THE BINDING OF n-BUTYL ISOCYANIDE TO HUMAN HEMOGLOBIN*

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Abstract

The binding of n-butyl isocyanide to hemoglobin has been investigated by ^{19}F-nuclear magnetic resonance spectroscopy. The ^{19}F-nmr spectrum of hemoglobin trifluoroacetonylated at cysteine β93 exhibits chemical shift changes on binding of ligands to the β chains. Comparison of these changes to the fractional change in the visible spectrum, shows that in the presence of diphosphoglyceric acid initial ligands bind preferentially to α chains. In the absence of DPG, ligation of β chains increases linearly with overall fractional ligation, indicating that binding to α and β chains is random under these conditions.

The classical models for ligand binding to hemoglobin treated the α and β chains as equivalent binding sites (1-3). Recently, kinetic (4) and structural (5) evidence has been presented which indicates that the subunits are nonequivalent, ligands being bound in a preferential order. Knowledge of the sequence in which binding occurs is of interest in understanding the molecular basis of the cooperative process, since analysis of cause and effect in the known structural changes is then possible.

Extensive studies on the subunit binding order have been conducted by Gibson and co-workers (4) for several ligands, most notably for n-butyl isocyanide (BIC).* The experimental results of these workers has been interpreted to show that BIC binds preferentially to β chains at low ligand concentrations. A series of studies were conducted in this laboratory on the

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*Abbreviations used are: BIC, n-butylisocyanide; nmr, nuclear magnetic resonance; DPG, diphosphoglyceric acid; Hb, hemoglobin; Hb^{TFA}, trifluoroacetonylated hemoglobin.

carbon monoxide binding process, comparing the overall ligation (observed in the visible spectrum) to the ligation of β chains (observed concurrently from the nmr spectrum of a fluorine label attached to the β chains) (8). The results of these experiments conflicted with the conclusions of Gibson on the order of BIC binding, so concurrent nmr-visible studies of BIC association with Hb were undertaken to determine whether differences exist in the order of binding of different ligands.

MATERIALS AND METHODS

Hemoglobin -- Human hemoglobin was prepared from freshly drawn citrated blood. The packed erythrocytes were washed three times with 0.9% sodium chloride solution, and lysed with distilled water and toluene. The stroma were removed by centrifugation and the supernatant was desalted by gel filtration through a BioGel P-2 column (2.5 x 45 cm) equilibrated with a buffer containing 0.05 M bistris and 0.1 M NaCl at pH 7.0. Hemoglobin solutions were stored at 4° and used within four days of isolation. Trifluoroacetonylated hemoglobin was prepared as described previously (6).

Reagents -- Diphosphoglyceric acid was obtained from Calbiochem as the pentacyclohexylammonium salt, and converted to the acid by being stirred with Dowex 50-X8. Bromotrifluoroacetone was a product of Peninsular Chem-research, Inc. n-Butyl isocyanide was a product of Aldrich Chemical Co.

Methods -- Visible and nmr spectra were obtained concurrently at various stages of ligand binding using an nmr tube with a cuvette fused to the top. This combination cell was made to order by Wilmad Glass Co. Deoxygenated hemoglobin solutions were introduced into the argon-filled tube through a small access hole in the side. The opening was covered with a rubber septum, and aliquots of BIC were injected with a microsyringe. Visible absorbances were determined with a Gilford Model 240 spectrophotometer. ^{19}F-nmr spectra were recorded using a Varian XL-100 spectrometer with fluorine Fourier transform capability. The temperature of the nmr probe was 27°; the

air temperature in the Gilford sample compartment was 26-27°. pH measurements were made using a Radiometer Copenhagen Model 26 pH meter. Magnitudes of nmr peaks were determined by gravimetric integration.

BIC Binding Experiments -- Nmr solutions contained 175 mg of Hb^{TFA} in 5 ml of bistris/NaCl buffer (pH 6.75, 0.05 M bistris, 0.1 M NaCl). A five-fold excess of DPG was introduced as a concentrated solution of the same pH. The hemoglobin solution was deoxygenated by repeated washing with nitrogen in a tonometer, and transferred to the argon-filled sample tube by means of a syringe. Absorbance of the solution was determined at 534 nm before and after each nmr spectrum was recorded. Aliquots of BIC were injected into the tube and the tube was rotated manually to permit mixing. When the absorbance had reached a constant value after each addition, the nmr spectrum was recorded.

RESULTS AND DISCUSSION

The ^{19}F-nmr spectrum of trifluoroacetonylated hemoglobin consists of a single absorption whose chemical shift changes depending on the ligand state of the molecule (6). Liganded Hb^{TFA} derivatives absorb about 480 cps upfield of trifluoroacetic acid, with oxyhemoglobin (Hb^{TFA}-O_2), carboxyhemoglobin (Hb^{TFA}-CO), cyanmethemoglobin (Hb^{TFA}-HCN) and Hb^{TFA}-BIC resonances appearing within the same 20 cps range. The Hb^{TFA}-deoxy resonance appears 50-70 cps upfield of the various liganded hemoglobin peaks. Partially liganded hemoglobin exhibits both the normal deoxy- and liganded hemoglobin peaks, in addition to a small intermediate resonance which appears 10 cps upfield of the liganded peak in BIC spectra.

More specifically, the changes observed in the nmr spectrum of Hb^{TFA} on ligand binding reflect the structure and ligation of the β chains. The ^{19}F labeling group is covalently attached to the β chains at cysteine β93. The magnetic environment of this group is influenced primarily by the equilibrium position of the β-chain carboxy terminal segment (7), which changes on ligand

binding. Experiments with the ligand state hybrid $\alpha_2^{III}CN\beta_2$, in which the α chains are locked in a liganded structure and the β chains can be deoxygenated independently, showed that the chemical shift of the fluorine probe is a function of the ligand state of the β chains (8). Therefore, changes in the relative magnitudes of the nmr peaks as ligand concentration is increased reflect ligand binding to the β chains.

Since the visible spectrum of hemoglobin reflects the ligation state of all subunits equally and independently, the overall fractional ligation (Y) could be compared to the β chain ligation (Y_β), determined from the combined magnitude of the liganded and intermediate peaks. As is shown in the binding curves in Figure 1 and in the plots of Y versus Y_β (Figure 2), in DPG bound hemoglobin no change appeared in the nmr spectrum until the visible spectrum change indicated that 15% of the subunits were liganded. In the absence of DPG (lower binding curve) this lag in Y_β disappeared. Thus it appeared that in the absence of DPG, BIC bound to α and β chains randomly, while DPG produced preferential ligand binding to α chains at low ligand concentrations.

These findings are similar to the results of equivalent experiments using oxygen and carbon monoxide as ligands (Huestis and Raftery, manuscript in preparation). In the presence of DPG, all ligands bound preferentially to α chains at low ligand pressures. Depending on the ligand and pH studied, the disparity in affinities of the two chains was altered or eliminated in the absence of DPG.

These results from modified hemoglobin are relevant to ligand binding in the native protein, since introduction of the fluorine moiety has little effect on allosteric parameters. The Hill coefficient of Hb^{TFA} is 2.5, compared with 2.7 for native hemoglobin; the oxygen affinity is very slightly increased (6), and the Bohr effect and DPG effect are normal (9). Thus it is unlikely that the fluorine label caused the observed depression of the β chain ligand affinity, since an increase in β-chain affinity would be the likely effect of serious modification.

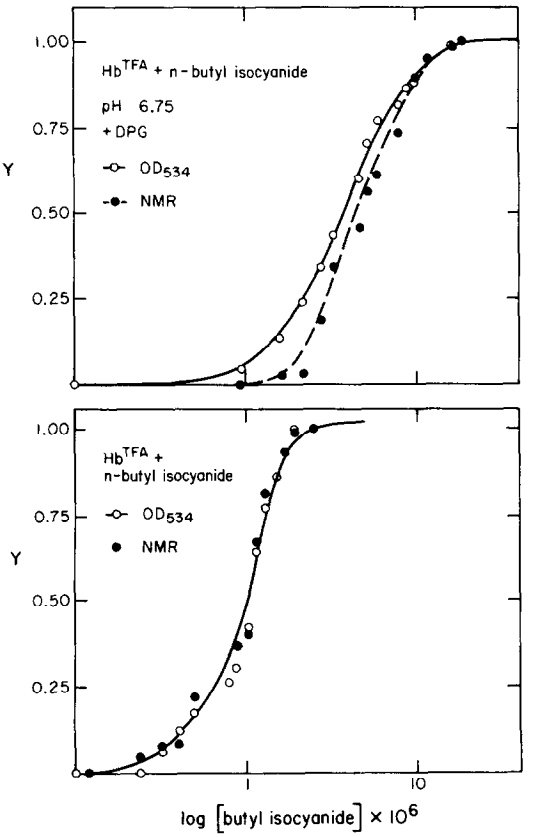

Fig. 1.

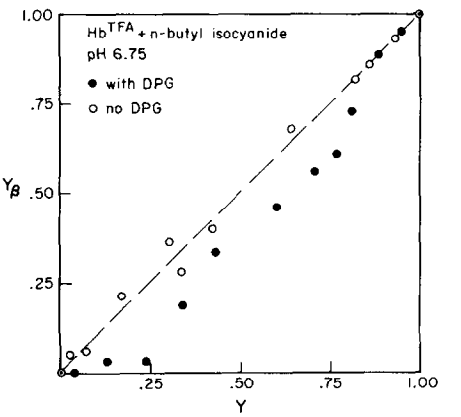

Fig. 2.

Figure I -- n-Butyl isocyanide binding curves observed concurrently in visible and nmr spectra. -o-, Y; -●-, Y_β. A. with DPG, B. without DPG.

Figure II -- Comparison of Y with Y_β for n-butyl isocyanide binding to Hb^{TFA}. ●, with DPG; o, without DPG.

These experiments show that in the early stages of ligand binding, the α chains of hemoglobin exhibit higher ligand affinity than the β chains provided DPG is present. In agreement with the results of Gibson et al. (4), BIC binds to all chains randomly if DPG is absent. However, it appears that the high affinity chains which give rise to an isosbestic point at low ligand pressures in

stopped flow studies of DPG-bound hemoglobin (10) must be α-chains. This conclusion is consistent with the known effect of DPG, which binds to deoxy β chains and stabilizes their low affinity conformation (11). DPG binding must also have an indirect effect on ligand affinity of the α chains, since DPG complexed hemoglobin exhibits decreased overall affinity. However, it is not surprising to find that the β chains are affected to a greater degree.

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