Chain Nonequivalence in Binding of Nitric Oxide to Hemoglobin*

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(Received for publication, February 2, 1978)

In the presence of inositol hexaphosphate (IHP), the ESR spectrum of each of the NO derivatives of the human HbA/cat HbA hybrid hemoglobins, α2β2′ and α2β2, resembles the spectrum of the parent hemoglobin that provides the α chain, particularly with respect to the intensity of the triplet hyperfine structure at $g = 2.009$. This observation gives support to the suggestion (Honry, Y., and Banerjee, R. (1973) J. Mol. Biol. 73, 469-482; Perutz, M. F., Kilmartin, J. W., Nagai, K., Szabo, A., and Simon, S. F. (1976) Biochemistry 15, 378-387) that the hyperfine structure is an α chain specific spin label within T state NO-hemoglobin.

The question of chain nonequivalence in the equilibrium binding of NO to T state hemoglobin was investigated using relative ESR resonance intensities at $g = 2.009$ and $g = 1.985$ to estimate the fraction of NO bound to the α-hemes. Partially saturated solutions of nitric oxide hemoglobin prepared by mixing solutions of NO and deoxyhemoglobin with IHP or of NO-hemoglobin and deoxyhemoglobin with IHP were allowed to equilibrate at room temperature prior to analysis by ESR spectroscopy at 77 K. The hyperfine structure at $g = 2.009$ was much more prominent in the spectra of solutions at low than at high degrees of saturation, indicating that a greater fraction of the NO was bound to the α than to the β chains at saturation. A time-dependent increase in the ESR hyperfine structure intensity occurs when a solution of saturated NO-hemoglobin is mixed with a solution of deoxyhemoglobin plus IHP and is allowed to equilibrate. This suggests that NO dissociates and then recombines preferentially with available α chain heme sites in T state hemoglobin. Half-times for attainment of equilibrium under the conditions of these experiments were of the order of 5 min at room temperature.

Hemoglobin liganded with nitric oxide can be induced to undergo an R to a T type of conformational transition in the absence of any change in the state of its ligation with the gas. When NO-hemoglobin interacts with the allosteric effector inositol hexaphosphate, changes are observed in optical properties (1-4) and in reactivity of sulfhydril groups (3-5) similar to those that occur in the R → T quaternary transition of oxyhemoglobin to deoxyhemoglobin. In addition, the ESR spectrum of NO-hemoglobin plus IHP* shows features that differ from that of NO-hemoglobin without IHP, the most prominent of which is the presence of a strong three-line hyperfine structure centered at $g = 2.009$ (6). The development of this hyperfine structure occurs only under conditions that tend to favor transition to T state NO-hemoglobin (3-5) and is apparently diagnostic of such a state.

Current interpretation of the change in the ESR spectrum of NO-hemoglobin brought about by its interaction with IHP is based on the observations of Wayland and Olson (7) and of Kon (8) on the differences in spectra of model synthetic pentacoordinated NO-heme complexes. The hexacoordinated NO-heme complex gives an ESR spectrum with resonances similar to those of NO-hemoglobin without IHP whereas the penta coordinated NO-heme complex gives a spectrum with an intense three-line hyperfine structure centered at $g = 2.009$ that is also present in the spectrum of NO-hemoglobin with IHP. Kon (8) and as well as Szabo and Perutz (9) have concluded that the IHP produces distortion or rupture of some of the heme-imidazole bonds within NO-hemoglobin to give them a pentacoordinate character; i.e. the spectrum of NO-hemoglobin with IHP represents a superposition of the spectra of penta- and hexacoordinated NO-hemes. By comparing the infrared spectra of NO-hemoglobin plus or minus IHP with those of synthetic penta- and hexacoordinated NO-hemes, Maxwell and Caughey (10) confirmed this interpretation and concluded further that about one-half of the NO-hemes in the NO-hemoglobin IHP complex shows pentacoordinate and the other half a hexacoordinate character.

That the ESR triplet hyperfine structure might be attributed to the α subunit within NO-hemoglobin was suggested earlier by Henry and Banerjee (11) from their analysis of the ESR spectra of isolated αNO and βNO subunits as well as NO-hemoglobin hybrids in which one type of chain was bound to NO and the partner chain in a variety of other ligation or valency states. More recently, Nagai et al. (12) confirmed these observations and showed further that the intensity of the IHP-induced ESR hyperfine structure is proportional to the fraction of the NO that is bound to the α subunit in T state NO-hemoglobin. It appears, therefore, that the ESR hyperfine structure is an α chain specific label which can be used to ascertain whether or not chain preference exists in the equilibrium binding of NO in T state NO-hemoglobin. Henry and Cassoly (13) examined the question of chain nonequivalence and concluded from ESR studies as well as indirect and stopped flow kinetic analyses that the apparent rate of NO binding was faster to the α subunit than to the β subunit. However, subsequent stopped flow kinetic analysis by Cassoly and Gibson (14) and ESR analysis coupled to a rapid mixing and freezing technique by Hille et al. (15) gave no evidence for the preferential binding of NO to either chain, at least initially. The latter workers also found that the ESR triplet hyperfine structure at $g = 2.009$, characteristic of T state NO-hemoglobin, develops only during a slower subsequent step. Since Moore and Gibson (16) found that the dissociation constant of NO from the T state hemoglobin is relatively large.

* This work was supported by Grant AM 16770 (P.T.), CA 20737 (W.E.A.), and RR-01009 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: IHP, inositol hexaphosphate; bis-Tris, 2,2'-bis(hydroxymethyl)-2,2'-nitriloethanol.

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HbA can be prepared and isolated in good condition. Their moglobin formation in these solutions even after several hours by mixing different proportions of 0.1 mM deoxyhemoglobin plus 1 μM IHP with saturated NO-hemoglobin solutions and allowing the reaction to proceed. Partially saturated NO-hemoglobin solutions were prepared by adding IHP in the same buffer. Appropriate amounts of deoxygenated buffer were added to the solution so that the final concentrations of hemoglobin and IHP were 0.1 mM each. The mixture was then added to the solution so that the final concentrations of hemoglobin and IHP were 0.1 mM each. The mixture was then added to the solution to ensure complete reaction. After mixing, aliquots were transferred to quartz tubes and frozen immediately in liquid nitrogen. Spectra were obtained at 77 K using a Varian E-9 spectrometer as described previously.

**RESULTS**

Hybrid hemoglobins derived from human HbA and cat HbA can be prepared and isolated in good condition. Their visible spectra are indistinguishable from the spectra of the parent hemoglobins and they are as sensitive as the parent hemoglobins to allosteric control by organic phosphates (not shown). Evidence to support the conclusion that the ESR triplet hyperfine structure can be attributed to the α2β2 subunit in T state NO-hemoglobin is provided by the spectra shown in Fig. 1. As reported in previous work (24), the ESR spectrum of cat NO-HbA with IHP shows a hyperfine structure at g = 2.009 that is only about one-half as intense as that found in the spectrum of human NO-HbA with IHP. The corresponding spectra of each of the two hybrid NO-hemoglobins (α2humanβ2cat and α2catβ2human) closely resembles that of the parent hemoglobin from which the α chains were derived; the intensity of the hyperfine structure in the spectrum of α2humanβ2cat is similar to that of human HbA and is about twice that of α2catβ2human and of cat HbA.

Fig. 2 shows spectra that are obtained when increasing increments of NO are added to solutions of deoxyhemoglobin plus IHP and the mixtures allowed to equilibrate at room temperature before freezing for ESR analysis. Clearly, a progressive change in the spectrum of NO-hemoglobin as a function of its saturation with NO is observed; the hyperfine structure being most prominent at low degrees of saturation and becoming less so with increasing saturation. At very low degrees of saturation, the spectra of NO-hemoglobin with IHP closely resemble those of pure pentacoordinate NO-heme complexes (7-9) suggesting that most of the NO is pentacoordinated in NO-hemoglobin as well. Furthermore, since the hyperfine structure associated with pentacoordinated NO-hemes is apparently an α chain specific spin label, this indicates that under these conditions, most of the NO is bound to the α subunits within partially saturated NO-hemoglobin.
FIG. 2. The ESR spectra at 77 K of human NO-hemoglobin plus IHP as a function of increasing saturation with NO. The percentage saturation from top to bottom was 25%, 50%, 75%, and 100% as estimated from the visible spectra. Solutions were frozen in liquid nitrogen after 5 or more min of equilibration at room temperature. Spectrometer settings were the same as in Fig. 2 except that the gain was 4 X 10^4.

With increasing saturation, the change in spectrum involves a progressive decline in hyperfine structure intensity at \( g = 2.009 \) with respect to an increase in the peak at \( g = 1.987 \) indicating increasing contribution of resonances from hexacoordinate \( \beta^{5+} \) hemes superimposed upon those from pentacoordinate \( \alpha^{5+} \) hemes. At saturation, the spectrum characteristic of fully saturated T state NO-hemoglobin comprised of contributions from equal proportions of both types of hemes (9) is observed. Therefore, these data support the conclusion that chain nonequivalence exists in the equilibrium binding of NO within partially saturated T state NO-hemoglobin; under these conditions there is preferential binding of NO to the \( \alpha \) subunits.

The same conclusion comes from analysis of the ESR spectra that are obtained when partially saturated NO-hemoglobin solutions are prepared by equilibrating mixtures of fully liganded NO-hemoglobin and deoxyhemoglobin solutions in the presence of IHP. If NO dissociates from NO-hemoglobin and is redistributed among available \( \alpha \) and \( \beta \) subunit sites so that preferential binding occurs at either the \( \alpha \) or \( \beta \) hemes, a time-dependent change in the ESR spectrum of the mixture reflecting the shift in proportion of pentacoordinate \( \alpha \) and hexacoordinate \( \beta \) NO-hemes might be expected. The results shown in Fig. 3, A and B indicate that this indeed occurs. Within 2 min after mixing solutions containing 90% deoxyHb/10% NOHb (10% saturation) or 50% deoxyHb/50% NOHb (50% saturation), changes in the ESR spectra indicate a shift towards a relative increase in the fraction of pentacoordinated NO-hemes. Equilibrium redistribution at room temperature of NO was attained at about 10 min after mixing as indicated by the absence of further changes in the spectrum.

FIG. 3. ESR spectra at 77 K of mixtures of NO-hemoglobin and deoxyhemoglobin plus IHP allowed to equilibrate at room temperature for various time periods before freezing in liquid nitrogen for analysis. A, spectra for a mixture containing 90% deoxyhemoglobin and 10% NO-hemoglobin initially and B, spectra for a mixture containing equal amounts of deoxy- and NO-hemoglobin initially. Spectrometer settings were the same as in Fig. 2 except that the gain was \( 2 \times 10^4 \) in A and \( 4 \times 10^4 \) in B.
Fig. 4. The relative concentrations of \( \alpha^{NO} \) and \( \beta^{NO} \) chains as a function of saturation of hemoglobin with NO in the presence of IHP. Calculations were made from resonances in the ESR spectra at \( g = 1.987 \) and \( g = 2.009 \) at various degrees of saturation. \( Y_{\alpha} \) and \( Y_{\beta} \) give the percentage of chains that are liganded \( \alpha \) and liganded \( \beta \) chains respectively (25).

beyond this time. Under the same conditions, no change occurred in the ESR spectrum of a saturated NO-hemoglobin solution. Spectra of solutions allowed to stand at room temperature for 5, 10, 30, and 60 min were typical of T state NO-hemoglobin with IHP and were indistinguishable from each other. For the 90% deoxyHb/10% NOHb mixture there is a relatively large excess of unoccupied NO-binding sites, practically all of the NO-hemes appear to be pentacoordinated at equilibrium; there is only a small peak at \( g = 1.987 \), and the hyperfine structure at \( g = 2.009 \) is very intense. The spectrum of a 50% deoxyHb/50% NOHb mixture (50% saturation) also showed increased hyperfine structure intensity with equilibration, indicating a shift in the proportion of pentacoordinated and hexacoordinated NO-hemes relative to that found in fully saturated NO-hemoglobin. In this case, however, a slightly larger fraction of hexacoordinated NO-heme remains as indicated by the more definite peak at \( g = 1.987 \). The spectrum is also identical with that observed (Fig. 2) when the 50% saturated solution was prepared by direct addition of NO to deoxyhemoglobin. Thus, these results also support the conclusion that NO is bound preferentially to the \( \alpha \) subunit within partially saturated T state NO-hemoglobin solutions.

Assuming that the resonance intensity at \( g = 2.009 \) is roughly proportional to the concentration of \( \alpha^{NO} \) heme and the intensity at \( g = 1.987 \) proportional to the concentration of \( \beta^{NO} \) heme, such as those shown in Figs. 2 and 3 can be used to estimate the fraction of \( \alpha(\overline{Y}_{\alpha}) \) and \( \beta(\overline{Y}_{\beta}) \) chains liganded with NO at various degrees of saturation (\( Y \)). The fractions of liganded \( \alpha \) and \( \beta \) chains were calculated using the relationship

\[
I_{\alpha^{NO}} = \frac{K[\beta^{NO}]}{K[\alpha^{NO}]}
\]

and the constant \( K/K' \), determined at 100% saturation where \( [\alpha^{NO}] - [\beta^{NO}] \). \( \overline{Y}_{\alpha} \) and \( \overline{Y}_{\beta} \) are plotted as a function of fractional saturation of the total hemes (25) in Fig. 4. It can be seen that at 50% saturation the affinity of the \( \alpha \) chain for NO is about 4 times greater than that of the \( \beta \) chain, corresponding to approximately 80% of the \( \alpha \) chains and 20% of the \( \beta \) chains being liganded. At lower degrees of saturation there is nearly exclusive binding of NO to the \( \alpha \) chains, the results being similar to the binding of oxygen to hemoglobin in the presence of IHP as observed by NMMH (26).

**DISCUSSION**

The present work demonstrates that there is chain non-equivalence in the equilibrium binding of NO to hemoglobin in the presence of IHP. Since Hille et al. (15) and Moore and Gibson (16) found, however, that the initial rate of binding of NO to the \( \alpha \) and \( \beta \) subunits of T state hemoglobin is identical, it must be concluded that following the initial binding of NO, subsequent changes result in redistribution of NO to favor its binding to the \( \alpha \) subunits at equilibrium. As evidence for this, Hille et al. (15) found that the ESR triplet hyperfine structure at \( g = 2.009 \) appeared relatively slowly following the initial binding of NO. There was a progressive increase in the ESR triplet hyperfine structure intensity with increasing incubation times of up to 30 s. It appears, that then, that following a rapid “on” rate for initial binding of NO, a slower “off” rate of release from the \( \alpha \) relative to the \( \beta \) subunit probably accompanies a conformational change in the structure of T state NO hemoglobin. The experiments using mixtures of NO-hemoglobin and deoxyhemoglobin in the presence of IHP confirm a slow re-equilibration of NO among available heme sites that ultimately results in its preferential binding to the \( \alpha \) chain hemes. The change in ESR spectra indicating the dissociation of NO from saturated NO-hemoglobin and its preferential binding to the \( \alpha \) chain hemes occurred with a half-time of about 5 min at room temperature. The rate constant for the re-equilibration, about \( 1 \times 10^{-3} \) s\(^{-1} \) at pH 7 and room temperature, is similar to the value of \( 2 \times 10^{-3} \) s\(^{-1} \) determined by Moore and Gibson (16) for the dissociation constant of NO from T state NO-hemoglobin under similar conditions. Thus, the difference in affinities of the chains for NO at equilibrium can be explained on the basis of a larger dissociation constant for the \( \beta \) chain relative to a NO-heme.
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