K_\beta x^3+c_{\alpha\alpha\beta\beta}K_\alpha^2K_\beta^2x^4 \quad (3)$$

where $x=[L]$, the ligand concentration in arbitrary units, K_α and K_β are the binding constants to the α and β chains when the other chains are unli-

ganded and the c's are the second, third and fourth order interaction constants between the four pair of chains. In applying Eq. (3) the same constraint regarding the concentrations of the symmetrical and asymmetrical diliganded intermediates was assumed as for Eq. (1), which reduced to seven the parameters to be calculated: K_α, K_β, $c_{\alpha\alpha}$, $c_{\beta\beta}$, $c_{\alpha\alpha\beta}$, $c_{\alpha\beta\beta}$, $c_{\alpha\alpha\beta\beta}$. Finally, to directly compare the parameters obtained from the analyses of the cyanomet analogue data in this work and the CO data from previously published work [8], the factor converting the CO concentration units to partial pressure in Torr units was also used for the analogue.

3. Results

3.1. Valency exchange intermediates in deoxy/cyanomet hemoglobin solutions

The species formed by the valency exchange reactions under anaerobic conditions at 20° using 2.5 mM hemoglobin solutions in 5 mM phosphate, 50 mM KCl, pH 7.0, were studied using two complementary approaches.

3.1.1. Mixtures of deoxy and cyanomet HbA_0

Equal amounts of deoxy and O_2-free cyanomet HbA_0 were mixed in gastight syringes and incubated for times varying from 15 min to 144 h. At the end of the incubation period part of the solution was exposed to air for the determination of the spectral components and part was analyzed by the cryogenic technique to determine the concentrations of the intermediates. The sum of the fractional values of the concentrations of the intermediates is plotted in Fig. 4 vs. the incubation time. The total fraction of the intermediates leveled off around a mean value of 0.23 ± 0.02 after 50 h incubation. The detailed compositions of the solutions in the 50–144 h time period are listed in Table 3. The intermediates $(\alpha^{CN}\beta)(\alpha^{CN}\beta)$ and $(\alpha\beta^{CN})(\alpha\beta^{CN})$ were resolved and their concentrations were measured. However, only their sum is listed in Table 3 for comparison with the sum of the concentrations of intermediates $(\alpha^{CN}\beta)(\alpha\beta^{CN})$ and $(\alpha^{CN}\beta^{CN})(\alpha\beta)$, which were not resolved. The HbCN fraction determined after each incubation

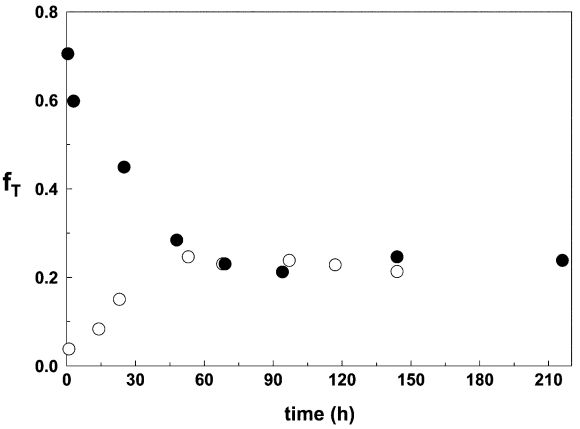

Fig. 4. Total fraction, f_T, of deoxy/cyanomet intermediates in 2.5 mM hemoglobin solutions in 5 mM phosphate, 50 mM KCl, pH 7, incubated anaerobically at 20°. (○) Equimolar mixtures of deoxy and cyanomet HbA_0. (●) Samples of 50% Hb and 50% HbCN prepared by random 50% ferricyanide oxidation of oxy HbA_0 in the presence of cyanide, equilibration with buffer and deoxygenation by argon tonometry.

experiment from the spectral analyses assuming two components, HbCN and HbO_2, was in the range 0.51–0.53, in agreement with the values, 0.51–0.54, calculated from the concentrations of the intermediates listed in Table 3. Assuming three components, HbCN, HbO_2 and Hb^+, the spectral analyses indicated the presence of Hb^+ in the 1–2% range, which was the error limit of the spectral resolution of the three species.

At short incubation times (15–60 min) the paucity of the intermediates precluded meaningful measurements of the concentrations of all the components. Thus some of them were pooled together. According to the data listed in Table 4 within 1 h the valency exchange between the two main species, $(\alpha\beta)(\alpha\beta)$ and $(\alpha^{CN}\beta^{CN})(\alpha^{CN}\beta^{CN})$, was very slight yielding only 1–1.5% of detectable mono- and triliganded intermediates. This suggests that most of the concentration of the observed asymmetrical diliganded intermediates was made up of species $(\alpha^{CN}\beta^{CN})(\alpha\beta)$, formed by the dimer exchange reactions between the parental species, which had reached the equilibrium within 15 min of incubation. Due to the error in the measurement of concentrations lower than 1.5% of the total (Table 4), it was not possible to monitor the

Table 3
Fractions (%) of the species in mixtures of deoxy and cyanomet HbA_0 at pH 7.0 and 20°, measured at various incubation times after reaching the equilibrium

Species	53 h	68 h	97 h	117 h	144 h	Average value
$(\alpha\beta)(\alpha\beta)$	35.1	34.2	33.6	37.0	35.2	35.0 ± 1.3 (41.8 ± 0.21)
$(\alpha^{CN}\beta)(\alpha\beta)$	6.63	5.39	7.16	6.20	5.44	6.16 ± 0.76 (2.37 ± 0.07)
$(\alpha\beta^{CN})(\alpha\beta)$	3.63	3.95	3.58	4.03	2.30	3.50 ± 0.70 (4.92 ± 0.35)
$(\alpha^{CN}\beta^{CN})(\alpha\beta)+(\alpha^{CN}\beta)(\alpha\beta^{CN})$	1.68	3.41	2.32	2.48	2.81	2.47 ± 0.66 (2.93 ± 0.22)
$(\alpha^{CN}\beta)(\alpha^{CN}\beta)+(\alpha\beta^{CN})(\alpha\beta^{CN})$	3.14	2.16	4.06	2.12	2.30	2.83 ± 0.78 (2.44 ± 0.27)
$(\alpha^{CN}\beta)(\alpha^{CN}\beta^{CN})$	6.21	4.67	4.00	5.58	5.27	5.15 ± 0.85 (3.84 ± 0.48)
$(\alpha\beta^{CN})(\alpha^{CN}\beta^{CN})$	3.28	3.41	2.71	2.48	3.15	3.01 ± 0.40 (4.68 ± 0.72)
$(\alpha^{CN}\beta^{CN})(\alpha^{CN}\beta^{CN})$	40.3	42.8	42.6	40.1	43.5	41.9 ± 1.6 (37.0 ± 1.58)

The total cyanomet fractions Y measured spectrophotometrically and calculated from the fractions of the intermediates were 0.52 ± 0.01 and 0.53 ± 0.01, respectively. The average values in parentheses refer to the CO equilibrium at total CO fraction of 0.48 under similar pH and temperature conditions [8].

formation of intermediate $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ in the 0–15 min time range.

Assuming that the concentration of diliganded asymmetrical intermediates listed in Table 4 was solely representative of the dimer exchange equilibrium concentration of $(\alpha^{CN}\beta^{CN})(\alpha\beta)$, we calculated the cooperative free energy, ΔG_C, for the transition from Hb to this intermediate. It has been shown that this quantity can be calculated from measurements of the ligand affinities and is also equal to the difference between the free energies of the dimer–tetramer equilibria of the intermediate and Hb [15]. The free energy of the dimer–tetramer equilibrium of the intermediate, $\Delta G^i_{(D\rightarrow T)}$, was calculated as follows. Scheme 1 depicts the kinetics of the dimer exchange reactions between Hb and HbCN. The accepted value of the rate of dimer association in any ligation state is $k_\alpha = 1.1\times10^{+6}\ M^{-1}\ s^{-1}$ [11]. The values of the rates of tetramer dissociation of Hb and HbCN under similar conditions are $k^0_d = 1.2\times10^{-5}\ s^{-1}$ and $k^4_d = 2.3\ s^{-1}$, respectively, [16]. By averaging the concentration values in the 15–60 min time range listed in Table 4 the value $k^i_d = 0.42\pm0.02\ s^{-1}$ was calculated for intermediate $(\alpha^{CN}\beta^{CN})(\alpha\beta)$. Notice that if $k^i_d = k^4_d$ the estimated value of the asymmetrical diliganded intermediate concentration would be $<0.3\%$.

Table 4
Fractions (%) of the species in mixtures of deoxy and cyanomet HbA_0 incubated for short periods at pH 7.0 and 20°

Species	15 min	30 min	45 min	60 min
$(\alpha^{CN}\beta)(\alpha\beta)+(\alpha\beta^{CN})(\alpha\beta)$	1.05 ± 0.27	1.22 ± 0.49	1.67 ± 0.49	1.55 ± 0.57
$(\alpha^{CN}\beta^{CN})(\alpha\beta)+(\alpha^{CN}\beta)(\alpha\beta^{CN})$	1.25 ± 0.37	1.30 ± 0.24	1.29 ± 0.16	0.99 ± 0.35
$(\alpha^{CN}\beta)(\alpha^{CN}\beta^{CN})+(\alpha\beta^{CN})(\alpha^{CN}\beta^{CN})$	0.92 ± 0.18	0.72 ± 0.35	0.50 ± 0.26	1.27 ± 0.31

The main species, Hb and HbCN at initial concentrations of $47\pm1.0\%$ and $49\pm1.0\%$, respectively, yielded the monoliganded and triliganded species by the valency exchange reactions and the asymmetrical diliganded species $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ by the dimer exchange reaction mainly. The symmetrical diliganded species $(\alpha\beta^{CN})(\alpha\beta^{CN})$ and $(\alpha^{CN}\beta)(\alpha^{CN}\beta)$ were not detectable. The data are the average of four independent blank-corrected experiments.

Scheme 1
Kinetic scheme of the dimer exchange reactions between deoxy- and liganded hemoglobin.

$$(\alpha\beta)(\alpha\beta) \underset{k_a}{\overset{k^o_d}{\rightleftharpoons}} 2(\alpha\beta) \qquad + \qquad \underset{k^i_d}{\overset{k_a}{\rightleftharpoons}} 2(\alpha\beta)(\alpha^L\beta^L)$$

$$(\alpha^L\beta^L)(\alpha^L\beta^L) \underset{k_a}{\overset{k^4_d}{\rightleftharpoons}} 2(\alpha^L\beta^L)$$

Using the calculated and published values of the rate constants in Scheme 1, we obtained the values of the free energy for the dimer–tetramer equilibrium of intermediate $(\alpha^{CN}\beta^{CN})(\alpha\beta)$, $\Delta G^i_{(D\rightarrow T)} = -8.6 \pm 0.6$ kcal/mol, and of the cooperative free energy for Hb ligation to yield this intermediate, $\Delta G_C = 6.17 \pm 0.3$ kcal/mol.

Mixtures containing different proportions of deoxy and cyanomet HbA_0 were also incubated for time periods of 60–70 h and then analyzed by the cryogenic technique. The fractions of the various species are plotted in Fig. 5a–d vs. the total HbCN fractional value, *Y*.

3.1.2. Mixture containing a large amount of intermediates

Oxy HbA_0 oxidized with half equivalents of ferricyanide in the presence of cyanide, freed of ferrocyanide and equilibrated with buffer was deoxygenated for 40 min by tonometry. Any slight loss of hemoglobin bound cyanide (<5%) was replenished before incubation under anaerobic conditions, as described in Section 2. Immediately after deoxygenation the total fraction of intermediates measured by the cryogenic technique was 70%. As indicated by the data in Fig. 4, such a value dropped to 23–24% after 60 h incubation and remained constant thereafter. The HbCN fractions yielded by the spectral analyses of the solutions and those calculated from the concentrations of the intermediates were in the 0.50–0.54 range. The fractions of the various species soon after deoxygenation and after 69–216 h incubation are listed in Table 5. The values relative to $[(\alpha^{CN}\beta)(\alpha^{CN}\beta)$ plus $(\alpha\beta^{CN})(\alpha\beta^{CN})]$ are listed in

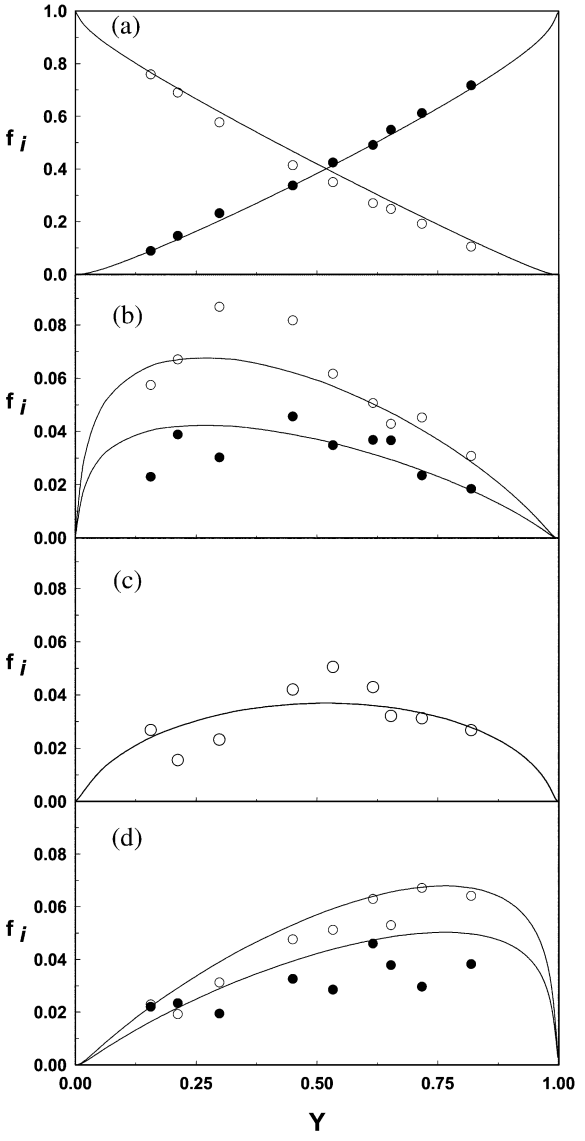

Fig. 5. Fractional values, f_i ($i = 0$–4), of the concentrations of the species isolated by the cryogenic technique in mixtures of varying proportions of deoxy and cyanomet HbA_0 incubated at 20° for >50 h. (a) (○), $(\alpha\beta)(\alpha\beta)$; (●), $(\alpha^{CN}\beta^{CN})(\alpha^{CN}\beta^{CN})$. (b) (○), $(\alpha^{CN}\beta)(\alpha\beta)$; (●), $(\alpha\beta^{CN})(\alpha\beta)$. (c) (○), total concentration of diliganded intermediates $(\alpha^{CN}\beta)(\alpha^{CN}\beta)+(\alpha\beta^{CN})(\alpha\beta^{CN})+(\alpha^{CN}\beta^{CN})(\alpha\beta)+(\alpha^{CN}\beta)(\alpha\beta^{CN})$. (d) (○), $(\alpha^{CN}\beta)(\alpha^{CN}\beta^{CN})$; (●), $(\alpha\beta^{CN})(\alpha^{CN}\beta^{CN})$. The solid curves were drawn using the values of the apparent stepwise binding constants calculated from the concentrations of the intermediates at $Y = 0.53$ (Table 3) according to the scheme in Fig. 3. The constants are expressed in $[L]^{-1}$ units: $k_{1\alpha} = 0.077$, $k_{1\beta} = 0.045$, $k_2 = 0.13$, $k_{3\alpha} = 3.4$, $k_{3\beta} = 2.2$, $k_{4\alpha} = 18$, $k_{4\beta} = 14$ (Table 6).

Table 5
Fractions (%) of the species in 50% Hb-50% HbCN solutions incubated anaerobically at 20°, pH 7

Species	0.5 h	69 h	94 h	144 h	216 h	Average value
$(\alpha\beta)(\alpha\beta)$	13.5	32.7	38.6	32.2	33.4	34.2 ± 2.9
$(\alpha^{CN}\beta)(\alpha\beta)$	16.2	6.98	4.76	8.20	7.28	6.80 ± 1.46
$(\alpha\beta^{CN})(\alpha\beta)$	10.7	3.57	3.34	3.16	2.37	3.11 ± 0.52
$(\alpha^{CN}\beta^{CN})(\alpha\beta)+(\alpha^{CN}\beta)(\alpha\beta^{CN})$	15.3	3.12	1.84	2.19	2.74	2.47 ± 0.57
$(\alpha^{CN}\beta)(\alpha^{CN}\beta)+(\alpha\beta^{CN})(\alpha\beta^{CN})$	5.2	2.67	1.50	2.51	2.85	2.38 ± 0.60
$(\alpha^{CN}\beta)(\alpha^{CN}\beta^{CN})$	11.0	4.61	6.94	5.39	5.58	5.63 ± 0.97
$(\alpha\beta^{CN})(\alpha\beta^{CN})$	12.1	2.08	2.84	3.14	2.98	2.76 ± 0.47
$(\alpha^{CN}\beta^{CN})(\alpha^{CN}\beta^{CN})$	16.0	44.3	40.2	43.2	42.8	42.6 ± 1.7

The solutions were prepared by deoxygenation of an oxy HbA_0 sample randomly oxidized with half equivalent of ferricyanide in the presence of cyanide and equilibrated with buffer. The values determined immediately after deoxygenation (0.5 h) are compared with those determined at various incubation times after reaching the equilibrium. The cyanomet fractions measured spectrophotometrically and calculated from the fractions of the intermediates were in the range 0.50–0.54.

Table 5 for comparison with those relative to $[(\alpha^{CN}\beta)(\alpha\beta^{CN})$ plus $(\alpha^{CN}\beta^{CN})(\alpha\beta)]$.

3.2. Data analysis

The seven apparent microscopic constants for the cyanomet analogue, calculated from the data on the equilibrium fractions of the intermediates at $Y=0.53$ (Table 3) according to the scheme in Fig. 3 and the simplification, $k_{2\alpha}=k_{2\beta}=k_2$, are listed in Table 6. Using these values the solid curves in Fig. 5a–d and the apparent saturation curve in Fig. 6 were calculated. The calculated value of Hill's n was 2.9. Listed in Table 6 are also the parameters calculated for the analogue at $Y=0.53$ and CO at $Y=0.48$ according to the model independent site-specific approach.

4. Discussion

4.1. Role of the valency exchange reactions

It has long been known that hemes and electrons are exchanged in mixtures of oxy and ferric hemoglobin [17]. More recently the same phenomenon has been observed under anaerobic conditions [10,18]. Aside from their physiopathological relevance [17], these reactions provide the rationale for the interpretation of the hemoglobin sigmoidal redox equilibrium curves [19]. In the case of O_2

Table 6
Equilibrium data analyses

A	B	
Cyanomet analogue, $Y=0.53$	Cyanomet analogue, $Y=0.53$	CO, $Y=0.48$
$k_{1\alpha}=0.077\times[L]^{-1}$	$K_\alpha=3.15\pm0.79$ Torr^{-1}	$K_\alpha=1.35\pm0.13$ Torr^{-1}
$k_{1\beta}=0.045\times[L]^{-1}$	$K_\beta=1.6\pm0.48$ Torr^{-1}	$K_\beta=2.7\pm0.28$ Torr^{-1}
$k_2=0.13\times[L]^{-1}$	$c_{\alpha\alpha}=16\pm5.0$	$c_{\alpha\alpha}=31\pm6$
$k_{3\alpha}=3.4\times[L]^{-1}$	$c_{\beta\beta}=4.0\pm1.2$	$c_{\beta\beta}=11\pm2$
$k_{3\beta}=2.2\times[L]^{-1}$	$c_{\alpha\alpha\beta}=870\pm280$	$c_{\alpha\alpha\beta}=990\pm220$
$k_{4\alpha}=18\times[L]^{-1}$	$c_{\alpha\beta\beta}=600\pm190$	$c_{\alpha\beta\beta}=600\pm110$
$k_{4\beta}=14\times[L]^{-1}$	$c_{\alpha\alpha\beta\beta}=(6\pm2)\times10^5$	$c_{\alpha\alpha\beta\beta}=(4\pm1)\times10^5$

A, Microscopic stepwise binding constants for ligand binding, expressed in arbitrary units of ligand concentration, calculated for the cyanomet analogue at $Y=0.53$ (Table 3) according to the Adair scheme (Fig. 3) [13], using the constraint $k_2=k_{2\alpha}=k_{2\beta}$. B, Comparison of the parameters calculated for the cyanomet analogue at $Y=0.53$ and CO at $Y=0.48$ (Table 3) according to a model independent site-specific approach [14]. As described in Section 2 the arbitrary concentration units adopted for the analogue were converted to apparent partial pressure in Torr units by using the same scaling factor of the CO data.

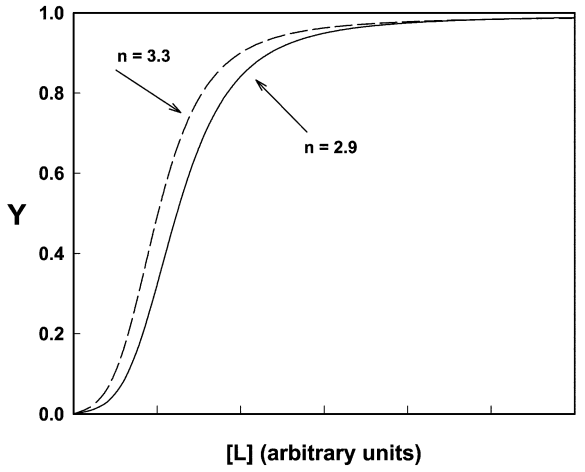

Fig. 6. (—), Apparent equilibrium curve for the cyanomet analogue constructed using the apparent stepwise binding constants calculated from the concentrations of the intermediates at $Y=0.53$ (Table 3) according to the scheme in Fig. 3 and listed in Table 6. (----), CO equilibrium curve constructed using the stepwise binding constants calculated from the concentration values of the CO intermediates at $Y=0.48$ listed in Table 3, using the same approach adopted for the analysis of the concentrations of the cyanomet analogue. The values of the Hill's coefficient n are indicated for each equilibrium curve.

and CO, the sigmoidal equilibrium curves are the macroscopic effect of non-statistical distributions of ligation intermediates generated by the protein allosteric mechanisms through the ligand association–dissociation reactions. Similarly, it is expected that non-statistical distributions of ferric intermediates make up the cooperative redox equilibrium curves. Thus the role of the valency exchange reactions should be to allow the ferric and deoxy chains to reach equilibrium distributions. Cyanide addition slows down the valency exchange reactions markedly [10,11] and prevents the measurements of the redox potential. However, the data in Fig. 4 indicate clearly that both a mixture of approximately equal amounts of Hb and HbCN with zero initial content of intermediates and a 50% oxidized solution containing approximately 70% of deoxy/cyanomet intermediates in a quasi random distribution slowly reached an equilibrium at which a similar stable total concentration of intermediates was observed. Furthermore, at equilibrium, as shown by the data in Tables 3 and 5, the same types of intermediates were observed in both cases and the average values of their concentrations were the same within the error of the analytical method. The curves of the distributions of the intermediates calculated from the average equilibrium data at $Y=0.53$ (Tables 3 and 6), and drawn in Fig. 5a–d, were consistent with the concentrations of the mono- (Fig. 5b) and diliganded (Fig. 5c) intermediates measured at Y values lower and higher than 0.53. The slightly less satisfactory description of the triliganded intermediates (Fig. 5d) was likely due to the constraints imposed in the data analysis approach due to the non-resolution of two intermediates, $(\alpha^{CN}\beta)(\alpha\beta^{CN})$ and $(\alpha^{CN}\beta^{CN})(\alpha\beta)$.

The value of Hill's $n=2.9$ indicates high cooperativity in the hemoglobin redox reaction in the presence of cyanide. These findings agree with previous studies of the hemoglobin redox potential [20]. Cooperativity in these reactions is pH-dependent. The values of Hill's n calculated from the curves of the redox potential vs. the Hb^+ fraction increase from 1.3 at pH 6.5, where a water molecule fills the cavity of the heme pocket, to approximately 2.5 at pH 8.5, where a hydroxyl ion binds the heme ferric iron. Thus the deoxy/cyanomet system may be assumed to represent a pH independent hemoglobin redox system in which a high affinity cyanide anion replaces the hydroxyl anion.

4.2. Analogies between the cyanomet analogue and carbon monoxide

The similarities between the equilibrium distributions of the cyanomet and CO intermediates show up in comparing the total intermediate content and the respective parameters of the model independent site-specific approach listed in Table 6. The total fraction of CO intermediates, 0.21 at $Y=0.48$ [8], is close to that of the analogue, 0.23 at $Y=0.53$. The values of the interaction constants, c, for the second, third and fourth order are also close in magnitude. The main difference regards the different affinities for the ligand of the α and β chains. Inspection of the microscopic binding constants listed in Table 6 shows that the analogue has an apparent greater affinity for the α chains in

the monoliganded state, with an affinity ratio of approximately 1.7 (see also Fig. 5b), and in the triliganded state, with an affinity ratio of approximately 1.5 (Fig. 5d). The apparent affinity ratio drops to 1.3 in the last binding. The situation is reversed for CO, as evidenced by comparing the respective K_α and K_β values in Table 6. Previous analyses of the CO distributions have stressed the crucial role of the nature and concentration of the diliganded intermediates in testing the allosteric model [7,8,21]. In this regard the analogies between CO and the cyanomet analogue are even more striking. At similar saturation values the concentrations of the asymmetrical diliganded intermediates unresolved by the cryogenic method, $[(\alpha^{CO}\beta)(\alpha\beta^{CO})$ plus $(\alpha^{CO}\beta^{CO})(\alpha\beta)]$ and $[(\alpha^{CN}\beta)(\alpha\beta^{CN})$ plus $(\alpha^{CN}\beta^{CN})(\alpha\beta)]$, are equal and, within the error of the analytical technique, equal also to the concentrations of the respective symmetrical diliganded intermediates. This finding indicates that the total concentration reached by the asymmetrical diliganded intermediates after equilibration for 50 h, $2.47 \pm 0.66\%$, contained approximately equal proportions of species $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ and $(\alpha^{CN}\beta)(\alpha\beta^{CN})$, and excludes major structural/functional differences between the symmetrical and asymmetrical diliganded intermediates. Additional evidence is provided by the analysis of the data obtained at short incubation times. During the initial process of equilibration of deoxy- and cyanomet hemoglobin (15–60 min), when the predominant reactions are the dimer exchanges between these parental species and the valency exchange reactions are minor, the total concentration of asymmetrical diliganded species was $1.20 \pm 0.28\%$ (Table 4), significantly lower than the value, $2.47 \pm 0.66\%$, found at equilibrium. This suggests that under these conditions species $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ was the only significant asymmetrical diliganded intermediate and that the calculated cooperative free energy value $\Delta G_C = 6.17 \pm 0.3$ kcal/mol refers to this intermediate. The ΔG_C value of the analogue is close to the value $\Delta G_C =$ 6.29 kcal/mol indicated as the most probable value for the transition from Hb to intermediate $(\alpha^{CO}\beta^{CO})(\alpha\beta)$ by the analysis of the equilibrium distributions of the CO intermediates [7]. Since $\Delta G_C = 7.11 \pm 0.14$ kcal/mol has been measured for the transition from Hb to HbCN, or HbCO, and, within 0.3–0.4 kcal/mol, for the transition from Hb to any cyanomet diliganded intermediate except $(\alpha^{CN}\beta^{CN})(\alpha\beta)$ [16], the estimated value of 6.17 kcal/mol for this intermediate indicates a release of 90% of the cooperative free energy in this binding step. Thus almost all the cooperative free energy is released upon the binding of two ligands irrespective of the nature of the diliganded intermediate. This is consistent with the hypothesis that diliganded hemoglobin is mostly in the R quaternary conformation [21,22].

Finally, both the equilibrium curves for the cyanomet analogue constructed from the concentrations of the intermediates at $Y = 0.53$ and that obtained from the CO intermediates at $Y = 0.48$, using the same data analysis approach, indicate high cooperativity in the processes involved (Fig. 6).

4.3. Implications for protein cooperativity

The data provided in this paper have several important implications for the study of hemoglobin cooperativity, which could partly be of paradigmatic value for the interpretation of protein cooperativity in general. Firstly, two apparently different phenomena through which hemoglobin exhibits cooperativity, heme ligation and redox reactions, present close analogies with regard to the reproducible, non-random distributions of the intermediates. Thus despite the different nature of the reactions that change the tertiary structure of the chains, i.e. ligation of a ferrous heme and heme oxidation to the ferric state, the mechanisms of the quaternary structural changes triggered by these reactions are the same. Secondly, the valency exchange reactions that allow the cyanomet analogue to reach the equilibrium are much slower than the dissociation and association reactions of O_2 and CO, the only ferrous heme ligand that has made so far accessible the study of the intermediates of native hemoglobin [6]. This makes the cyanomet analogue a key tool for the direct study of the properties of some intermediates, which would be inaccessible using O_2 and CO. Thirdly, our work supports the results of part of previous studies using the analogue, such as the studies on

the Bohr effect of the intermediates [22] and the work on the energetics of the cyanomet analogues presumably carried out under experimental conditions excluding a major contamination by the products of the valency exchange [9]. These studies, in agreement with the work on the CO intermediates [7,21,22], indicate that hemoglobin retains the T quaternary structure at the stage of monoligation, but switches to the R conformation upon binding a second ligand molecule. It cannot be excluded that different pathways of intersubunit and/or interdimeric communication promote such a switch [23]. However, the bulk of our studies are not consistent with a mechanism assuming that the asymmetrical intermediate $(\alpha^L\beta^L)(\alpha\beta)$ is energetically hyper-stabilized with respect to the other diliganded intermediates.

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