Asymmetric Cooperativity in a Symmetric Tetramer: Human Hemoglobin

This paper is available online at www.jbc.org

Published, JBC Papers in Press, January 19, 2006, DOI 10.1074/jbc.R500019200

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A longstanding challenge in macromolecular biochemistry has been the question of how the four “active site” hemes of human hemoglobin (Hb) interact cooperatively over widely separated intramolecular distances (i.e. 24–37 Å) to regulate the O2 affinity of the molecule. In the modern era of Hb research, beginning with the availability of crystal structures for both the deoxy and fully ligated tetramers by the late 1960s, the problem of subunit-subunit communication has been addressed by extensive static and dynamic structural characterization of the end-state tetramers and of the equilibrium and kinetic ligand binding reactions for numerous mutant and chemically modified Hbs. Throughout this period, the approach to characterizing Hb structure-function relationships has been dominated by the structural symmetry of the tetramer, in that structural symmetry has been assumed to dictate functional symmetry. For this reason, Hb research has, by default, been based on the perturbation of both αβ dimers in the tetramer equally and simultaneously, i.e. by mutations and chemical modifications, the binding of numerous small molecules, and the wide variation of solution conditions.

The idea that all heme sites of Hb respond uniformly to the ligation of any one heme site was formalized in 1965 by Monod, Wyman, and Changeux by their proposed two-state concerted model (reviewed in Ref. 1). The binding of O2 (as well as allosteric effectors, such as protons and organic phosphates) altered the relative stabilities of the T (low O2 affinity) and R (high O2 affinity) states. Thus, each of the four subunits retained the same O2 affinity at each stage of ligation. The two-state model was given an explicit structural interpretation by Perutz in 1970, in which O2 binding to the heme iron resulted in conformational change on the opposite side of the heme (the proximal side), which was communicated to the dimer-dimer interface, weakening the T state relative to the R state (as reviewed in Ref. 1). The Perutz interpretation favored the concerted model over the sequential model of Koshland et al. (2), and by 1970, the molecular mechanism of cooperativity in Hb was considered by many to be largely solved. However, many researchers continued to doubt that cooperativity and allostery could operate by such a simple, if not simplistic, concerted two-state mechanism.

In keeping with T/R-based models, the properties of the partially ligated intermediates (with only one, two, or three ligated heme sites) were assumed to be linear combinations of properties pertaining to the oxy and deoxy end states. Early approaches to the study of Hb intermediates (3, 4) demonstrated that these partially ligated tetramers were labile and not readily accessible to study. The experimental difficulties in stabilizing the intermediates are primarily the result of the strong cooperativity of Hb and the lability of bound O2, which favor solutions of oxy-Hb instead of partially ligated Hbs. Aside from chemically cross-linking the two dimers to prevent the exchange of αβ dimers among tetramers (5, 6), the intermediates could only be studied as mixtures (7, 8), from which individual binding constants cannot be resolved with reliability. These barriers were overcome in the late 1980s through the combined application of stable (i.e. non-labile) heme site analogs and thermodynamic linkage analysis, permitting the measurement of binding constants for each partially ligated intermediate (9). This work revealed a dimer-based distribution of cooperativity among the partially ligated tetramers, which was not uniform among the four heme sites. Properties that had historically been attributed to the quaternary T and R states of the Hb tetramer may have been due to the dimers within the tetramer instead. This was possible, in principle, because the tetramer had not been systematically perturbed in an asymmetric fashion during the decades of Hb research.

This review focuses on the principal findings from studies on individual partially ligated Hb intermediates, which demonstrate that the symmetric tetramer responds in an asynchronous fashion to asymmetric ligation and mutation. Thus, communication among its four heme sites is not uniform, and tetrameric symmetry does not have to be maintained over the course of O2 binding and release.

A Dimer of Dimers

Perhaps the most familiar property of Hb is the sigmoidal shape of its binding isotherm, a characteristic of positive cooperativity (Fig. 1). The low affinity of the deoxytetramer is converted to an affinity several hundredfold higher in the oxytetramer; thus, the binding energy for oxygenation of one subunit is transduced into a higher affinity for binding to neighboring subunits. The relatively low O2 affinity of deoxy-Hb is due to structural constraints within the tetramer, which are broken upon oxygenation, resulting in the observed high affinity that is characteristic of the oxy-Hb molecule (9). The sigmoidal shape of the O2 binding curve results from the specific manner in which this free energy transduction is distributed among the subunits that comprise the Hb molecule.

A closer look at the tetramer reveals that it is organized as a symmetric dimer of αβ dimers, possessing two distinct kinds of subunit-subunit interfaces. The two intradimer interfaces (α1β1 and α2β2) are composed of hydrophobic interactions and do not dissociate appreciably under most in vitro conditions. In contrast, the dimer-dimer interface (α1β2) contains polar residues and water and readily dissociates in vitro and in vivo. To maintain symmetry within the tetramer at each binding step, T/R model(s) have posited that ligand binding to one subunit is communicated to the other three subunits via weakening the dimer-dimer interface. The weakened interface promotes a shift in quaternary T to R equilibrium, i.e. from low to high affinity.

Recent discoveries of multiple quaternary crystal forms of T and R (as reviewed in Ref. 10) have been confirmed for ligated Hb in solution (as reviewed in Ref. 11). The presence of multiple quaternary structures permits the original two-state model to be expanded to include classes of T and R states (consistent with the symmetry rule model (12) and the global allostery model (13)). However, within each variant of the quaternary structure, the dimers still are implicitly assumed to remain synchronized through the strength of the dimer-dimer contacts. This can be true even when intradimer coupling is considered (14), in that intradimer cooperativity can be postulated to occur simultaneously in both dimers within the tetramer.

Asymmetric Ligation

In the course of binding O2 ligands to the four heme sites of human Hb, as many as eight unique partially ligated intermediates, or microstates, are gener-

![FIGURE 1. Oxygen binding, micro- and macroconstants. O2 saturation isotherm for binding to the four subunits within the native Hb tetramer (crystal structures obtained under low salt conditions (26, 27)), measured at pH 7.4, 0.1 mM Tris, 0.18 mM NaCl, 1 mM EDTA, 21.5 °C (28) are shown. The experimental curve (dotted line) is compared with that calculated from the microstate binding constants, which were evaluated by their thermodynamic linkages with the dimer to tetramer assembly constants (solid line) (16). The microstate constants are illustrated in Fig. 2A. The partially ligated intermediates are positioned along the binding curve in illustrative positions only.](image-url)
As a result of the high cooperativity with which Hb binds O₂, the lability of the bound O₂, and the fact that Hb binds and releases O₂ close to equilibrium in vivo, the relative abundance of any one microstate intermediate is very low. Fortunately, the assembly of the Hb tetramer from dimers is thermodynamically linked to oxygenation (15), and this property can be exploited to permit measurement of the binding constants for each microstate species. Results of these measurements have shown that placing one ligand on each dimer is essentially noncooperative under the experimental conditions (see Fig. 1), whereas placing two ligands on a single dimer occurs with positive cooperativity. A, microstate constants predicted by a simple two-state model in which both dimers within the tetramer respond equivalently to ligation.

Throughout the 1990s, the application of thermodynamic linkage analysis was pursued employing a range of non-labile heme site analogs and solution conditions (reviewed in Ref. 1). This research demonstrated that the binding constant for the asymmetric doubly ligated species was indeed unique, and this was found to be independent of the type of heme site analog employed or the specific conditions of pH, temperature, chloride concentration, or water concentration. This work extended the experimental basis for a microstate model of cooperativity proposed in 1992, the symmetry rule model, in which the Hb tetramer functions as a dimer of cooperative dimers (reviewed in Ref. 12). In this model, the transition from T to R occurred only when each dimer had bound at least one ligand. Thus, the symmetry rule model permitted the dimers to function asynchronously within the T structure.

The unique binding constant of this microstate species relative to the other intermediates in the Zn/FeO₂ analog system has recently been confirmed by stopped-flow experiments (16) and by an extensive analysis of CO recombination kinetics in normal Hb (17, 18). Thus, the respective roles of the ligation intermediates were found to be incompatible with the simple two-state model. The seemingly singular importance of this particular asymmetrically ligated microstate has now been largely supplanted by recent findings with asym-
metrically modified Hb (19), which show characteristic heme site-residue coupling modes to be manifested not just in a single microstate but throughout the binding cascade.

Asymmetric Mutation

The structural basis of Hb cooperativity originally developed by Perutz was based on the loss of noncovalent bonds (i.e. salt bridges) in the dimer-dimer interface upon ligation of any one of the heme sites. One of the most convincing arguments that binding O₂ results in weakening the dimer-dimer interface contacts in the deoxy-T structure was the observation that removal of a dimer-dimer hydrogen bond by mutation or chemical modification resulted in an increase in oxygen affinity and a decrease in cooperativity (as reviewed in Ref. 19). The removal of the C-terminal Arg of the α-subunits, for example, results in a 30-fold enhancement of the first O₂ binding constant (Fig. 3). According to the T/R model, the increase in O₂ affinity is due to a weakened dimer-dimer interface in T, permitting the tetramer to spend more time in the high affinity R conformation. The experimental result, in different formats, has been reproduced in numerous studies and is certainly robust. However, the experiment has always (with very few exceptions) been conducted as a symmetric modification, with the mutation on both dimers, i.e. in both α- or both β-subunits.

According to the T/R model, modification of only a single α-subunit must also result in enhanced O₂ affinity, although to a lesser degree than the double modification. However, most importantly, the enhanced affinity must be observed uniformly, in the wild type dimer as well as the modified dimer. When the same classic modification was applied to only one of the two α-subunits, the asymmetrically modified hybrid tetramers revealed a clear asynchronous response to heme site ligation (Fig. 4). The wild type deoxydimer was found to exhibit the same low affinity found in the wild type tetramer, while the dimer containing the modified α- or β-subunit displayed the same affinity as the classic doubly modified tetramer. Thus, the observed effect of mutation, which had always been interpreted as an effect on quaternary dimer-dimer coupling, was actually reporting on tertiary or intradimer coupling. The asymmetric response to single subunit mutations has been observed in all mutations or modifications studied to date, at residues located throughout the dimer-dimer interface (19).

The Synchrony Assumption

Models that incorporate multiple quaternary states and tertiary changes nested within quaternary changes, including the microstate-based symmetry rule model of 1992, do not adequately address asynchronous dimer function. Asymmetric structural properties of the intermediates, which underlie the mechanism of Hb cooperativity cannot, unfortunately, be extrapolated with confidence from crystal structures or assignments based on spectroscopic properties of the symmetric deoxy and oxy end states. It is time to identify and move beyond the ubiquitous assumption of dimer synchrony upon which experimental design and analysis continues to be based, even in modern studies of Hb cooperativity.

The utility of concepts such as T and R has been greatly diminished as multiple T and R states, to which a wide range of functional and structural properties have been ascribed, are increasingly required to satisfy the analysis of global experimental data (i.e. data based only on averages of the microstate constants of the system) (20). These wide ranging states of T and R lack the specificity needed for an unambiguous, testable model that would be applicable to characterization of the microstate cooperativity in Hb. A greater emphasis on sequential mechanisms (2), as incorporated into the symmetry rule model (9), is likely to be more relevant to analysis of the microstate intermediates.

The Functional Dimer

The results of the work on partially ligated intermediates of Hb conducted over the last 2 decades form a functional picture of the mechanism of Hb cooperativity that is substantially influenced by intradimer coupling. The two αβ dimers within the tetramer exhibit autonomous functions while remaining energetically coupled with one another as well. Even the long held dogma of an inert intradimer interface (21) has come under question, based on the NMR work of Russu (22) and Ho (23), the vibrational spectroscopic analysis of Spiro (14), the crystallographic work of Arnone (10), and the computational studies of Rikfink (24) and of Ten Eyck (25). Key energetic questions also remain to be answered, particularly with respect to the intradimer versus dimer-dimer modulation of cooperativity by heterotropic effectors (particularly the Bohr protons and organic phosphates). Obtaining these answers will require new approaches to structural and evolutionary characterization of this classic molecule and a fresh outlook toward the interpretation of both existing and new data bases.

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