CO Ligation Intermediates and the Mechanism of Hemoglobin Cooperativity*

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Direct experimental resolution of the ligation intermediates for the reaction of human hemoglobin with CO reveals the distribution of ligated states as a function of saturation. At low saturation, binding of CO occurs with slightly higher affinity to the β chains, but pairwise interactions are more pronounced between the α chains. At high saturation, the two chains tend to behave identically. The sequence of CO ligation reconstructed from the distribution of intermediates shows that the overall increase in CO affinity is 588-fold, but it is not distributed uniformly among the ligation steps. The affinity increases 16.5-fold in the second ligation step, 4.6-fold in the third ligation step, and 7.7-fold in the fourth ligation step. This pattern and the detailed distribution of ligated states cannot be immediately reconciled with the predictions of either the concerted allosteric model of Monod-Wyman-Changeux or the sequential model of Koshland-Nemethy-Filmer and underscore a more subtle mechanism for hemoglobin cooperativity.

Hemoglobin has long served as a paradigm for cooperative proteins (1, 2) and continues to provide new and important insights into how allostery is exploited in processes of pathophysiological importance (3). Two proposed models of hemoglobin cooperativity have made a significant impact in our understanding of structure-function relations in this and other proteins. The MWC model (4) assumes that hemoglobin exists in two quaternary structures, T and R, that differ in their affinity for oxygen. The low affinity T state is predominant in the absence of oxygen. Binding of oxygen progressively drives the hemoglobin tetramer to the high affinity R state, thereby bringing out the cooperative nature of the binding curve that is key to the function in vivo. A key postulate of the MWC model is that site-site interactions are not direct but are mediated indirectly through a concerted allosteric transition from T to R involving the molecule as a whole. An alternative allosteric model, known as the KNF model (5), assumes that each subunit is capable of tertiary conformational changes upon oxygen binding. These changes are directly transmitted to neighbor subunits through pairwise interactions and lead to sequential changes in the affinity for oxygen. Cooperativity ensues as a result of direct communications.

There is much kinetic and structural evidence that the MWC model best represents the behavior of hemoglobin under a variety of conditions (6), but the validity of this model has been challenged (7, 8), and the molecular code for hemoglobin cooperativity remains to be defined (9, 10). The highly cooperative nature of the oxygen binding isotherm naturally suppresses the contribution of the singly, doubly, and triply ligated species, making it very difficult to discriminate models of cooperativity (11). It has long been recognized that the overall shape of the oxygen binding curve is consistent with many models of cooperativity (5) and does not provide conclusive discrimination. The precise distribution of ligation intermediates that makes up the oxygen binding curve is more informative, but efforts to resolve these intermediates have been largely confined to model systems where hemoglobin or its ligands bear significant chemical perturbations (10). Results from these model systems are not consistent with either the MWC or the KNF model (8, 10), but their relevance to the properties of native hemoglobin reacting with oxygen has been questioned (6, 9). Resolution of the ligation intermediates of native hemoglobin reacting with a gaseous ligand is therefore highly desirable. These measurements are presented here for the first time and provide important new information on the mechanism of hemoglobin cooperativity.

EXPERIMENTAL PROCEDURES

Equilibration of Hemoglobin with CO—Weighted samples of HbA0 (5 g/dl) in 0.1 M KCl, deoxygenated in a tonometer using a stream of humidified nitrogen, were introduced into Hamilton gas-tight syringes and mixed with weighted samples of the same solutions exposed to CO. The syringes were kept for 20 h in a closed cylinder filled with water containing dithionite and thermostatted at 20 °C to let the hemoglobin solution equilibrate with CO. The CO saturation was calculated from the sample weights. The methemoglobin content after equilibration was 1–2%. The pH of the mixture was measured anaerobically after the attainment of equilibrium. A constant value of 7.0 ± 0.05 was obtained by adjusting the pH of the hemoglobin solution with 0.1 M KOH before equilibration. The P50 equilibrium value of the mixtures was calculated from the CO binding isotherm obtained previously under the same conditions of solvent, pH, protein concentration, and temperature by equilibrating solutions of HbA0 with CO/N2 mixtures of known composition (12).

Isolation of Intermediates—Samples of hemoglobin solutions (100 μl) equilibrated with CO were injected anaerobically into a stirred cryosolvent (60% v/v ethylene glycol, 40% v/v 22 mM phosphate buffer, pH 7.5, at 20 °C) containing ferricyanide and cooled at −30 °C. Ferricyanide oxidizes the unliganded subunits of hemoglobin yielding a mixture of partially oxidized species that can be separated by cryofocusing at −25 °C on gel tubes. Only nine species are detected by this procedure because the products of the oxidation of the two possible doubly ligated αβ pairs (Fig. 1) have identical isoelectric points. Gels were then moved from the glass tubes and sliced at the level of the colored protein components, which were eluted and assayed. The procedures for the separation and analysis of the isolated intermediates and all the relevant controls are described in detail elsewhere (13). Resolution of the intermediates α1α2β1β2 and α1α2β1β2 (Fig. 1) was obtained by noncryogenic focusing of the mixture of oxidized species isolated as a single component by cryofocusing, as described (14).

Theory and Data Analysis—The hemoglobin subunits α and β assembled in the tetramer form two structurally nonequivalent αβ contacts and yield 10 possible ligation states (Fig. 1). These states define the

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‡ The abbreviations used are: MWC, Monod-Wyman-Changeux; KNF, Koshland-Nemethy-Filmer.

Equilibrium of Hemoglobin with CO—Weighted samples of HbA0 (5 g/dl) in 0.1 M KCl, deoxygenated in a tonometer using a stream of humidified nitrogen, were introduced into Hamilton gas-tight syringes and mixed with weighted samples of the same solutions exposed to CO. The syringes were kept for 20 h in a closed cylinder filled with water containing dithionite and thermostatted at 20 °C to let the hemoglobin solution equilibrate with CO. The CO saturation was calculated from the sample weights. The methemoglobin content after equilibration was 1–2%. The pH of the mixture was measured anaerobically after the attainment of equilibrium. A constant value of 7.0 ± 0.05 was obtained by adjusting the pH of the hemoglobin solution with 0.1 M KOH before equilibration. The P50 equilibrium value of the mixtures was calculated from the CO binding isotherm obtained previously under the same conditions of solvent, pH, protein concentration, and temperature by equilibrating solutions of HbA0 with CO/N2 mixtures of known composition (12).

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Theory and Data Analysis—The hemoglobin subunits α and β assembled in the tetramer form two structurally nonequivalent αβ contacts and yield 10 possible ligation states (Fig. 1). These states define the
model-independent partition function for ligand binding to the four hemes of hemoglobin as follows (11).

\[
\Psi = 1 + 2(K_a + K_b)x + [c_{aa}K_a^2 + 2(c_{ab} + c_{bb})K_aK_b + c_{bb}K_b^2]x^2
+ 2(c_{aab}K_a + c_{abb}K_aK_b + c_{bab}K_b^2)x^3 + c_{aab}K_aK_b^2 + c_{abb}K_a^2K_b + c_{bab}K_aK_b^2 (Eq. 1)
\]

where \(x\) is the ligand concentration or partial pressure, \(K_a\) and \(K_b\) are the binding constants for the \(a\) and \(b\) subunits when all other subunits are unligated, \(c_{aa}, c_{ab}, c_{bb}\) are the second order interaction constants between the four possible pairs of chains, \(c_{aab}\) and \(c_{abb}\) are the third order interaction constants involving the two possible triplets of chains and \(c_{bab}\) is the fourth order interaction constants involving all chains. The \(c\) values reflect the presence of positive \((c > 1)\), negative \((c < 1)\) or no \((c = 1)\) cooperativity. For example, a value of \(c_{aa} = 10\) means that ligand binding to the second \(a\) chain is enhanced 10-fold when the first \(a\) chain is bound. The population of each intermediate is determined by the ratio of the term in the partition function related to the intermediate divided by \(\Psi\). These functions were used to analyze the distribution of CO ligation intermediates obtained experimentally.

The site-specific description of ligand binding to the four hemes of hemoglobin is related to the classical Adair description (15) that takes into account the five possible ligation states without discriminating between hemoglobin chains. The partition function for this description is

\[
\Psi = 1 + 4k_1x + 6k_2x^2 + 4k_3x^3 + k_4x^4 (Eq. 2)
\]

where \(k_i\) is the stepwise binding constant for the \(i\)th ligation step. These constants can be written in terms of the site-specific parameters as follows (11).

\[
k_1 = \frac{K_a + K_b}{2} (Eq. 3)
\]

\[
k_2 = \frac{1}{3}\left(\frac{c_{aa}K_a^2 + 2(c_{ab} + c_{bb})K_aK_b + c_{bb}K_b^2}{K_a + K_b}\right) (Eq. 4)
\]

\[
k_3 = \frac{3(c_{aab}K_a + c_{abb}K_aK_b + c_{bab}K_b^2)}{c_{aa}K_a^2 + 2(c_{ab} + c_{bb})K_aK_b + c_{bb}K_b^2} (Eq. 5)
\]

The distribution of CO-ligated intermediates was also analyzed in terms of the MWC and KNF models (4, 5). The partition function of the
FUNCTION FOR THE KNF MODEL IS AS FOLLOWS.

BINDING CONSTANTS TO THE WHITE BARS ARE THE ANALOGOUS BINDING CONSTANTS FOR THE MWC MODEL IS AS FOLLOWS.

PARAMETERS USING EQUATIONS 3–6 IN THE TEXT (MAN HEMOGLOBIN.

SHOWN ARE THE VALUES DERIVED FROM THE SITE-SPECIFIC ANALYSIS INVOLVING THE LIGATED PAIRS OF CHAINS. NO DISTINCTION WAS NECESSARY TO DESCRIBE INTERACTIONS RELATIVE TO THE R STATE IN THE ABSENCE OF LIGAND, \( K \).

FOR THE FIRST TWO LIGATION STEPS (TABLE I). BINDING TO THE \( a \) CHAINS IN THE T AND R STATES, AND \( K \) ARE THE BINDING CONSTANTS TO THE \( \alpha \) CHAINS IN THE T AND R STATES, AND \( K_1 \) AND \( K_2 \) ARE THE ANALOGOUS BINDING CONSTANTS FOR THE \( \beta \) CHAINS. THE PARTITION FUNCTION FOR THE KNF MODEL IS AS FOLLOWS.

\[
\begin{align*}
\Psi &= L(1 + K_1 x)^2(1 + K_2 x)^2 + (1 + K_3 x)(1 + K_4 x)^2 \\
&= \frac{L}{1 + 1} \quad \text{(Eq. 7)}
\end{align*}
\]

WHERE \( L \) IS THE ALLOSTERIC CONSTANT REFLECTING THE POPULATION OF THE T STATE RELATIVE TO THE R STATE IN THE ABSENCE OF LIGAND, \( K_1 \) AND \( K_2 \) ARE THE BINDING CONSTANTS TO THE \( \alpha \) CHAINS IN THE T AND R STATES, AND \( K_3 \) AND \( K_4 \) ARE THE ANALOGOUS BINDING CONSTANTS FOR THE \( \beta \) CHAINS. THE PARTITION FUNCTION FOR THE KNF MODEL IS AS FOLLOWS.

\[
\begin{align*}
\Psi &= 1 + 2(K_1 + K_3) x + (c_{\alpha \beta} K_2^2 + 4c_{\alpha \beta} K_1 K_3) x^2 + 2(c_{\alpha \beta} K_1 K_3) x^3 + c_{\alpha \beta} K_1 K_3 x^4 \\
&= \frac{L}{1 + 1} \quad \text{(Eq. 8)}
\end{align*}
\]

WHERE THE \( K \) VALUES ARE THE BINDING CONSTANTS OF THE TWO CHAINS AND THE \( c \) VALUES ARE THE PAIRWISE INTERACTION CONSTANTS BETWEEN THE POSSIBLE PAIRS OF CHAINS. NO DISTINCTION WAS NECESSARY TO DESCRIBE INTERACTIONS INVOLVING THE LIGATED \( \alpha \beta \) PAIRS (SEE "RESULTS"). THE TWO MODELS HAVE THE SAME NUMBER OF INDEPENDENT PARAMETERS AND COULD BE COMPARED DIRECTLY.

RESULTS AND DISCUSSION

IT FOLLOWS FROM DEFINITION OF THE ADAIR CONSTANTS IN EQUATIONS 3–6 THAT DIFFERENT COMBINATIONS OF SITE-SPECIFIC PARAMETERS REFLECTING THE CONTRIBUTION OF THE TEN LIGATED STATES OF HEMOGLOBIN CAN TRANSLATE IN THE SAME PATTERN OF STEPWISE CONSTANTS. THIS IS BECAUSE ANALYSIS OF THE BINDING CURVE IN TERMS OF THE ADAIR FORMALISM CONTAINS FOUR INDEPENDENT PARAMETERS, AS OPPOSED TO NINE IN THE SITE-SPECIFIC FORMALISM (11). THEREFORE, THE DISTRIBUTION OF THE 10 LIGATED INTERMEDIATES IN THE NATIVE MOLECULE REACTING WITH A GASEOUS LIGAND PROVIDES A VERY ACCURATE TEST OF ANY PROPOSED MOLECULAR CODE OF HEMOGLOBIN COOPERATIVITY.

A HIGH RESOLUTION CRYOGENIC TECHNIQUE (13) HAS MADE IT POSSIBLE TO RESOLVE THE DISTRIBUTION OF INTERMEDIATES FOR THE REACTION OF CO WITH NATIVE HUMAN HEMOGLOBIN (FIG. 2). BECAUSE CO SHARES WITH OXYGEN HIGH BINDING AFFINITY AND COOPERATIVITY (11, 12), IT REPRESENTS AN IDEAL LIGAND FOR DISSECTING HEMOGLOBIN COOPERATIVITY AT THE SITE-SPECIFIC LEVEL, WHICH CANNOT BE ACHIEVED WITH OXYGEN IN VIEW OF ITS FAST KINETICS OF DISSOCIATION (16). ANALYSIS OF THE DISTRIBUTION OF CO-LIGATED INTERMEDIATES AS A FUNCTION OF SATURATION REVEALS STRONG COOPERATIVITY ALREADY IN THE FIRST TWO LIGATION STEPS (TABLE I). BINDING TO THE \( \beta \) CHAIN IS SLIGHTLY PREFERRED IN THE SINGLE LIGATED SPECIES, BUT SUCH PREFERENCE DISAPPEARS IN THE TRIPLY LIGATED SPECIES, INDICATING THAT CONFORMATIONAL CHANGES TAKING PLACE AT HIGH SATURATION TEND TO ABDOMINATE THE DIFFERENCES BETWEEN THE CHAINS. ONCE THE FIRST CHAIN IS LIGATED, THE SECOND BINDING EVENT TAKES PLACE WITH SIGNIFICANTLY HIGHER AFFINITY. INTERACTIONS ARE STRONGER BETWEEN \( \alpha \) CHAINS AND COMPENSATE FOR THE LOWER AFFINITY OF THESE CHAINS COMPARED WITH THE \( \beta \) CHAINS, THEREBY PRODUCING DOUBLY LIGATED INTERMEDIATES OF COMPARABLE MAGNITUDE (FIG. 2). NO SIGNIFICANT
Mechanism of Hemoglobin Cooperativity

The distribution of ligated intermediates has peculiar features that are difficult to reconcile with current models of hemoglobin cooperativity. The significant increase in affinity at the second step of ligation populates the doubly ligated intermediates well beyond the expected value (0.2–0.5%) in the absence of interactions. The MWC model does not account for this important feature of CO ligation (Table I). The best fit parameter values obtained from the analysis of the predictions predict the T to R transition to take place upon binding of the third CO molecule. This provides a satisfactory fit for all but the doubly ligated intermediates, predicted to be only 1%, as opposed to almost 5% found experimentally. The KNF model yields a better fit but again fails to correctly reproduce the distribution of doubly ligated intermediates and the high degree of cooperativity in the first two ligation steps (Table I). Combinatorial switch mechanisms for hemoglobin cooperativity, like the one implied by the “symmetry rule,” also fail to describe the distribution of doubly ligated intermediates, because ligation of an αβ pair is not dependent on the location of the chains in the tetramer.

The discrepancies of the MWC and KNF models are also illustrated by the cooperativity pattern embodied by the four stepwise Adair constants (Table I), as shown in Fig. 3. Both models significantly underestimate the affinity of the second ligation step, but the MWC model also overestimates the affinity of the third ligation step. However, the overall saturation curves predicted by these models from the analysis of the distribution of intermediates are practically indistinguishable from each other and from the curve predicted by the model-independent analysis (Fig. 4). The excellent agreement of the models with experimental data in Fig. 4 is deceiving, because the shape of the binding isotherm is notoriously insensitive to the contribution of poorly populated intermediates. The limitations of the MWC and KNF models are observed only when the properties of these intermediates are measured directly (Table I). Hence, overall binding isotherms cannot and should not be used to assess the validity of mechanistic models or to discriminate among them.

The distribution of CO ligation intermediates obtained experimentally and the pattern of stepwise constants derived from them reveal a more subtle mechanism for hemoglobin cooperativity than those predicted by the MWC and KNF models. The KNF model fits the data significantly better than the MWC model, but it does not capture the large enhancement in binding affinity in the first two ligation steps. An unambiguous interpretation of the pattern in Fig. 3 is not possible, because of the limitations intrinsic to the Adair description. It should also be pointed out that the pattern in Fig. 3 applies specifically to the experimental conditions in the present study and may be modified by allosteric effectors and physical variables. For these reasons, we limit ourselves to emphasize the phenomenological significance of the results. A modified MWC model with direct pairwise interactions within the T state would be consistent with the experimental data, but so would a modified KNF model with interactions higher than second order. More information must be gathered on the ligation intermediates of native hemoglobin reacting with a ligand that closely mimics oxygen, before the code of hemoglobin cooperativity can be formulated conclusively. The results reported here represent an important first step in this direction.

REFERENCES
1. Perutz, M. F. (1970) Nature 228, 726–739
2. Perutz, M. F. (1989) Q. Rev. Biophys. 22, 139–236
3. Gow, A. J., and Stamler, J. S. (1998) Nature 391, 169–173
4. Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol. 12, 88–118
5. Koshland, D. E., Némethy, G., and Filmer, D. (1966) Biochemistry 5, 365–385
6. Henry, E. R., Jones, C. M., Hofrichter, J., and Eaton, W. A. (1997) Biochemistry 36, 6511–6528
7. Ackers, G. K., Doyle, M. L., Myers, D., and Dougherty, M. A. (1992) Science 255, 54–63
8. Hå, C. (1992) Adv. Protein Chem. 43, 153–312
9. Perutz, M. F., Wilkinson, A. J., Paoli, M., and Dodson, G. G. (1998) Annu. Rev. Biophys. Biomol. Struct. 27, 1–34
10. Ackers, G. K. (1998) Adv. Protein Chem. 51, 185–253
11. Di Cera, E. (1995) Thermodynamic Theory of Site-specific Binding Processes in Biological Macromolecules, Cambridge University Press, Cambridge, UK
12. Perrella, M., Colosimo, A., Benazzi, L., Ripamonti, M., and Rossi-Bernardi, L. (1990) Biophys. Chem. 37, 211–223
13. Perrella, M., and Rossi-Bernardi, L. (1994) Methods Enzymol. 232, 445–460
14. Perrella, M., Ripamonti, M., and Caccia, S. (1998) Biochemistry 37, 2017–2028
15. Adair, G. S. (1925) J. Biol. Chem. 63, 529–545
16. Olson, J. S., Anderson, M. E., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5919–5923