^{31}P-NMR STUDIES OF THE RELEASE OF DIPHOSPHOLYGERIC ACID ON CARBON MONOXIDE BINDING TO HEMOGLOBIN*

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Summary

The binding of 2,3-diphosphoglycerate to human hemoglobin at intermediate stages of ligand saturation has been studied by visible and ^{31}P-nuclear magnetic resonance spectroscopy. Release of diphosphoglycerate was found to lag behind the ligand binding, supporting the previous finding that α chains bind ligands before the β chains, and suggesting that the resulting intermediate molecules may still bind diphosphoglycerate. Comparison of the fraction of free diphosphoglycerate at any stage of fractional ligation to the fraction of molecules containing four, or three and four, bound ligands showed that the dissociation of diphosphoglycerate does not occur completely during any single step of ligand binding.

2,3-Diphosphoglycerate is an allosteric effector which lowers the ligand affinity of mammalian hemoglobin by binding preferentially to the unliganded conformation of the protein (1). The stereochemistry of the deoxyhemoglobin complex has been determined (2), and the effect of complex formation on the Bohr effect and cooperative ligand binding have been studied extensively (3,4). Concerted models for allostery contend

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Abbreviations: DPG, 2,3-diphosphoglycerate; nmr, nuclear magnetic resonance; Y_β, fractional ligand saturation of β chains; Y, fractional ligand saturation of all chains.

that DPG binding contributes to the crucial step in the cooperative process, acting in determining the position of the R-T equilibrium (5,6).

The work reported here was undertaken to determine the stage of ligand binding at which DPG is expelled. The fraction of DPG bound was measured by ^{31}P-nmr spectroscopy for a series of partial carbon monoxide saturation values. The fractional ligand saturation was determined by concurrent measurement of the visible spectrum, for which changes reflect ligand binding to all subunits equally and independently. The fraction of unbound DPG was compared to the overall fraction of carboxyhemoglobin and to the fraction of hemoglobin containing three and four ligand molecules, determined from ^{19}F-nmr (7).

Experimental

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° C and used within four days of preparation.

Reagents: Diphospholyceric acid was obtained from Calbiochem as the penta-cyclohexylammonium salt, and converted to the acid by being stirred with Dowex 50-X8. Bistris was a product of Aldrich Chemical Co.

Methods: Visible and nmr spectra were observed concurrently at various stages of carboxygenation using an nmr tube with a cuvette fused to the top, previously described (7). Visible absorbances were determined with a Gilford Model 240 spectrophotometer. ^{31}P-nmr spectra were recorded using a Varian XL-100 spectrometer with 10 mm sample tubes and ^{31}P-Fourier transform capability. The temperature of the nmr probe and the air temperature in the Gilford sample compartnemt was 32°. pH measure-

ments were made using a Radiometer Copenhagen Model 26 pH meter.

Carbon Monoxide Binding Experiments: Nmr solutions contained 325 mg of hemoglobin in 2.5 ml of bistris/NaCl buffer (pH 6.75 or 7.40, 0.05 M bistris, 0.1 M NaCl). DPG was introduced as a concentrated solution of the appropriate pH, in either a 1:1 molar ratio (pH 6.75) or a 1.5:1 molar ratio to hemoglobin. One mg of EDTA was added to each solution. The sample solution was deoxygenated by washing with nitrogen. Absorbance of the solution at 650 nm was determined before and after each nmr spectrum was recorded. Aliquots of carbon monoxide were introduced by syringe, and the solution was allowed to equilibrate with the gas mixture before the nmr spectrum was recorded.

Results

The ^{31}P-nmr spectra of 2,3-DPG in solution with fully carboxygenated hemoglobin, and bound to deoxyhemoglobin, are shown in Figure 1a and c. Binding to the protein effects chemical shift changes of 0.425 and 0.75 ppm in the two resonances, with no detectable change in the linewidths (2 cps). Partially liganded solutions yielded intermediate chemical shifts, as shown in Figure 1b. The change in chemical shift with increasing ligand pressure was not proportional to the fraction of liganded subunits, as determined from the visible spectrum. As shown in Figure 2 and 3, carbon monoxide binding led the release of DPG by 5-10% throughout most of the ligation range. Since DPG is known to bind to the β chains of deoxyhemoglobin (2), this result indicates that the initial stages of ligand binding do not perturb the required deoxy conformation of the β chains. Studies on binding of ligands to trifluoroacetonylated hemoglobin (7,8) showed that initial ligands bound to α chains, and the belated release of DPG is consistent with the binding sequence thus indicated.

Discussion

From previous ^{19}F-nmr studies of carbon monoxide binding to

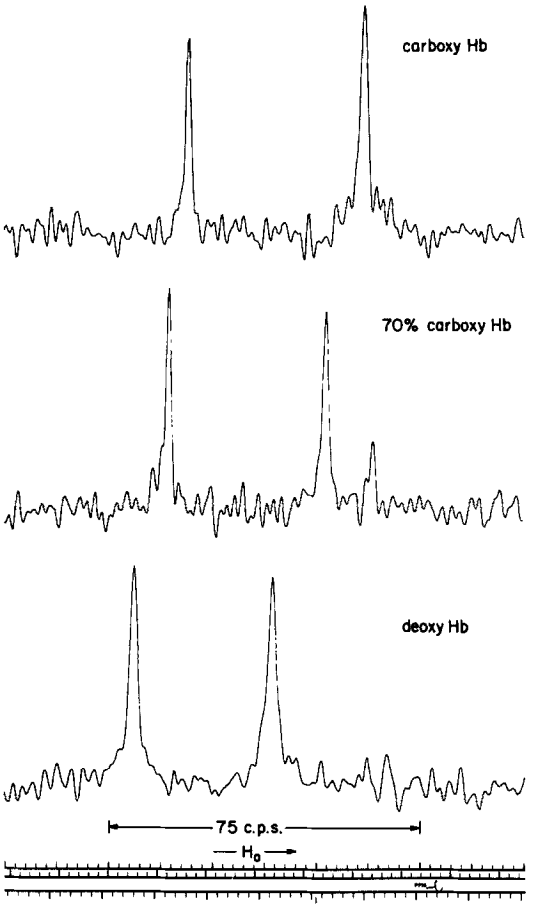

Figure 1: ^{31}P-nmr spectra of DPG at pH 7.4, 3 x 10^{-3} M, + hemoglobin A, 2 x 10^{-3} M. a) deoxyhemoglobin, b) 70% carboxyhemoglobin, c) 100% carboxyhemoglobin.

trifluoroacetonylated hemoglobin, the fractions of species containing none, two, three, and four ligand molecules were measured for fractional saturations between 0 and 1.0 (7). In Figure 4, the release of DPG with ligand binding is compared with the fraction of species containing four ligands (open circles), with the fraction of species containing three and four ligands (crosses), and with the fractional saturation of β chains (filled circles). (The fractional saturation of β chains is determined as the sum of the species containing four ligands and half the species containing three ligands, the latter having

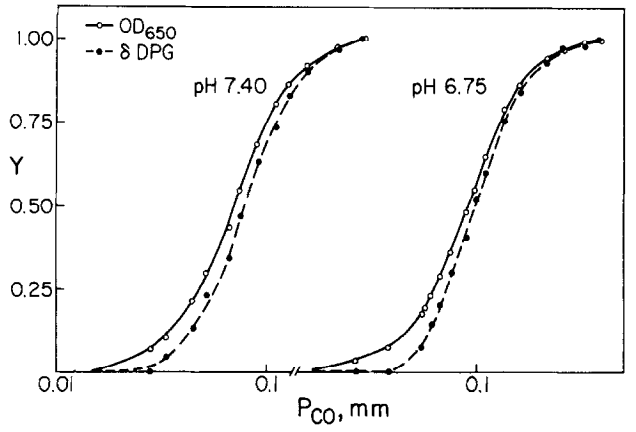

Fig. 2.

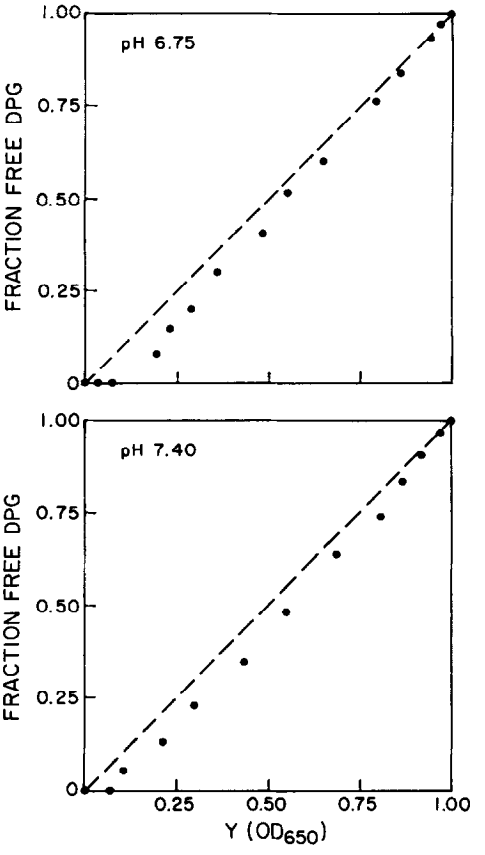

Fig. 3.

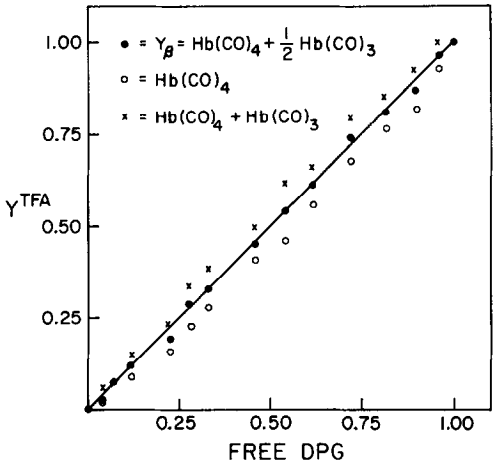

Fig. 4.

Figure 2: Carbon monoxide binding curves obtained concurrently from fractional change in the visible spectrum (-●-) and fractional change in the ^{31}P chemical shift (-o-).

Figure 3: Fraction of liganded subunits vs. fractional change in ^{31}P chemical shift.

Figure 4: o, Fraction of hemoglobin molecules containing four liganded chains vs. fractional ^{31}P chemical shift; X, sum of molecules containing four or three liganded chains vs. fractional ^{31}P chemical shift; ●, fraction of liganded β chains vs. ^{31}P chemical shift.

been identified as $\alpha_2^{CO}\beta^{CO}\beta^{deO_2}$. See reference 7 for this identification and for further discussion.) The release of DPG is exactly proportional to the fraction of liganded β chains, leading the production of $Hb(CO)_4$ and lagging behind the sum of $Hb(CO)_4$ and $Hb(CO)_3$. Thus DPG does not appear to dissociate completely at a single stage of the ligation process; rather, it must bind with intermediate affinity to the partially liganded species (3 liganded). This result is in qualitative agreement with the intermediate binding constants found by Ogata and McConnell for binding of DPG analogs to artificial intermediates ($\alpha_2^{III}CN_\beta^{deO_2}$) (5), but does not support concerted ligand binding models (9) which propose an "all or nothing" switch between R and T forms--two conformations which bind DPG strongly or not at all.

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