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What the intermediate compounds in ligand binding to hemoglobin tell about the mechanism of cooperativity

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The populations of the intermediates in concentrated solutions of hemoglobin A_0 equilibrated at various P_{CO} values, pH 7.0, 0.1 M KCl, and 20 °C, have been determined using cryogenic methods. Data on CO saturations and distributions of intermediates were analysed in terms of the free energies of dimer-tetramer assembly of the intermediates (G.K. Ackers and F.R. Smith, Annu. Rev. Biophys. Chem. 16 (1987) 583). The cooperative free energy value of the singly ligated species was approximately one-half the total cooperative energy. The cooperative free energy value of the doubly ligated species was not significantly different from that of carboxyhemoglobin. Because of experimental error, the observed difference in concentrations among the populations of the doubly ligated species cannot be taken as indicative of their functional heterogeneity. Additional studies on some NO intermediates have emphasized that $(\alpha^1\beta^1)(\alpha^2\beta^2)X$, a key intermediate in the formulation of the 'third-state' hypothesis in the deoxy/cyanomethemoglobin system, has a free energy value for dimer-tetramer assembly which is critically dependent on the nature of the ligand X as suggested by Ackers and Smith (reference as cited above).

1. Introduction

Macromolecular assemblies of protein subunits which react with ligands in a cooperative way undergo transitions from an unligated T structure to a ligated R structure, having the same symmetry [1]. The model of concerted allosteric transitions developed by Monod, Wyman and Changeaux (MWC) [2] has provided a theoretical framework for the interpretation of this cooperative phenomenon.

The MWC model explains the conservation of symmetry by assuming that only two quaternary structures of the same symmetry exist in equilibrium at all stages of ligation. However, this does not exclude tertiary structural changes of the subunits which occur at intermediate states of ligation in going from the unligated to the ligated state [3].

Hemoglobin, as the most extensively studied cooperative protein, is a prototype for testing theories of cooperativity.

Hemoglobin has a built-in asymmetrical connotation. The two different α- and β-subunits assemble in the cooperative tetramer as two pairs of identical $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers so that the interdimer contacts $\alpha^1\beta^2$ and $\alpha^2\beta^1$ are different from the contacts within each dimer. This allows the protein to dissociate only along the interdimer contacts $\alpha^1\beta^2$ and $\alpha^2\beta^1$ under physiological conditions.

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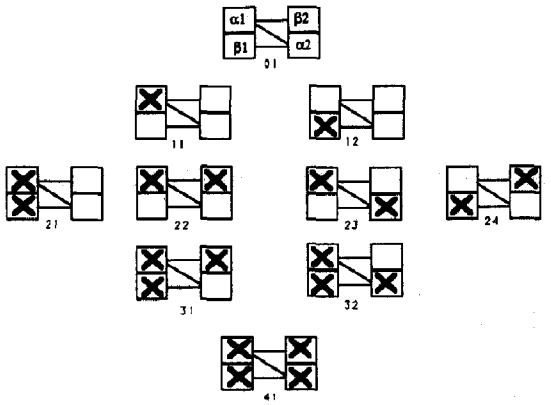

Fig. 1. Topology of ligand (×) distribution among the hemoglobin intermediates, according to Ackers and Smith [14].

These structural features also lead to degeneracy of the molecular ligation states, as shown in fig. 1 [14]. The asymmetrical arrangement of the α- and β-subunits in the tetramer allows for different ways of combination with two ligands, yielding species 21 and 22. These dissociate into different dimers, whereas the other two doubly ligated species, 23 and 24, dissociate into identical dimers.

A great deal of the experimental evidence on hemoglobin can be explained within the theoretical framework of the concerted MWC model. Most of this evidence comes from studies of the unligated and ligated protein or solutions in which these two components prevail upon the species in an intermediate state of ligation.

Evidence that directly supports a mechanism of hemoglobin cooperativity, in line with the MWC model, derives from studies of the intermediates 23 and 24 in which the ligand is carbon monoxide (CO) or cyanide ion (CN^-) bound to the ferric heme. The kinetics of CO binding to these intermediates yield biphasic curves and the interpretation is that the two phases are in part due to slow equilibration between the two quarternary structures T and R [5,6].

The NMR spectrum of $\alpha_2^{+CN}\beta_2$ has been shown to result from the superposition of the spectra of the T structure of deoxyhemoglobin and the R structure of cyanomethemoglobin whose ratio in the equilibrium mixture depends on pH and concentration of 2,3-diphosphoglycerate. The spectrum of $\alpha_2\beta_2^{+CN}$ changes to that of deoxyhemoglobin only in the presence of the more potent effector, inositol hexaphosphate [7].

A large proportion of the data in disagreement with the predictions of the basic formulations of the MWC model also arises from studies of the intermediates:

(1) The NMR spectra of some hemoglobin intermediates in which the tetramers were stabilized by cross-linking the $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers have not been found to result from the superposition of the spectra of the unligated and ligated protein. This has been interpreted as indicating the existence of more than two quaternary structures [8,9].

(2) The free energies of assembly of dimers into tetramers for the deoxy/cyanomethemoglobin hybrids, in which a stable heme-ligand interaction is mimicked by CN^- bound to the ferric hemes, have been shown to group into three distinct levels. One represents the lowest energetic state of deoxyhemoglobin, another the highest energetic state of cyanomethemoglobin and species 23, 24, 31 and 32. An intermediate, third level comprises the energetic states of 11, 12 and 21 [4]. Species 22 initially assigned to this level was later found to group with species 23 and 24 [10].

Similar observations were reported for artificial intermediates in which ligation was mimicked by substituting the heme Fe(II) with Mn(III) [11]. All of this suggested the possibility of a unique structure for each of the three energetic states.

(3) The isolation and identification of the intermediates in the equilibrium reaction between hemoglobin and CO have demonstrated a functional heterogeneity among the doubly ligated species, a feature not assumed by the MWC model [12].

Because of the prototypic role of hemoglobin, it is of crucial importance to assess whether the conflicting data quoted above can be avoided by modifications of the basic formulation of the MWC model to comply with the structural peculiarities of hemoglobin or whether a different theory of allostery needs to be formulated in order to explain the discrepancies.

Cryogenic methods have opened new perspectives in the clarification of these problems, since

they allow direct evaluation of the composition of complex systems of reacting hemoglobin molecules [13]. They do so by slowing down the two reactions that generate the instability of the intermediates: tetramer-dimer and ligand dissociations. Moreover, these methods can be applied under conditions of high protein concentration which leads to further simplification of the system by reducing the concentration of hemoglobin dimers to a negligible quantity.

This paper reports novel experimental data on the populations of the intermediates of hemoglobin A_0 equilibrated at various values of P_{CO}. Since the data are accurate, but are neither sufficiently precise nor numerous, a comparative test of different models was not attempted. Instead, we used the approach of Ackers and Smith [14], who tested the MWC model through analysis of the saturation data in terms of the free energy of dimer-tetramer assembly of the intermediates. This type of data analysis allowed us to account also for the observed diversity in the concentrations of the doubly ligated species. The analysis does not provide conclusive evidence for the third-state hypothesis formulated by these authors as a mechanism of hemoglobin function.

It is demonstrated that the energetic state of species 21 and 22 depends not only on the configuration of the ligated subunit in the tetramer, but also on the nature of the ligand, as suggested by Ackers and Smith [14].

2. Materials and methods

2.1. Gas mixtures

Standard N_2/CO gas mixtures were purchased from Sapio Industrie (Milan). The supplier guarantees the composition of these standards and the relative precision (± 0.2 ppm below and ± 0.1 ppm above 9.9 ppm) as determined by gas chromatographic procedures using mixtures supplied by the U.S. National Bureau of Standards for absolute calibration.

2.2. Hemoglobin (Hb) preparations

$HbA_0(O_2)$ and $HbC(O)_2$ were obtained from red blood cell hemolysates and purified by ion-exchange chromatography [15]. $MetHbA_0$ was prepared by oxidation of $HbA_0(O_2)$ at pH 6.8 using 20% excess ferricyanide and removal of the products of incomplete oxidation by ion-exchange chromatography. Purified samples were stored in liquid N_2. Preparation of $\alpha_2\beta_2^{NO}$ and $\alpha_2^{NO}\beta_2$ was carried out as follows. A sample of $HbA_0(O_2)$ was oxidized using 50% of the stoichiometric amount of ferricyanide either at 20 °C for 1 h or at 0 °C for 1–2 min. $HbA_0(O_2)$, $MetHbA_0$, $\alpha_2^+\beta_2^{O_2}$ and $\alpha_2^{O_2}\beta_2^+$ were separated by ion-exchange chromatography [16]. The partially oxidized products were recovered via a batch procedure. The zone of the resin containing each product was removed from the column bed and eluted by suspending the resin in a buffer containing cyanide. Deoxygenation of $\alpha_2^{+CN^-}\beta_2^{O_2}$ and $\alpha_2^{O_2}\beta_2^{+CN^-}$ by tonometry yielded $\alpha_2^{+CN^-}\beta_2$ and $\alpha_2\beta_2^{+CN^-}$, respectively, which were rapidly exposed at 0 °C to a stream of N_2 containing 1% NO purged through NaOH. The cyanomet hemes were then reduced by the addition of a 50-fold excess of dithionite. These derivatives were prepared before use and those of HbC were prepared similarly.

2.3. Equilibration of HbA_0 with CO

Samples (10 ml) of $HbA_0(O_2)$ (5 g/dl) in 0.1 M KCl were equilibrated with gas phases containing N_2 and 10–50 ppm CO in a 2 l glass bottle rotating in a water bath at 20.0 ± 0.2 °C. The flow rate of the gas was kept at 10–15 l/h for the time required to attain equilibrium and analysis of the solution. Before equilibration, catalase and superoxide dismutase were added and the pH was adjusted to account for the change due to the Bohr effect. During equilibration, slight adjustments of the pH were made by the addition of microliter amounts of 20 mM KOH in 0.1 M KCl or 0.3 M lactate (pH 3). At equilibrium the pH was 7.00 ± 0.005 and the metHb content below 1%.

2.4. Measurement of CO saturation

The approach to equilibrium was followed by the spectrophotometric determination of CO saturation as follows. Samples (approx. 0.2 ml) of hemoglobin solutions were withdrawn into a gas-tight Hamilton syringe containing 10 μl of 20

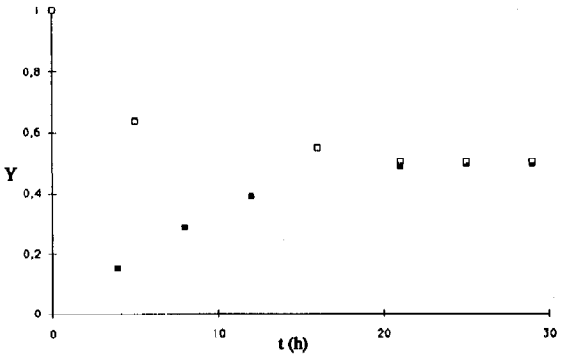

Fig. 2. Rate of attainment of equilibrium between HbA_0 and CO at 20 °C, pH ~ 7. A sample of hemoglobin was deoxygenated and equilibrated with an N_2/CO mixture containing approx. 25 ppm CO as described in the text (■). After 30 h, the solution containing 1% MetHb was exposed to pure CO and re-equilibrated with the same N_2/CO mixture (□). The final MetHb content was 2.5%.

mg/ml dithionite solution and passed through a cuvette (0.1 mm optical path length) monitored on a Beckman DU 6 spectrophotometer. Absorbances were recorded at 539, 555, 568 and 590 nm. The precision of saturation measurements was ±2% saturation. Equilibrium was attained after 20 h under all conditions of P_{CO} as shown in fig. 2. At equilibrium, saturation was also measured by the gas volumetric method of Peters and Van Slyke [17] to a precision of ±1% saturation.

2.5. Isolation, identification and quantitation of the intermediates

Samples (0.15 ml) of the equilibrated hemoglobin solution were quenched into a hydro-organic buffer at −30 °C containing ferricyanide under anaerobic conditions to trap the intermediates by a chemical-thermal quenching procedure described elsewhere [18]. Samples (15 μl) of the quenched solution of intermediates oxidized at the unligated hemes were focused at −25 °C and the separated components were identified as previously described [19].

Quantitation of the partially oxidized intermediates was carried out according to two methods. (a) Color slides of gel tubes were taken at the end of the isoelectric focussing (IEF) separation and the slides scanned. This method requires a calibration procedure, since the spectra of the bands of metHb and carboxyHb depend on the batch of film, light exposure, conditions for film development, etc. The calibration consisted in the quenching of various known amounts of deoxy- and carboxyHb separately in order to avoid hybridization, separation by IEF and color slides of the gel tubes when a new film was used. (b) The gels were sliced in correspondence to the hemoglobin components and the protein was eluted and assayed by the pyridine-hemochromogen method [10,20]. In general, this procedure is as accurate as the scanning method as shown in fig. 3. However, when used to quantitate the components in equilibrium mixtures at CO saturations greater than 50%, slight overlapping of bands for carboxyHb and species 31 does not permit one to distinguish between the two species correctly. This results in underestimation of the concentration of the triply ligated species and overestimation of that of carboxyHb.

In order to evaluate accurately the concentrations of intermediates, a blank must be subtracted. The blank is obtained according to the same method as for film calibration. The blank accounts for trace amounts of products of incomplete oxidation of unligated hemes, particularly of deoxyHb; trace amounts of products of oxidation of the ligated hemes, particularly carboxyHb; denatured protein or artifacts arising from the sep-

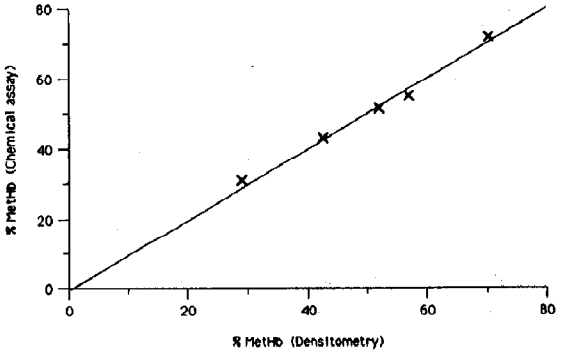

Fig. 3. Partially oxidized solutions of carboxyHb containing differing amounts of MetHb were focused and the MetHb fraction (×) was determined by the densitometric and chemical assay methods. The continuous plot represents the identity line.

aration which result in the gel having a slightly colored background. An accurate estimate of such a blank by using the scanning method is difficult to obtain due to the absence of well-defined peaks, whereas the chemical assay does give an accurate estimate of the background protein in those regions of the gel where the oxidized intermediates focus.

2.6. Data analysis

It has been shown that the Adair binding constant for ligand, A_{4i}, and the intrinsic binding constant, A_X [14], are related through eq. 1:

$$A_{4i} = A_X^i \sum_j g_{ij} e^{-\Delta G_c/RT} \quad (1)$$

where g_{ij} represents the statistical degeneracy of species ij (see fig. 1) and ΔG_c the cooperative free energy of ligand binding [14]. Since $\Delta G_c = {}^i\Delta G_{2j} - {}^0\Delta G_{21}$, where ${}^0\Delta G_{21}$ and ${}^i\Delta G_{2j}$ are the free energies of dimer-tetramer assembly of deoxyHb and the species with i ligands, respectively, the cooperative free energy is a quantity that can be determined by measuring dimer-tetramer equilibrium constants at different levels of ligand saturation.

The fractional population, f_{4i}, of the i-ligated intermediate can be calculated as a function of the total fractional saturation, Y, from eq. 2:

$$f_{4i} = \frac{A_{4i}[X]^i}{\sum_{i=0}^{4} A_{4i}[X]^i} \quad (2)$$

where [X] denotes the ligand concentration.

We have used the above approach to analyse our data on the values of CO saturation of HbA_0 and the corresponding populations of intermediates. The procedure was as follows.

Values of ${}^4\Delta G_{21}$ and ${}^0\Delta G_{21}$, the free energies of dimer-tetramer assembly of carboxy- and deoxyHb, respectively, were taken from published data in which the conditions were as close as possible to ours [21]. The values of free energy of intermediates 22–24, 31 and 32 were assumed to be the same as that of carboxyHb. The free energy values of intermediates 11 and 12, assumed to be equal, the free energy value of intermediate 21 and the value of the intrinsic binding constant A_X were determined by a trial-and-error procedure so that the calculated CO binding isotherm and the intermediates' distribution curves described qualitatively the experimental data of CO saturations Y and the intermediate concentrations vs Y.

Calculations were carried out on a MacIntosh II microcomputer equipped with a 4 megabyte memory and a program for simulation fitting problems on dynamic systems [22].

3. Results

3.1. CO equilibrium curve of hemoglobin A_0

The data on hemoglobin CO saturation, Y, vs P_{CO} are plotted in fig. 4. The Y values as determined via the spectrophotometric and gas-volumetric methods agree within the error limits.

3.2. Distribution of intermediates versus Y

The data on the relative concentrations of intermediates at various levels of CO saturation are plotted in fig. 5 together with their estimated errors.

Subunit functional heterogeneity of species 11, 12 and 31, 32 was ignored, since it was found to be only slight in extent, in agreement with previ-

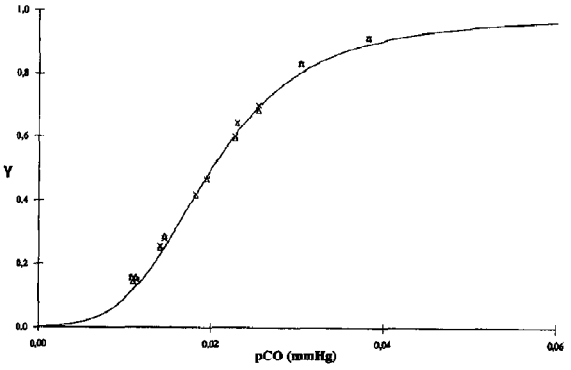

Fig. 4. Plot of CO saturation (Y) vs p_{CO}. (△) Data from gas volumetric analysis. (×) Data from spectrophotometric analysis. For the meaning of the full line, see text (section 3.4).

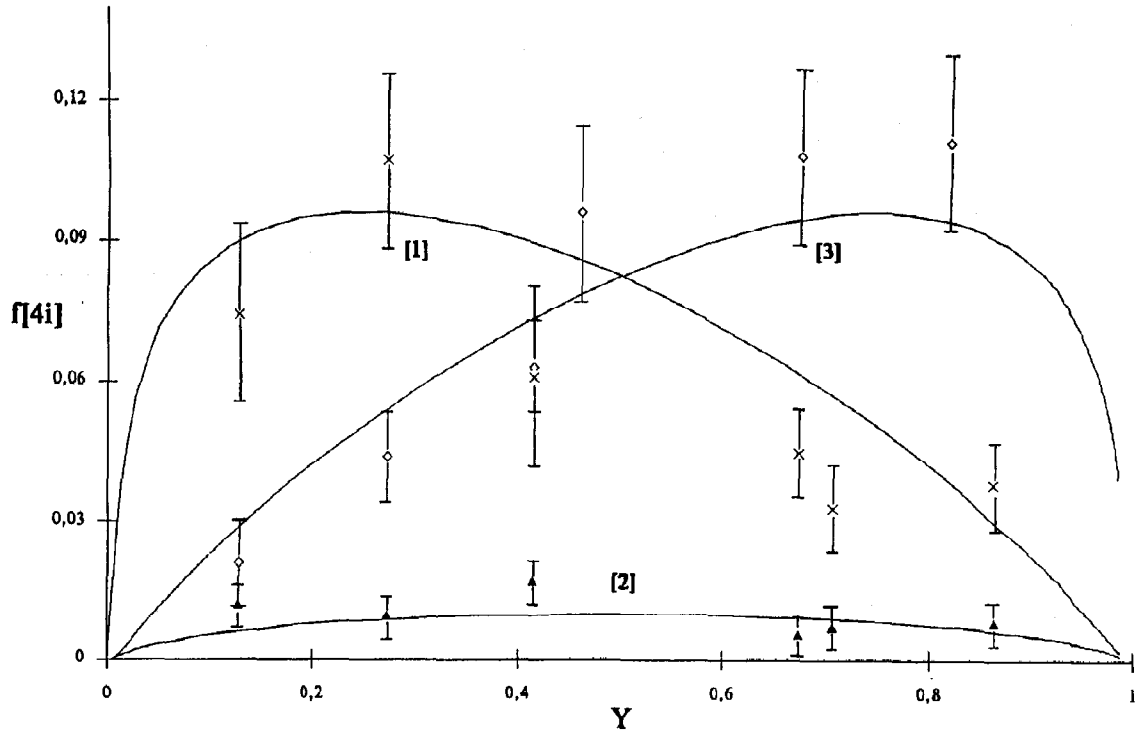

Fig. 5. Plot of fractional CO saturation $f[4i]$ of the intermediates i. ($i = 1$–3) vs total CO saturation. (×) Species 11 and 12. (▲) Species 21 and 22. (◇) Species 31 and 32. Vertical bars indicate the maximal error. For the meaning of full curves, see text.

ous estimates [12], and difficult to quantitate precisely. The concentrations of species 23 and 24 were found to be too low to be determined by either method of quantitation used in this work (< 0.5% of the total). The concentration of doubly ligated species shown in fig. 5 represents the sum of species 21 and 22, since they cannot be separated by IEF.

The Y values calculated from the distributions of the intermediates agree to within ±1–2% saturation with those shown in fig. 4.

3.3. Tetramer-dimer dissociation properties of the NO-ligated intermediates 21 and 22

The study of the tetramer dissociation reaction of the intermediates is made exceedingly difficult due to interference by heme-ligand reactions, unless ligation is mimicked by the complex of cyanide with the ferric heme. Since NO dissociates from nitrosylHb very slowly [23], we carried out the following experiments in order to obtain qualitative information on the tetramer dissociation reactions of the NO-ligated intermediates 21 and 22.

3.3.1. Experiment 1

A 1 : 1 mixture of nitrosylHbA$_0$ and metHbA$_0$ was rapidly mixed at 25° C, pH 7.4, with a 50-fold excess of dithionite in a continuous-flow apparatus. Under these conditions, the ferric hemes were reduced in less than 1 s [12]. After reaction times varying from 1 s to 3 min, the NO intermediates were trapped by using the same procedure as in the study of CO intermediates [12]. Table 1 summarizes the data on time-dependent changes in concentrations for the various intermediates.

Fig. 6 shows a kinetic simulation of the decay of species 21 according to the following scheme, based on dimer exchange reactions only:

$$2[21] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \begin{matrix} 2(\alpha^1\beta^1)\text{deoxy} & \underset{k_6}{\overset{k_3}{\rightleftharpoons}} [01] \\ 2(\alpha^2\beta^2)\text{NO} & \underset{k_4}{\overset{k_5}{\rightleftharpoons}} [41] \end{matrix}$$

Table 1

Time-dependent concentrations of the intermediates in a partially NO-ligated hemoglobin solution incubated under anaerobic conditions at 25 °C and pH 7.4

t (s)	Concentrations of NO intermediates (%) [a]					Fraction of ligated hemes (%)
	[01]	[11]+ [12]	[21]	[31]+ [32]	[41]	
1	21	4.1	30.6	9.0	33.2	56.3
35	20.4	7.5	26.8	9.2	34.8	57.0
120	18.8	17.4	16.7	13.5	31.4	54.2
160	14.8	16.6	18.2	15.3	32.5	57.0

[a] The % composition of the mixture before reduction with dithionite was: MetHb (18.4), singly ligated species (4.9), triply ligated species (9.4), nitrosylHb (31.2), asymmetrical doubly ligated species (34.7) plus traces of symmetrical doubly ligated species (1–2).

Values of the rate constants $k_2 = k_3 = k_5 = 10^3 s^{-1}M^{-1}$, $k_4 = 0.6\ s^{-1}$, and $k_6 = 2 \times 10^{-6}\ s^{-1}$ were taken from ref. 4. The value of k_1, the rate constant for tetramer-dimer dissociation of intermediate 21, was varied to give curves 1 ($k_1 = 4 \times 10^{-4}\ s^{-1}$), 2 ($k_1 = 4 \times 10^{-3}\ s^{-1}$) and 3 ($k_1 = 0.04\ s^{-1}$).

Fig. 6 shows that, in the absence of ligand reactions, the decay of intermediate 21 could be reproduced if $k_1 = 4 \times 10^{-3}\ s^{-1}$, the same value observed for the dissociation reaction of the corresponding deoxy/cyanometHb intermediate [4]. However, the data of table 1 show that such a decay is not paralleled by an increase in concentrations of deoxy- and nitrosylHb. Instead, the concentration of nitrosylHb remains constant, that of deoxyHb decreases and those of the singly and triply ligated species increase. This behavior indicates that the dissociation of NO from 21, followed by NO recombination to the two main reactive species present in solution, i.e., deoxyHb, to form 11 and 12, and species 21, to form 31 and 32, is responsible for most of the observed reduction in concentration of the NO-ligated intermediate 21.

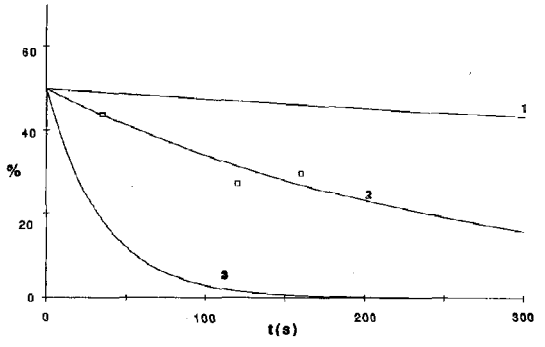

Fig. 6. Plot of concentration (%) of NO intermediate 21 vs time (t (s)) calculated according to a kinetic simulation (see text). (□) Experimental data on the concentration of 21 from table 1 normalized to the value obtained after 1 s.

3.3.2. Experiment 2

The cryogenic method described by Perrella et al [10] for studying the hybridization reactions of deoxy/cyanometHb intermediates was used to observe hybrid formation between the two NO-ligated intermediates, species 23 of HbA_0 and 24 of HbC.

Derivatives $\alpha_2^{NO}\beta_2^{+CN^-}$ and $\alpha_2^{+CN^-}\beta_{c2}^{NO}$ were prepared as described in section 2. They were reduced separately by treatment at 0 °C with a 50-fold excess of dithionite for 30 s, rapidly mixed and incubated at 20 °C in gas-tight syringes before quenching samples at −30 °C to stop dimer exchange and ligand reactions. The three hemoglobin components HbA_0, HbC and the resulting hybrid were separated by nonequilibrium IEF at −25 °C and quantitated.

Assuming that the parent species are pure and that no ligand reactions occur during incubation of the mixture at 20 °C, these components should be species 23 of HbA_0, 24 of HbC and the hybrid species 22. If the free energies of dimer-tetramer assembly of the three species are equal, their concentrations at equilibrium should conform to a binomial distribution [10].

Fig. 7 shows that the concentration of the hybrid in a 1:1 mixture of the parent species reached a plateau value of 40% of the total within 0.5 min.

The value of the hybrid concentration predicted from the binomial distribution (50% of the total) was not reached due to possible contamination by deoxyHb (~ 5%), which exchanges dimers at a slow rate. As shown in fig. 7, a concentration close to 50% of the total was noted when derivative 23 of HbC was incubated for 4 min with derivative

Table 2

Free energy values ($^{i}\Delta G_{(2j)}$) for dimer-tetramer assembly used to describe the CO saturation curve and the equilibrium distribution of CO intermediates of hemoglobin

Ligation state	Degeneracy	$^{i}\Delta G_{(2j)}$ (kcal/mol)	$^{i}\Delta G_{(2j)}$ (kcal/mol) [a]	Cooperative free energy (kcal/mol)
[01]	1	−14.8	(−14.4±0.1, $i=0$)	0
[11]	2	−11.3±0.3	(−11.5±0.2, $i=1$)	3.5
[12]	2	−11.3±0.3		3.5
[21]	2	−8.3±0.5	(−8.6±1.3, $i=2$)	6.4
[22]	2	−7.8		7
[23]	1	−7.8		7
[24]	1	−7.8		7
[31]	2	−7.8	(−7.2±0.4, $i=3$)	7
[32]	2	−7.8		7
[41]	1	−7.8	(−8.0±0.1, $i=4$)	7

[a] Values calculated by Ackers and Smith [14] from oxygen binding data of Chu et al. [31] in 0.1 M Bis-Tris-HCl, 0.1 M NaCl, pH 7.4, 21.5 °C, for the states of ligation $i=0$–4.

24 of HbA_0. This indicates less contamination by deoxyHb in the preparation of these species.

3.4. Data analysis

Ackers and Smith [14] have shown how the ligand-hemoglobin equilibrium can be analysed on the basis of the free energies of dimer-tetramer assembly of the intermediates, $^{i}\Delta G_{2j}$. The criteria we followed for partitioning the intermediates among a limited number of energy levels will be discussed later.

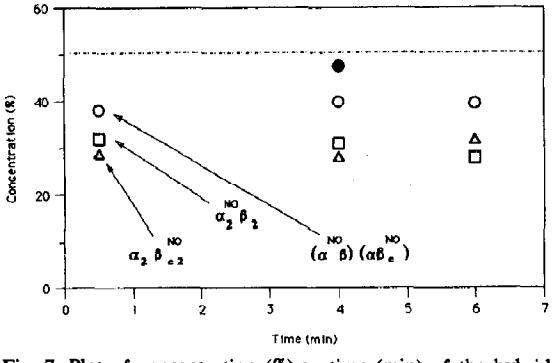

Fig. 7. Plot of concentration (%) vs time (min) of the hybrid ($\alpha^{NO}\beta$) ($\alpha\beta^{NO}$), (○) between NO symmetrical intermediates, in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4. (□) Species 23 from HbA_0. (△) Species 24 from HbC. (●) Hybrid ($\alpha\beta^{NO}$) ($\alpha^{NO}\beta_c$) between species 24 from HbA_0 and species 23 from HbC.

Some of these energies were assumed from published data, others being chosen to reproduce the data on both CO saturation and distribution of intermediates.

Thus, the value $^{4}\Delta G_{21} = -7.8$ kcal/mol at 20 °C, in 0.1 M KCl, pH 7.0, was assumed on the basis of the free energy value measured by Chu and Ackers [21] for oxyHb in 0.1 M Bis-Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.0, 21.5 °C, corrected for the $[Cl^-]$ difference, but neglecting the temperature difference. A value of $^{0}\Delta G_{21} = -14.8$ kcal/mol was similarly assumed for deoxyHb under our conditions.

Species 22–24, 31 and 32 were assigned to the same energy level of carboxyHb. The values of free energy of dimer-tetramer assembly of intermediates 11, 12 and 21 and their estimated errors, given in table 2, were obtained by the qualitative procedure described in section 2. The results of the calculations shown in fig. 8A, B indicate that these values provide an acceptable preliminary estimate. However, a statistical analysis of the data will be presented elsewhere.

Fig. 8A shows the dramatic effect of ±0.5 kcal/mol change in the free energy of the singly ligated intermediates, with respect to the value given in table 2, on the calculated distributions of these species. A similar change in free energy value of species 21 alters the total population of doubly ligated species by ±50%, without signifi-

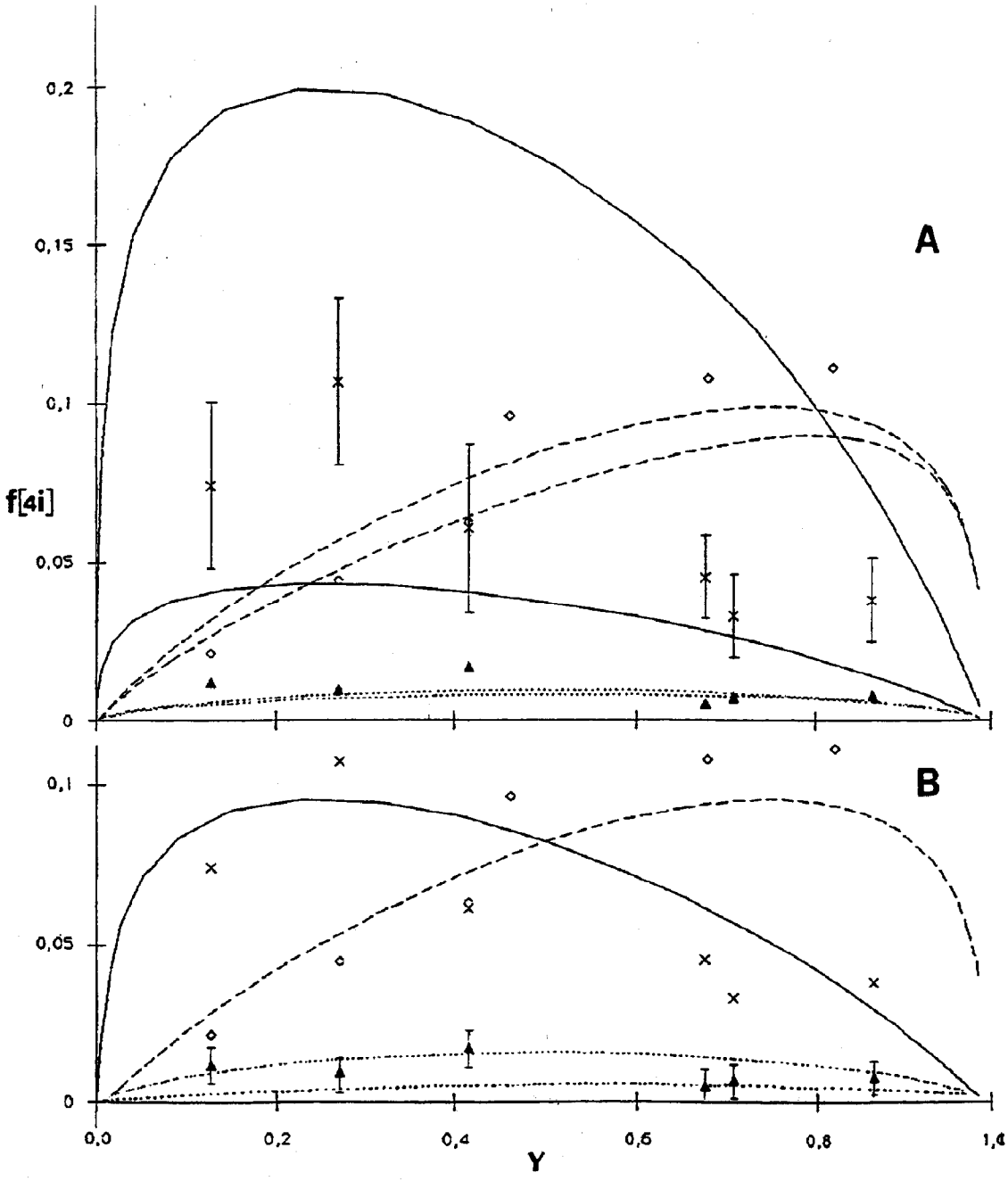

Fig. 8. Distributions of the intermediates calculated for a ±0.5 kcal/mol change in free energy values of the singly (A) and doubly (B) ligated species, with respect to the values given in table 2. (———) Singly ligated species; (— — —) triply ligated species; (- - -) doubly ligated species. (B) Calculated full and dashed lines coincide with those in fig. 5.

cant effects on the distributions of the other species, as shown in fig. 8B. However, such a change is comparable with the experimental error in the determination of the total population of doubly ligated species.

4. Discussion

4.1. Comparison with previous reports

This work marks a significant improvement over previous reports on the isolation of intermediates in the equilibrium reaction between hemoglobin and CO [12,18]. The populations of intermediates were measured in solutions equilibrated with gas phases of known CO concentration over a wide range of CO saturations and by the use of two independent methods for quantitation of the intermediates.

The accuracy of our measurements is supported by the agreement between (1) the two methods of quantitation of intermediates, except in the determination of the triply ligated species at $Y > 0.5$; (2) the values of CO saturation calculated from the populations of the intermediates and those measured directly by spectrophotometric and gas-volumetric methods. Also, the value for the Hill coefficient of $n = 3.4$ as determined from the maximal slope of the saturation curve agrees with that calculated from the corresponding populations of intermediates [12].

The total amount of intermediates at $Y = 0.5$ was 17%, as compared with 12–14% reported previously for identical conditions [12]. The difference is due to the use of the protein assay method to obtain the baseline correction for both methods of quantitation; the uncorrected data were identical.

We have confirmed that the singly and triply ligated species are significantly populated at all values of CO saturation studied, however, we have not attempted to confirm the slight chain heterogeneity previously reported [12]. It has not been possible to quantify with precision the concentrations of the symmetrical doubly ligated species 23 and 24, since the amount of these species was comparable with the error in the baseline correction.

Ackers and collaborators, measuring the free energies of dimer-tetramer assembly of the deoxy/cyanomet- and metal-substituted intermediates, have discovered a third thermodynamic state in addition to those of deoxy- and ligated hemoglobin [4] and have suggested a novel mechanistic interpretation of ligand binding to this protein [14].

Our accurate data on the hemoglobin-CO equilibrium allowed us to test the third-state hypothesis on a system likely to be functionally more akin to the hemoglobin-oxygen system than the model compounds studied by others [4,11].

4.2. Criteria for partitioning the CO intermediates among free energy levels

4.2.1. Species 23, 24, 31 and 32

The deoxy/cyanomet intermediates, the most thoroughly studied compounds, were found to group into three distinct free energy levels as follows: the lowest level (−14.4 kcal/mol, under the standard conditions adopted by Ackers et al.) is occupied by deoxyHb, the highest (−8.5 kcal/mol) by cyanometHb and intermediates 23, 24, 31 and 32. The third level (−11.4 kcal/mol) is occupied by intermediates 11, 12, 21 and 22 [14].

For the qualitative purpose of our analysis we decided to select the species that could have a free energy of dimer-tetramer assembly different from those of deoxy- and carboxyHb on the basis of the following assumptions: (1) the two species in the singly and triply ligated state have the same energies; (2) the energy of species ij, where i indicates the number of ligands, can be equal to, lower than, but not greater than the energy of species $(i+1)j$; (3) species 23, 24, 31 and 32 have the same energy as carboxyHb.

We made the first assumption since chain functional heterogeneity in the singly and triply ligated species was too small in extent to allow precise quantification via our methods.

The second and third assumptions were based on experimental evidence on the deoxy/cyanomet intermediates [4]. These assumptions are also partially and qualitatively supported by other evi-

dence reported in the literature. It has been shown that most of the structural transition from T → R occurs at the stage of triple ligation [24] and model 23 and 24 compounds show greatly reduced cooperativity [5,7,25–29].

4.2.2. Species 21 and 22

Smith and Ackers [4] measured the same free energy of dimer-tetramer assembly for the deoxy/cyanomet intermediates 21 and 22. We have assigned 22 to the energy level of carboxyHb and let 21 have a different energy for the following reasons.

Intermediate 21 appears to have properties distinct from those of intermediates 23 and 24 in various model compounds. Perrella et al. [10] confirmed, under true thermodynamic conditions, that the free energy of the deoxy/cyanomet intermediate 21 differs from those of deoxy- and cyanometHb. Cassoly [29] has shown that the NO-ligated intermediate 21 has particular optical properties with CO-binding kinetics indicating a T-like behavior, in contrast to species 23 and 24 behaving in an R-like manner.

The data on the NO-intermediate 21 presented in this paper also indicate a T-like behavior in the tetramer-dimer dissociation reaction. The decay in the concentration of this intermediate shown in fig. 6 could apparently be explained by a dimerization reaction with a rate constant equal to that of the corresponding deoxy/cyanomet intermediate [4], but as shown in table 1, such decay is in fact due to ligand reactions. This indicates qualitatively that the free energy of this intermediate is lower, i.e., more T-like, than that of the corresponding deoxy/cyanomet intermediate.

In contrast, both cyanomet- and NO-ligated intermediates 22 undergo dimerization reactions indicating a free energy similar to the R-like intermediates 23 and 24.

A hybridization study by Perrella et al. [10] of the deoxy/cyanomet intermediates 23 and 24 has shown that these derivatives yield a concentration of the resulting hybrid species 22 equal to 40% of the total after a short incubation time (~ 1 min) and the binomial value (50%) after a period longer than 30 h. These results indicate that although the solution was contaminated by deoxyHb, the resulting hybrid had the same free energy of the R-like parent species. If species 22 had the same energy as 21 or even lower, a concentration of hybrid greater than 90% would have been observed.

The present work on the hybridization of the NO-ligated intermediates 23 and 24 followed the same technical and conceptual approach and identical results were obtained. These data are less conclusive, since no evidence on the ligand reactions of intermediates 22–24 is available. However, significant dissociation of NO from R-like molecules is very unlikely to occur particularly during a 30 s time period.

4.3. Is there a third state in hemoglobin?

The analysis of the data, using the criteria discussed above for partitioning the CO intermediates among a limited number of energy levels, shows that the cooperative free energy of the singly ligated intermediates, $\Delta G_c = 3.3 \pm 0.3$ kcal/mol, is one-half of the total energy. A similar observation was made for the same deoxy/cyanomet intermediates [4]. However, the free energy of the dimer-tetramer assembly of species 21, or any other doubly ligated intermediate, cannot be set equal to the free energy of the singly ligated species. The free energy value of intermediate 21, selected among the four doubly ligated intermediates for the reasons discussed above, is not significantly different from that of carboxyHb.

The free energy value for the singly ligated species, intermediate between those of deoxy- and carboxyHb, does not support per se a third-state model as opposed to the two-state MWC model. Ackers and Johnson [30] have demonstrated that a third state could result from a restatement of the MWC model, which assigns different ligand affinities to dimers and R-state tetramers or different free energies of dimer-tetramer assembly to R-state tetramers in various ligation states.

Ackers and Smith [14] have also demonstrated for this modified MWC model that the stepwise ligand binding constants increase monotonically with ligation. In the deoxy/cyanomet system they identified a particular reaction pathway, 01 → 11 → 21 → 31 → 41, along which the stepwise bind-

ing constants, calculated from the corresponding cooperative free energies, do not fulfill this requirement. This was because species 11 and 21 have the same cooperative free energy values. This finding forms the basis of their claim of the existence of a third state independent of the MWC model and the formulation of a global switch mechanism in ligand binding to hemoglobin.

The existence of the third state can neither be confirmed nor be contradicted by our data on the hemoglobin-CO equilibrium, analysed on the basis of the assumptions made above. However, the finding that the energy of intermediate 21 is very close, if not equal, to that of carboxyHb does not provide a strong case in support of this hypothesis as being of general validity for the interpretation of the mechanism of hemoglobin cooperativity.

Since the free energies of dimer-tetramer assembly of the doubly ligated species are similar, their concentration depends on the state of degeneracy. As shown in table 2, the sum of intermediates 23 and 24 contributes one-third of the total population of doubly ligated species, the sum of 21 and 22 providing the remaining two-thirds.

In the experimental range of CO saturations that we have studied, the sum of 21 and 22 was approx. 1% of the total, which suggests an estimate of 0.5% as the sum of the concentrations of species 23 and 24. Such an amount of protein could be present, though undetectable by our methods. Therefore, the difference in concentrations among the populations of the various doubly ligated intermediates reported in this work and elsewhere [12] is not clearly indicative of functional heterogeneity among these species, as concluded erroneously by Perrella et al. [12].

Table 2 shows that the free energies of dimer-tetramer assembly, calculated by Ackers and Smith [14] from the oxygen equilibrium data of Chu et al. [31], parallel the case of the hemoglobin-CO equilibrium. They used the free energy values of the deoxy/cyanomet intermediates to calculate the distributions of intermediates and the O_2 saturation curve by the same procedure as used in this work [14]. Their results were similar to ours in indicating a large population of doubly ligated species and a total misfit of the O_2 saturation data.

Clearly, the deoxy/cyanomet- or metal-substituted hemoglobin systems are not satisfactory as representatives of the O_2- or CO-hemoglobin system and caution should be exercised in the extrapolation of the results of the analysis of these model compounds to the physiological conditions of ligation.

In this work, we have shown that, in agreement with other studies [4,10,29], intermediate 21 can have functional properties distinct from those of intermediates 22–24. Also, some of these properties are critically dependent on the nature of the ligand. The structural basis for this is unknown. However, it is likely that the explanation lies in the different effects on the tertiary structure of hemoglobin by ligands, such as NO, CN^-, CO and O_2 [32], and in the still unclear relationship between tertiary and quaternary structural changes.

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