CHAIN NON-EQUIVALENCE IN NITRIC OXIDE BINDING TO HEMOGLOBIN

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Summary : The ratio of the apparent rates of ligand binding to the α and β subunits of human hemoglobin on mixing with non-saturating amounts of nitric oxide has been measured by two independent methods. Electron spin resonance measurements permit direct determination of the ratio of the amounts of the respective chains bound by NO. In stopped-flow kinetics measurements, use was made of the known difference in the kinetic constants of α and β chains in hemoglobin in the reaction with n-butyl isocyanide. Both methods concur in indicating that the apparent association rate constant of NO is greater for the α than for the β chain.

Introduction

In the study of the mechanism of heme-heme interaction occuring upon ligand binding to deoxyhemoglobin, chain non-equivalence has recently attracted much attention. Well-defined structural differences between the two types of chains, mostly located in the heme pocket, were shown by Perutz (1) both in the deoxy and the oxy forms. By use of nuclear magnetic resonance Ho and co-workers have studied deoxyhemoglobin (2) and carboxyhemoglobin (3) and have demonstrated in both cases the non-equivalence of the two types of chains. Studies of various mutant hemoglobins have led to tentative assignment of the resonances to a specific chain and to the location of the structural differences in the heme vicinity. More recently by use of electron spin resonance unequivocal resonance assignment to the two types of chains have been given for nitrosylhemoglobin (4).

As for the functional non-equivalence between the chains, Perutz (1) predicted from his atomic model of deoxyhemoglobin that ligands should first bind to the α hemes. Experimental search for chain differences, in a large variety of conditions and with different methods, led to different results depending on the ligand used (5 - 8). Some difficulties arise in the identification of the subunits with respect to their binding properties.

A divergence between authors in case of n-butyl isocyanide has already appeared (6, 7, 9).

Taking advantage of the direct and unequivocal structural non-equivalence of the α and β chains in nitrosylhemoglobin as demonstrated by E S R * (4), and of the slow rates of dissociation of nitric oxide from nitrosyl heme complexes, we have studied the association reaction of nitric oxide to deoxyhemoglobin. Furthermore we have studied by the stopped-flow method the association and dissociation reactions of B I C * with hemoglobin partially ligated with nitric oxide. Good agreement was obtained between the two independent methods, showing that nitric oxide binds first to the α hemes.

Materials and methods

Materials : Human hemoglobin was obtained from fresh hemolysates of erythrocytes washed three times with 0.9 % NaCl. The stroma was eliminated with toluene. All experiments were performed in 0.1 M phosphate buffer pH 6.5. n-Butyl isocyanide solutions were made according to Olson and Gibson (6). Stocks of NO and CO solutions were prepared by equilibrating buffer with one atmosphere of gas. Deoxygenated buffer was obtained by bubbling pure nitrogen.

Partial saturation of deoxyhemoglobin with NO : 400 to 800 µM of deoxy-hemoglobin was mixed with deoxygenated buffer containing a known amount of NO. The mixing is made volume per volume at 20° by means of a T mixing jet in the absence of oxygen. Dithionite (10 % of the total heme) was added to the protein side in order to prevent any possible oxidation. Controls in which the speed of mixing was varied by a factor of 100 did not result in significant differences in the results. The fraction of NO bound site ($\bar{Y}_{NO}$) was determined spectrophotometrically on the samples further saturated with CO. All the experiments were performed less than 30 minutes following the mixing of NO with hemoglobin. The mixtures were chilled to 0°C immediatly after their preparation in order to prevent NO dissociation.

E S R measurements : The E S R spectra were recorded at 77°K with a Varian E-3 X band spectrometer with 100 kHz field modulation. The microwave power

* Abbreviations used :
E S R : electron spin resonance
B I C : n-butyl isocyanide

was attenuated to 25 mW and the field modulation was 1 gauss. As was established previously (4), the E S R spectrum of HbNO at 77°K is within experimental error the same as would result from a sum of the isolated αNO and βNO subunits. The E S R spectra of the artificial nitric oxide hybrids $\alpha_2NO\beta_2CO$ and $\alpha_2CO\beta_2NO$ have been shown to be identical respectively to those of the isolated αNO and βNO chains. This indicates that the NO bound chains preserve in tetrameric molecules their respective spectral characteristics. The E S R spectra were analysed as a linear combination of the spectra of αNO and βNO to obtain the ratio $\bar{Y}_{\alpha NO} = \frac{\alpha NO}{\alpha NO + \beta NO}$. In this analysis we used the ratio $A'g/A'g_i$ of the absorption derivatives A'g at a given g-value, actually g = 2.077 and g = 2.038, to that $A'g_i$ at the isosbestic g-value, g_i = 2.064, where the three species αNO, βNO and HbNO have the same absorption derivative, for a given frequency, field modulation and detector gain. The ratio $\bar{Y}_{\alpha NO}$ was determined on hemoglobin partially ligated with nitric oxide; remaining free sites were saturated with carbon monoxide in order that the resulting hemoglobin be fully saturated and to exclude the existence of intermediates of the form αNOβdeoxy which present slightly altered spectra (4).

Kinetic experiments : A Durrum-Gibson stopped-flow apparatus with a 2 cm length path cell was used. Two sets of experiments were performed. The binding of B I C to solutions of deoxyhemoglobin partially saturated with NO, and the dissociation reaction of B I C from the same sample saturated with B I C. In the last experiment the displacing ligand was CO in large excess. For hemoglobin, both reactions are strongly biphasic. This heterogeneity reflects, as shown by Olson and Gibson (6), an inequivalence in the reactivity of α and β chains, the β chain being the most reactive. In the present experiments, in the possible case where NO is bound preferentially to the α or to the β subunit of hemoglobin, this will be reflected in the B I C kinetic experiments by a decrease in the fraction of one of the kinetic phases as compared to hemoglobin. It is then possible to determine the distribution of NO between the α and β sites. The calculations have been made (for reasons given in the Discussion section) only on the B I C dissociation reaction.

The fraction $\bar{Y}_{\alpha NO} = \frac{\alpha NO}{\alpha NO + \beta NO}$ is equal to $\frac{F_S - F'_S (1 - \bar{Y}_{NO})}{\bar{Y}_{NO}}$, where $\bar{Y}_{NO}$ is the fraction of hemoglobin sites bound to NO, F_S and F'_S are the fractions of the slow kinetic phase measured, respectively, in the dissociation reaction of B I C from B I C - hemoglobin and from hemoglobin partially saturated with NO and subsequently combined to B I C. F_S and F'_S are obtained by

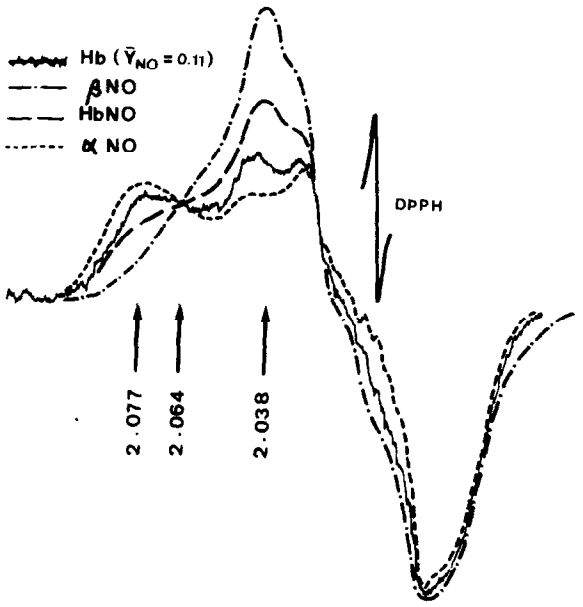

Figure 1. E S R spectrum at 77°K of hemoglobin partially ligated with nitric oxide ($\bar{Y}_{NO}$ = 0.11) as compared to the spectra of αNO, βNO and HbNO computed for the same nitrosyl-heme concentration (0.026 mM) and for identical apparatus settings. Conditions were 0.1 M phosphate buffer, pH 6.5 DPPH, 1,1-diphenyl-2-picryl-hydrazyl stable radical.

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extrapolation of the slow kinetic phase to time zero. This simple procedure is justified by the 10 fold difference in the kinetic rates of the two phases of the reaction (6).

Results and discussion

The remarkably high affinity of NO for Hb is chiefly due to the slow rate of dissociation of NO from HbNO. The half-time for dissociation of the NO molecule from $Hb_4(NO)_4$ at 19°C and pH 6.0 is about three hours (10). It was also found to be very large for the nitric oxide hybrids (4). Therefore, in the present experiments where the E S R and stopped-flow studies were made quickly after the mixing of deoxy Hb and NO, the uneven distribution of NO between the two types of chains reflects differences in the association rate constants on the chains.

As shown in Figure 1, when nitric oxide is mixed with deoxyhemoglobin in non-saturating amount the resulting E S R spectrum is the sum of the spectra of much higher amounts of αNO sites than of βNO. In the example shown ($\bar{Y}_{NO}$ = 0.11) the relative amounts are 0.8 for αNO and 0.2 for βNO. Figure 3 shows the dependence of $\bar{Y}_{\alpha NO}$ on $\bar{Y}_{NO}$ and indicates a preferential

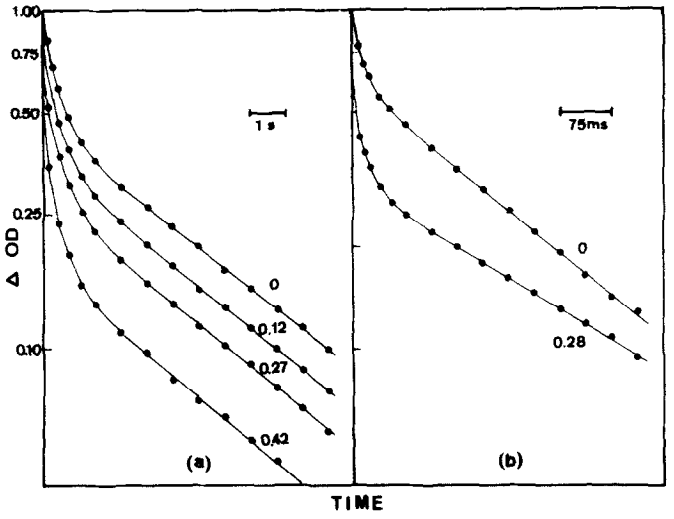

Figure 2. Time course for the reactions of dissociation and association of n-butyl isocyanide to hemoglobin and hemoglobin partially saturated with NO. The data are normalized to a total absorbance change of 1 for hemoglobin. All reactions were performed at 20°C in 0.1 M Phosphate buffer, pH 6.5. The values of $\overline{Y}_{NO}$ are given on the figure.

a) Dissociation reaction : conditions were 436 nm, 0.005 mM heme, 1.25 mM B I C and 0.46 mM CO (after mixing).

b) Association reaction : conditions were 444 nm, 0.020 mM heme and 2.3 mM B I C (after mixing). The calculated fractions of the fast kinetic phase are respectively 0.40 and 0.52 for $\overline{Y}_{NO}$ = 0 and 0.28.

association of NO to the α chains. The preferential binding to α is also indicated in the E S R spectrum obtained when no additional CO was mixed to hemoglobin partially ligated with NO; the hyperfine structure around g = 2.00 is altered in the same way (4) as in the artificial hybrid $\alpha_2^{NO}\beta_2$.

Similar results derive from kinetic experiments. Figure 2a shows the time course of the dissociation reaction of B I C from hemoglobin and hemoglobin partially saturated with NO. The fraction of the fast kinetic phase increases with the amount of NO bound. From Olson and Gibson (6), the fast phase in the B I C dissociation reaction of hemoglobin is due to the β chain. Its increase in the samples of hemoglobin partially saturated with NO indicates that NO is preferentially bound to the α chains. The computed values of $\overline{Y}_{\alpha NO}$ are plotted in Figure 3 together with those obtained from the E S R measurements.

The B I C association experiments shown in Figure 2b are in qualitative agreement with these results. When hemoglobin is partially saturated with NO, there is an increase in the fraction of the fast kinetic phase present in its

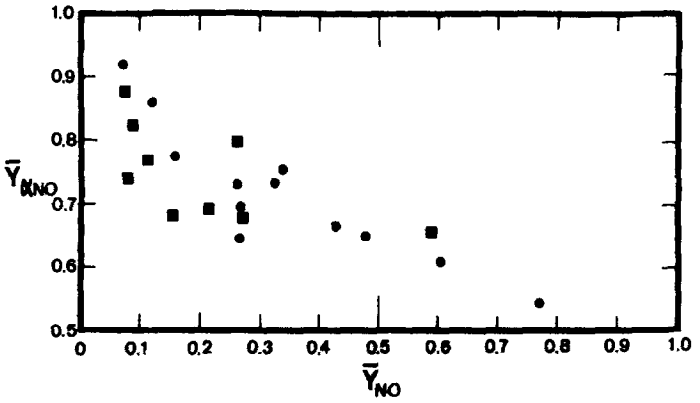

Figure 3. Preferential binding of NO to α chain of hemoglobin. $\bar{Y}_{\alpha NO} = \frac{\alpha NO}{\alpha NO + \beta NO}$ is plotted as a function of the partial saturation $\bar{Y}_{NO}$ of hemoglobin. ■ and ● refer respectively to E S R and stopped-flow measurements.

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reaction with B I C as compared with deoxyhemoglobin. If according to Olson and Gibson (6) the fast phase is due to the β chains, this result confirms the preferential binding of NO to the α subunit. A quantitative study of this reaction has not been attempted because of uncertainties in the kinetic and spectral properties of nitrosyl hemoglobin intermediates, although Cassoly and Gibson have shown that in artificial intermediates (valency hybrids) (11) the difference in the chain reactivity for B I C is maintained.

The data shown in Fig. 3 leave no doubt as to the validity of our main conclusion, i.e. preferential NO binding to the α chain. Nevertheless, it exhibits a large dispersion that could be due to the accumulation of errors inherent in the different measurements involved in the $\bar{Y}_{NO}$ and $\bar{Y}_{\alpha NO}$ assays and also in the mixing procedure. No precise results can be given at this point on the variation of $\bar{Y}_{\alpha NO}$ with respect to $\bar{Y}_{NO}$ that could be interpreted in terms of subsequent association rate constants. Although we have evidence for a preferential dissociation of NO from the β chain of fully saturated nitrosyl hemoglobin (unpublished results), the results do not yield directly the respective equilibrium constants of the subunits in the tetramer. For small values of $\bar{Y}_{NO}$ ($\leqslant 0.10$), the binding of NO to the first site of hemoglobin is predominent over the binding to subsequent sites. If species such as αNOαNOββ, αNOαβNOβ, ααβNOβNO, etc, are neglected, the values found for $\bar{Y}_{\alpha NO}$ correspond to a very high ratio $j'_{1\alpha}/j'_{1\beta}$ of the association rate constants to the first site, lying between four and ten. For the highest values the preceding assumption is less valid, as the second α site is competitive with the first β site, even at the lowest $\bar{Y}_{NO}$ values.

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References

(1) Perutz, M.F., Nature 228, 726 (1970)
(2) Davis, D.G., Lindstrom, T.R., Mock, N.H., Baldassare, J.J., Charache, S., Jones, R.T. and Ho, C., J. Mol. Biol., 60, 101 (1971)
(3) Lindstrom, T.R., Norén, I.B.E., Charache, S., Lehmann, H. and Ho, C., Biochemistry 11, 1677 (1972)
(4) Henry, Y. and Banerjee, R., J. Mol. Biol., 73, 469 (1973)
(5) Gray, R.D. and Gibson, Q.H., J. Biol. Chem., 246, 7168 (1971)
(6) Olson, J.S. and Gibson, Q.H., J. Biol. Chem., 247, 1713 (1972)
(7) Lindstrom, T.R., Olson, J.S., Mock, N.H., Gibson, Q.H. and Ho, C., Biochem. Biophys. Res. Comm., 45, 22 (1971)
(8) Lindstrom, T.R. and Ho, C., Proc. Nat. Acad. Sci. USA 69, 1707 (1972)
(9) Huestis, W.H. and Raftery, M.A., Biochem. Biophys. Res. Comm., 48, 678 (1972)
(10) Gibson, Q.H. and Roughton, F.J.W., J. Physiol., 136, 507 (1957)
(11) Cassoly, R. and Gibson, Q.H., J. Biol. Chem., 247, 7332 (1972)