Affinity Labeling of the Polyphosphate Binding Site of Hemoglobin†

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ABSTRACT: Pyridoxal phosphate and 2,3-diphosphoglycerate have analogous effects on oxygen binding by hemoglobin and they compete for the same site on the molecule in the deoxy conformation. Other pyridoxal derivatives are inactive, showing that both the aldehyde and the phosphate are essential for a bifunctional interaction with the active site. The Schiff's base of pyridoxal phosphate and deoxyhemoglobin was reduced with sodium borohydride to give a hemoglobin with a permanently lowered oxygen affinity which is uninfluenced by 2,3-diphosphoglycerate. The binding site was identified as the N-terminal valine of the β chain. Reaction of pyridoxal phosphate with oxyhemoglobin, on the other hand, led to labeling of the N terminus of the α chains.

It is now well recognized that organic polyphosphates profoundly decrease the affinity of hemoglobin for oxygen (Benesch and Benesch, 1969). In the red cell, P_2glycerate[1] and ATP thus act as powerful regulators of oxygen release to the tissues (deVerdier *et al.*, 1969; Benesch and Benesch, 1970; Brewer, 1970). We have shown (Benesch *et al.*, 1968a) that this allosteric effect is brought about by specific binding of the organic phosphates to deoxyhemoglobin tetramers. The stoichiometry of the reaction (1 mole of P_2glycerate/hemoglobin tetramer) and the release of P_2glycerate on oxygenation suggested the entrance to the central cavity along the diad axis of symmetry as a possible binding site (Benesch *et al.*, 1968b). Perutz (1970) concluded that P_2glycerate would fit very well into this site in deoxyhemoglobin but would be squeezed out as the size of the cavity decreases in the oxy conformation.

Since the interaction of P_2glycerate and related molecules with the protein is electrostatic in nature, unequivocal location of the binding site by direct chemical methods is not feasible. A solution to this problem presented itself with the discovery that pyridoxal-P has a very similar effect on ligand binding by hemoglobin as the organic phosphates of the red cell. Pyridoxal-P is a bifunctional organic phosphate which has important cofactor activity for many enzymes (Fasella, 1967) where it is bound to the ϵ-amino groups of lysine residues. Reaction of pyridoxal-P with proteins usually involves the reversible formation of a Schiff's base (imine) with amino groups. The imine can be reduced with sodium borohydride to a stable secondary amine, so that the amino acid residue which binds pyridoxal-P can be identified by classical methods of peptide analysis (Fischer *et al.*, 1958). Thus, if pyridoxal-P and P_2glycerate react at the same site on hemoglobin, pyridoxal-P provides a convenient affinity label for this active site.

Materials and Methods

Preparation of Hemoglobin. Hemoglobin was prepared from freshly drawn blood, kindly donated by Dr. Arthur Rowe of the New York Blood Center. For the earlier experiments the method of purification described previously (Benesch *et al.*, 1968a) was used, but for the bulk of the work the following procedure was employed.

The plasma was removed after 5-min centrifugation at 1.100*g*. The red cells were washed and the white cells removed by suspension in cold 0.9% NaCl and centrifugation at 120*g* for 5 min. This was repeated three times. The red cells were then packed by centrifugation at 1.100*g* for 5 min. The cells were lysed by the addition of two volumes of cold water. After 5 min a volume of 5% NaCl equal to one-fifth the volume of water used was added to precipitate the membranes, which were then removed by centrifugation at 48.000*g* for 30 min. The resulting hemoglobin solution was adjusted to pH 7.4 with 0.1 N NaOH and freed of phosphates as described by Berman *et al.* (1971).

For periods of storage of longer than 24 hr all solutions of normal and chemically modified hemoglobin were frozen in liquid nitrogen in the form of small droplets and kept in a liquid nitrogen refrigerator (Cryogenics Service Division, Union Carbide) until ready for use. No change in physical or functional properties occurred during such storage for several months.

The concentration of the hemoglobin solutions was determined after conversion to methemoglobin cyanide using the molar extinction coefficient of 4.6×10^4 at 540 nm. All hemoglobin concentrations are expressed on a molar (mol wt 64,500) basis.

Other Materials. Pyridoxal-P was either obtained from Calbiochem (A grade) or a highly purified sample kindly donated by Dr. T. Kuroda was used. Pyridoxal, pyridoxamine phosphate, and pyridoxamine were purchased from Sigma Chemical Co. Pyridoxylvaline was a gift from Dr. E. E. Snell or synthesized in this laboratory by Dr. Bonnie Bray by the

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[1] Abbreviations used are: P_2glycerate, 2,3-diphosphoglyceric acid; pyridoxal-P, pyridoxal phosphate; bisTris, *N,N*-bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane.

method of Severin *et al.* (1969). Samples of pyridoxyllysine were obtained through the courtesy of Dr. Hannah Pocker and Miss Helena Bertola. The cyclohexylammonium salt of P_2glycerate (Calbiochem) was converted to the free acid as described previously (Benesch *et al.*, 1968a). Tris was Schwarz-Mann Ultra Pure grade and bisTris was a recrystallized sample from General Biochemicals. Nitrogen was prepurified grade from which traces of oxygen were removed by the method of Meites and Meites (1948).

Chromatographic Materials. UR-30 resin was obtained from the Spinco Division, Beckman Instrument Co. Carboxymethylcellulose (Bio-Rad Cellex CM) was washed with 0.2 N NaOH and water, followed by equilibration with buffer. The most satisfactory grade of phosphocellulose of high capacity proved to be Whatman P-11 (Reeve Angel Co.). It was pretreated in the same way as carboxymethylcellulose. The source of Pronase and trypsin was Calbiochem and sodium borohydride was purchased from Matheson Coleman & Bell. All organic compounds, enzymes, etc., were stored in desiccators at +5° and the pyridoxal derivatives were also protected from light. All standard chemicals used were reagent grade.

Methods. The oxygen equilibrium curves were determined by the method of Benesch *et al.* (1965). For the experiments involving the effect of pyridoxal-P on oxygen binding it was found necessary to extend the equilibration time to 20 min. The binding of pyridoxal-P to hemoglobin was measured by ultrafiltration as described previously (Benesch *et al.*, 1968a) and the free concentration in the ultrafiltrate was determined spectrophotometrically. The molar extinction coefficient of pyridoxal-P in 0.1 M Tris buffer (pH 7.3) was found to be 6.5×10^3 at 413 nm. Whenever necessary, ferrihemoglobin was converted to ferrohemoglobin as described by Dixon and McIntosh (1967) but 0.05 M bisTris buffer (pH 7.3) which was 0.1 M in Cl^- was used instead of phosphate.

Reaction of Hemoglobin with Pyridoxal-P. Preliminary studies on the reaction between pyridoxal-P and hemoglobin in bisTris buffer showed that pyridoxal-P was bound at more than one site under these conditions. In Tris buffer, on the other hand, the reaction was found to be highly specific. Tris forms a Schiff's base with pyridoxal-P, with a characteristic absorption maximum at 413 nm, which then reacts with hemoglobin in a transimination reaction.

A typical experiment for the covalent attachment of pyridoxal-P to deoxyhemoglobin was conducted as follows.

Stripped hemoglobin (3 μmoles) and 1 M Tris buffer (0.7 ml; pH 7.3 at 10°) in a total volume of 11 ml were deoxygenated by bubbling nitrogen through the solution. The temperature of the mixture was maintained at 10° and 50 μl of caprylic alcohol was added to prevent foaming. After deoxygenation was complete, 6 μmoles of pyridoxal-P in 0.5 ml of 1 M Tris buffer was added and the pH of the mixture was adjusted to 6.8 by slow addition of 0.1 N HCl (about 0.5 ml). The concentrations at this stage were thus: Hb, 2.5×10^{-4} M; pyridoxal-P, 5.0×10^{-4} M; Tris, 0.1 M. After 30 min at 10° under nitrogen, 60 μmoles of $NaBH_4$ in 0.5 ml of 10^{-3} M NaOH was added. Hydrolysis of the excess $NaBH_4$ raises the pH to 7.1. The nitrogen bubbling was continued at 10° for 1 hr after which air was admitted. For the reaction with liganded hemoglobin, carbon monoxide or oxygen was substituted for nitrogen throughout.

The mixture was then dialyzed for 2 hr at 5° against four changes of 500 ml of 0.02 M phosphate buffer (pH 6.8) in two-dimensionally stretched $^5/_8$-in. Visking casing (Craig, 1967) in the apparatus described by Englander and Crowe (1965). After centrifugation for 20 min at 48.000*g*, it was applied to a 1.5 × 24 cm column of P-11 which had been equilibrated with 0.02 M phosphate buffer (pH 6.8) at 5°. The column was eluted at a rate of 30 ml/hr with a 16-hr linear gradient of pH 6.8 phosphate buffer from 0.02 to 0.2 M, using a programmed gradient pump (Isco Dialagrad, Model 380). Fractions of 5 ml were collected and assayed by the absorption at 540 nm. The various fractions from the chromatographic separations were freed of noncovalently bound phosphate by dialysis through two-dimensionally stretched Visking casing. Phosphate was determined by the method of Ames and Dubin (1960). For the analyses of the phosphate content of hemoglobin samples, "stripped" hemoglobin at the same concentration was used as a blank.

The modified hemoglobin was separated into α and β subunits by the method of Bucci and Fronticelli (1965) and Tyuma *et al.* (1966). Mercury was removed from the β subunits as described by Benesch *et al.* (1968b) and from the α chains by the method of DeRenzo *et al.* (1967). Heme was removed by the method of Anson and Mirsky (1930) and occasionally by that of Teale (1959).

For trypsin hydrolysis and the separation of the tryptic peptides, the procedures described by Jones (1964) were used.

Globin was hydrolyzed with Pronase by incubating a mixture containing 5 mg of globin and 5 mg of Pronase per 1 ml of 0.1 M ammonium acetate buffer (pH 8) for 24 hr at 37°. The initial drop in pH was corrected by the addition of 1 M ammonia. After 24 hr the clear solution was evaporated to dryness in a stream of nitrogen.

Peptides were hydrolyzed with 6 N HCl *in vacuo* at 110° for 22 and 72 hr. Amino acid analysis was carried out on a Spinco amino acid analyzer Model 120.

Separation and Identification of Pyridoxyl Amino Acids and Peptides on UR-30 Resin. A jacketed 0.9 × 58 cm column was used at 55°. It was eluted at 84 ml/hr at a pressure of 150–300 lb with a Beckman Accu-Flo pump. For analytical separations the citrate buffer system of Spackman *et al.* (1958) was used, but for preparative purposes the volatile pyridine–acetic acid gradients of Schroeder (1967) were substituted. The fractions were monitored either by the absorption at 325 nm or by fluorescence (excitation at 330 nm and emission at 400 nm) using an Aminco-Bowman spectrofluorometer. In this way, amounts down to 0.01 μmole of pyridoxyl derivatives could easily be detected. For the quantitative estimation of the amount of each component the absorbance at 325 nm was always used, since the extinction coefficients of pyridoxyllysine and pyridoxylvaline are very similar under the conditions used in these experiments.

The Pronase hydrolysate of $\alpha_2(\beta^{PLP})_2$-globin (PLP = pyridoxal-P) (Figure 7, fraction II) was purified in two steps. Portions corresponding to 60 mg of globin were applied to the column and eluted with 0.30 M pyridine–acetate buffer (pH 5.4). The elution pattern is similar to that shown in the bottom part of Figure 5. The major fraction was lyophilized and rechromatographed with 0.35 M pyridine–acetate (pH 5.4) to remove the remaining free lysine and histidine. On relyophilization a pyridoxyl peptide free of ninhydrin-positive material was obtained. It was then analyzed by nuclear magnetic resonance (nmr) spectroscopy and, after acid hydrolysis, by the chromatographic methods described above.

The pyridoxyl peptides of the α chains, resulting from the reaction of liganded hemoglobin with pyridoxal-P, were prepared by Pronase hydrolysis of the pyridoxyl α-globin. A preliminary purification of the Pronase hydrolysate was carried out on UR-30 resin with a 2-hr gradient which was

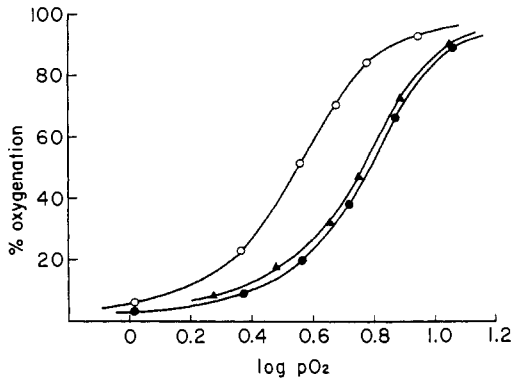

FIGURE 1: Effect of pyridoxal-P and P_2glycerate on the oxygen equilibrium curve of hemoglobin. (○) Stripped hemoglobin; (●) 2×10^{-4} M P_2glycerate; (▲) 2×10^{-4} M pyridoxal-P. Hemoglobin concentration, 5×10^{-5} M, in 0.1 M Tris buffer (pH 7.3), total chloride (0.1 M); temperature 20°.

TABLE I: Effect of Pyridoxal Analogs on the Oxygen Affinity of Hemoglobin.[a]

Pyridoxal Analog (2×10^{-4} M)	Log p_{50}		
	No P_2-glycerate	2×10^{-4} M P_2-glycerate	4×10^{-4} M P_2-glycerate
None	0.55	0.79	0.84
Pyridoxal phosphate	0.77	0.84	
Pyridoxal	0.54	0.76	
Pyridoxamine phosphate	0.56	0.77	
Pyridoxamine	0.57	0.78	
Pyridoxol	0.56	0.77	

[a] Hemoglobin (5×10^{-5} M), Tris buffer (pH 7.30, 0.1 M), and total chloride (0.1 M), temperature 20°.

formed by mixing 0.2 M pyridine–acetate (pH 3.15) (solution A) with 0.2 M pyridine–acetate (pH 4.85) (solution B). The proportion of solution B was increased linearly from 0 to 50% during the first 30 min and from 50 to 100% during the subsequent 90 min. An LKB Ultrograd 11300 was used for this purpose. The fluorescent components were pooled, lyophilized, and rechromatographed in 0.1 M pyridine–acetate (pH 4.05). Remaining ninhydrin-positive material was removed from the main fraction by high-voltage paper electrophoresis.

Results and Discussion

Pyridoxal-P as a P_2glycerate Analog. The evidence that pyridoxal-P lowers the oxygen affinity of hemoglobin like P_2glycerate is illustrated in Figure 1. The stabilization of the deoxy form, as in the case of P_2glycerate, was found to be due to strong preferential binding of pyridoxal-P to deoxy- as compared to oxyhemoglobin (Figure 2). The reduced binding of pyridoxal-P in the presence of P_2glycerate shows that the two compounds compete for the same binding site. In contrast to P_2glycerate, however, pyridoxal-P also binds to oxyhemoglobin, but with a much lower affinity than to deoxyhemoglobin ($K_{diss}^{PLP} = 4.5 \times 10^{-4}$ M for oxyhemoglobin and 2×10^{-5} M for deoxyhemoglobin).

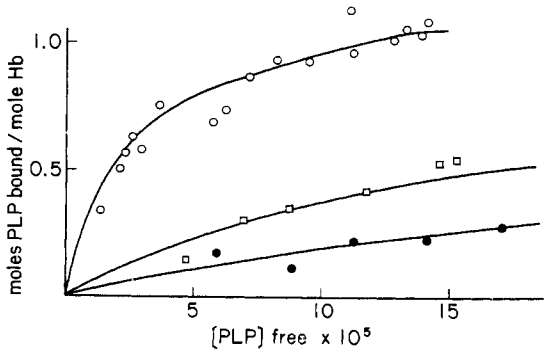

FIGURE 2: Binding of pyridoxal-P to hemoglobin. (○) Deoxyhemoglobin; (□) deoxyhemoglobin plus 4×10^{-4} M P_2glycerate; (●) oxyhemoglobin. Hemoglobin concentration, 0.5×10^{-4} to 1.2×10^{-4} M in 0.1 M Tris buffer (pH 7.3), 0.1 M total chloride; temperature 23°

The resulting free-energy difference then produces a shift which is very similar in magnitude to that of P_2glycerate (DPG) which under these conditions binds only to deoxyhemoglobin, but somewhat more weakly than pyridoxal-P (K_{diss}^{DPG} 4.7×10^{-5} M).

The specificity of the binding site for the pyridoxal compound becomes apparent from Table I. Only pyridoxal-P with its two interacting groups, *i.e.*, the aldehyde and the phosphate, is effective in lowering the oxygen affinity. The compounds which lack either or both of these groups are inactive and do not interfere with the normal P_2glycerate response. Pyridoxal-P and P_2glycerate can substitute for one another, since the same oxygenation curve is obtained in 2×10^{-4} M P_2glycerate plus 2×10^{-4} M pyridoxal-P as in 4×10^{-4} M P_2glycerate. This again argues for a competition of pyridoxal-P and P_2glycerate for the same polyfunctional binding site.

Covalent Attachment of Pyridoxal-P to Hemoglobin. When pyridoxal-P was coupled irreversibly to deoxyhemoglobin, it was incorporated exclusively in the β chains. This is illustrated in Figure 3A which shows a separation of the modified hemoglobin into α and β subunits. The three peaks observed correspond successively to β chains containing 1 mole of pyridoxal-P/chain, normal β chains and normal α chains. The presence of 1 mole of pyridoxal-P/chain in the first component was confirmed by phosphate analysis and by the characteristic absorption maximum at 325 nm after removal of the mercury and the hemes.

By contrast, the specificity for the β chains is lost when the reaction is carried out with carbonmonoxy- or oxyhemoglobin, as shown by the appearance of a new peak (fractions 28–36) which results from pyridoxal-P coupling to α chains (Figure 3B). This appears to be only partial, since during the reduction some deoxyhemoglobin is inevitably formed. Exclusive reaction with the α chains can, however, be achieved when the reduction is performed in the presence of P_2glycerate, which blocks the β site (Figure 3C).

It may seem surprising that liganded hemoglobin incorporates so much pyridoxal-P in view of the weak binding to oxyhemoglobin shown in Figure 2, but it must be remembered that the binding measurements reflect reversible imine formation, whereas the irreversible attachment of pyridoxal-P involves a reduction step which displaces the equilibrium in favor of the bound form.

TABLE II: Amino Acid Analysis of Pyridoxal-P Octapeptide.[a]

Amino Acid	Found	βT-1
Val	0	1
His	0.34	1
Leu	1.00	1
Thr	0.94	1
Pro	0.99	1
Glu	1.94	2
Lys	1.05	1

[a] The experimental amino acid values were normalized to leucine.

Location of the Binding Sites. DEOXYHEMOGLOBIN. Chromatography of the tryptic hydrolysate of the modified β-globin showed only a single fluorescent peptide with an absorption maximum at 325 nm (Benesch *et al.*, 1971). The amino acid composition of the acid hydrolysate of this peptide corresponds closely to that of the N-terminal octapeptide of the β chain of hemoglobin with the exception of valine and histidine (Table II). Valine is, of course, absent since it is replaced by *N*-pyridoxylvaline. This compound was identified in the hydrolysate by comparison to an authentic sample (Figure 4, first peak in A, B, C, and D). During acid hydrolysis a decomposition product with a slower mobility is formed and this is present both in the hydrolysate of pure *N*-pyridoxylvaline and in that of the octapeptide (Figure 4, 2nd peak in B, C, and D). The low value for histidine is due in part to the extraordinary resistance of the substituted N-terminal dipeptide to acid hydrolysis. It will be shown below that the last component in Figure 4C,D is *N*-pyridoxylvalylhistidine. A comparison of chromatograms C and D in Figure 4 demonstrates that the amount of this dipeptide decreases on prolonged hydrolysis with the formation of pyridoxylvaline and its decomposition product. Another factor which may account for the loss of histidine is the finding of Rippa and Pontremoli (1969) that pyridoxal-P can act as a photosensitizer for the oxidation of histidine residues in its vicinity.

The unusual stability of the peptide bond in *N*-pyridoxylvalylhistidine is also reflected by the results obtained with Pronase. When globin derived from deoxyhemoglobin coupled with pyridoxal-P was hydrolyzed with this enzyme, only one major component (Figure 5) with the same mobility as the last peak in Figure 4C,D was observed. On acid hydrolysis this fraction yielded only pyridoxylvaline and histidine. Nuclear magnetic resonance analysis (Figure 6), carried out by Dr. Kurt Wüthrich, independently confirmed the structure of this dipeptide. The intensities of the methyl resonance of valine compared to the low-field lines of pyridoxal and histidine are in a ratio of 6:1 as expected for pyridoxyl:Val:His 1:1:1. Moreover, time averaging of the low-field part of Figure 6 shows that the intensities of His C_2 and His C_4 and the pyridoxal ring H are in the expected ratio of 1:1:1 (Figure 6). This identification of *N*-pyridoxylvalylhistidine provides the most direct proof that the reaction of deoxyhemoglobin with pyridoxal-P takes place at the N-terminal residues of the β chains.

The minor component in Figure 5 was identified as pyridoxyllysine by comparison to the synthetic compound. Under the conditions used, reaction with lysine residues never amounted to more than 5–10% of the total. Since pyridoxal-P as an enzymatic cofactor, is invariably found on the ε amino groups of lysine (Fasella, 1967), it is remarkable that in the case of deoxyhemoglobin at least 90% is incorporated at the N-terminal amino group.

LIGANDED HEMOGLOBIN. The site of attachment of pyridoxal-P to the α chains was again found to be the N-terminal valine. Pronase hydrolysis of modified α-globin gave rise to three

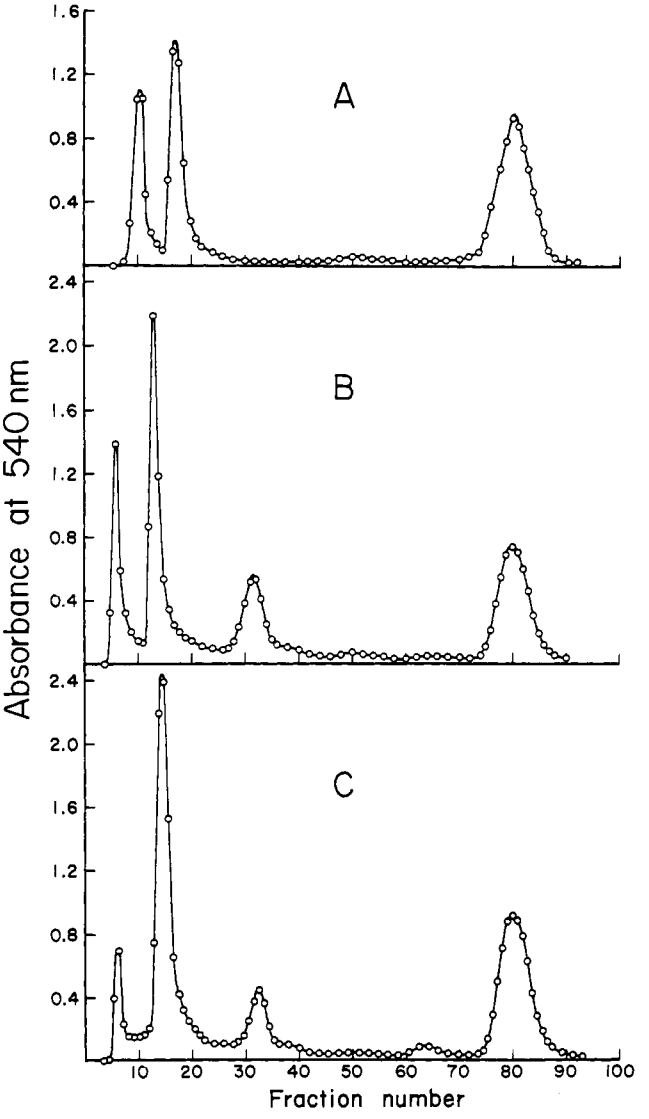

FIGURE 3: Separation of hemoglobin into subunits after covalent attachment of pyridoxal-P. The conditions for reaction with pyridoxal-P and sodium borohydride reduction are described in the text. The product was treated with *p*-mercuribenzoate and chromatographed as described by Bucci and Fronticelli (1965). (A) Deoxyhemoglobin plus 1 mole of pyridoxal-P/mole of Hb; (B) COHb plus 2 moles of pyridoxal-P/mole of Hb; (C) COHb plus 2 moles of pyridoxal-P and 20 moles of P_2glycerate per mole of Hb. The relative amounts of the fractions (in per cent) were

	A	B	C
pyridoxyl β chains	18	11	3
normal β chains	30	33	44
pyridoxyl α chains	0	19	12
normal α chains	43	27	31

The values for pyridoxal β chains were corrected by the amount of the fast component of the same mobility found in unmodified Hb under the same conditions (4%).

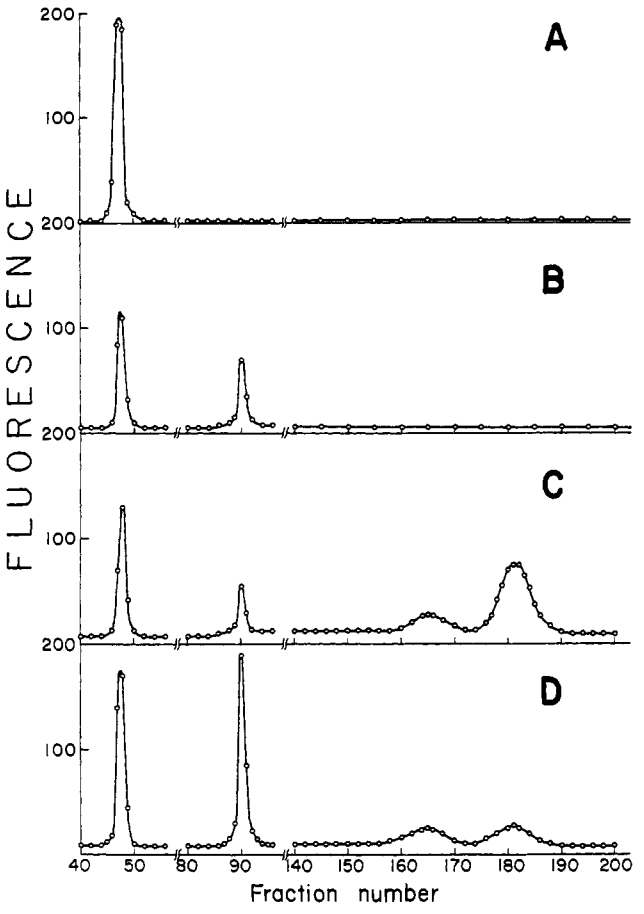

FIGURE 4: Chromatography of the acid hydrolysate of the fluorescent tryptic peptide of the β chain. (A) Pyridoxylvaline; (B) pyridoxylvaline after 72-hr hydrolysis with 6 N HCl at 105°; (C) 20-hr HCl hydrolysate of the fluorescent tryptic octapeptide; (D) 72-hr HCl hydrolysate of the same octapeptide. Elution with 0.2 N citrate buffer (pH 3.25) from fractions 1–17, 0.2 N citrate buffer (pH 4.25) from fractions 17–76, and thereafter with 0.35 N citrate buffer (pH 5.25).

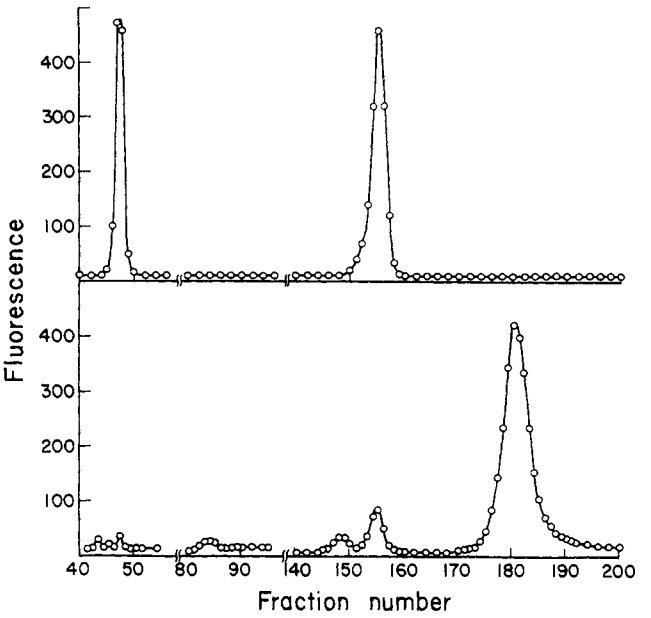

FIGURE 5: Chromatography of the Pronase hydrolysate of globin prepared from the deoxyhemoglobin–pyridoxyl-P reaction. Upper chromatogram: pyridoxylvaline (1st peak) and pyridoxyllysine (2nd peak); lower chromatogram: Pronase hydrolysate of 5 mg of globin prepared from fraction II of Figure 7. Conditions of chromatography as in Figure 4.

fluorescent components. The major one was identified as the N-terminal pentapeptide of the α chain (Table III). The two minor components were found to be the N-terminal tetrapeptide and pyridoxylvaline, respectively. As in the case of deoxyhemoglobin, lysine substitution was negligible compared to reaction at the α N terminus.

TABLE III: Amino Acid Composition of the Main Fraction of the Pronase Hydrolysate of Pyridoxyl α Chains.

Amino Acid	Found	α N-Terminal Pentapeptide
Val	0	1
Leu	0.93	1
Ser	0.99	1
Pro	1.00	1
Ala	1.00	1

[a] The value for serine was corrected to zero hydrolysis time and for leucine 72-hr hydrolysis was used. In view of the resistance of pyridoxylvalylleucine to hydrolysis, the analyses were normalized to alanine.

The selective affinity of pyridoxal-P for the N-terminal β residues in deoxy- and the α-N-terminal residues in oxyhemoglobin is best understood in terms of the stereochemical model of Perutz (1970). In deoxyhemoglobin the α-N-terminal residues form salt bridges with the C-terminal residues of the other α chain and are therefore unreactive. The β N terminals, on the other hand, provide an easily accessible binding site for a molecule of pyridoxal-P which can then form additional salt bridges with strategically placed positive residues such as lysine EF6β or histidine H21β. The change in the dimensions of the central cavity on oxygenation greatly decreases the affinity of pyridoxal-P for the N-terminal end of the β chains. At the same time, the salt link blocking the α N-terminal valine is broken, permitting reaction at this locus.

It is worth noting that methemoglobin cyanide failed to incorporate any pyridoxal-P at all. The absence of reaction at the β locus is consistent with the observation that methemoglobin cyanide does not bind P_2glycerate (Benesch *et al.*, 1968a). The unusual properties of methemoglobin cyanide are reflected by its extraordinary resistance to denaturation (Simko and Kauzmann, 1962) and by its failure to dissociate into α and β subunits on mercuration (unpublished observations). These features illustrate the special nature of methemoglobin cyanide in contrast to other liganded hemoglobins with which it is often compared.

Properties of Deoxyhemoglobin Coupled with Pyridoxal-P. The irreversible attachment of pyridoxal-P to deoxyhemoglobin was studied at different initial ratios of the reactants. The total incorporation as a function of pyridoxal-P concentration is shown in Table IV and a typical chromatogram on phosphocellulose in Figure 7. Apart from a small fast component (I) containing 3–4 moles of pyridoxal-P/mole of hemoglobin, two major fractions are observed. One of these

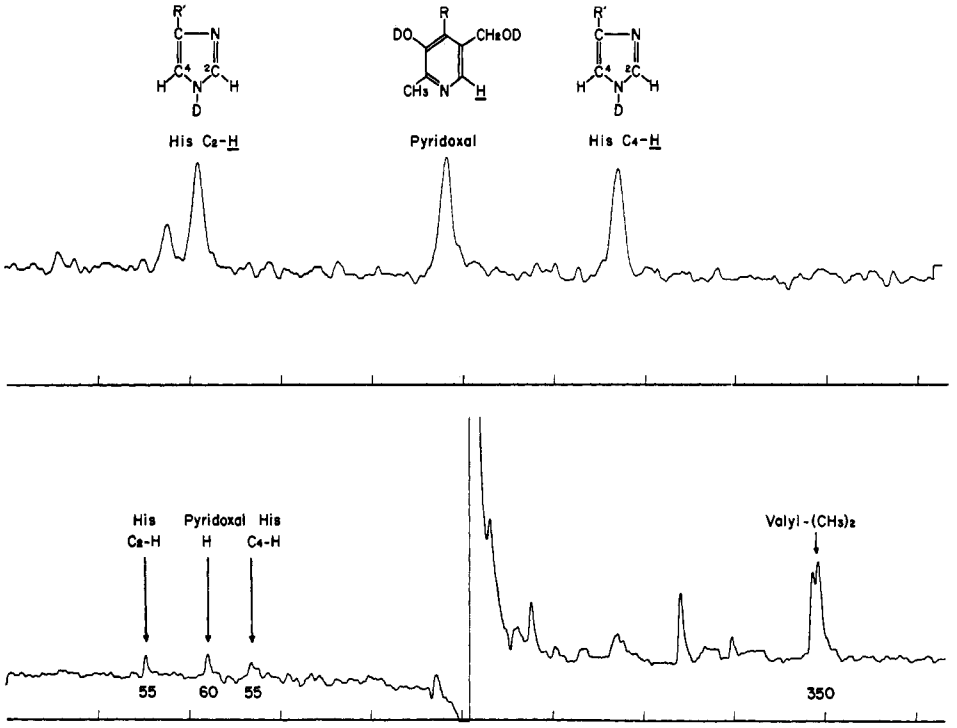

FIGURE 6: Nuclear magnetic resonance spectra of the fluorescent Pronase peptide derived from coupled deoxyhemoglobin. The peptide (major fraction of Figure 5) was purified as described in the text and dissolved in D_2O, 0.1 M phosphate (pD 7.0). The top spectrum is the low-field portion of the bottom one after time averaging in a computer (CAT). The relative intensities of the resonances in the lower spectrum are indicated on the figure.

(II) consists of molecules with exactly 2 moles of pyridoxal-P/mole of hemoglobin and the other, slower one (IV) contains only traces of pyridoxal-P. Between these two components, a small peak having one pyridoxal-P per mole is also evident (III).

The appearance of these species during chromatography is interpreted as follows. The formation of a single 1:1 pyridoxal-P–deoxyhemoglobin complex under anaerobic conditions, revealed by the binding curves in Figure 2, is analogous to the reaction of P_2glycerate with deoxyhemoglobin. Reduction of this imine with sodium borohydride must give rise to a tetramer composed of three different kinds of chains, *i.e.*, $\alpha_2\beta\beta^{PLP}$.

It was first pointed out by Guidotti *et al.* (1963) that tetramers of this type cannot be isolated under conditions where any dissociation into $\alpha\beta$ dimers takes place, since the following rearrangement will occur:

$$2\alpha_2\beta\beta^{PLP} \rightleftharpoons 2\alpha\beta + 2\alpha\beta^{PLP}$$
$$\downarrow\uparrow \qquad\qquad \downarrow\uparrow$$
$$\alpha_2\beta_2 \qquad \alpha_2(\beta^{PLP})_2$$

This equilibrium is displaced in favor of the symmetrical tetramers by the separation method itself. The major products are therefore $\alpha_2(\beta^{PLP})_2$ and $\alpha_2\beta_2$ in equal amounts, while the original $\alpha_2\beta\beta^{PLP}$ is discernible between them, indicating that rearrangement is not entirely complete under the conditions of the experiment.

Since deoxyhemoglobin does not dissociate into $\alpha\beta$ dimers, it should be possible to isolate $\alpha_2\beta\beta^{PLP}$ as the major product of the reaction only in the absence of oxygen. This has been accomplished and will be described in a separate article.

As expected, hemoglobin with both β chains blocked with pyridoxal-P (Figure 7, fraction II) does not bind P_2glycerate and has an irreversibly lowered oxygen affinity ($\log p_{50} = 0.90$ as compared to 0.58 for unmodified hemoglobin at pH 7.3 and 20°) which is uninfluenced by P_2glycerate.

TABLE IV: Reaction of Deoxyhemoglobin with Pyridoxal-P.[a]

Pyridoxal-P Added (Moles/Mole of Hb)	Pyridoxal-P Irreversibly Attached (Moles/Mole of Hb)
1.2	0.83
1.2	0.86
1.35	1.04
1.35	0.99
1.35	0.97
1.35	0.95
1.50	1.09
2.0	1.40
2.0	1.24

[a] Hemoglobin concentration, 2.5×10^{-4} M, reaction conditions as described in the text.

General Conclusions

The main conclusion from the results reported here is that the polyphosphate binding site in deoxyhemoglobin involves the N-terminal groups of the β chains. This result is also borne out by the absence of a P_2glycerate effect on the oxygen affinity in acetylated fetal hemoglobin (Bunn and Briehl, 1970) and acetylated cat hemoglobin (Taketa *et al.*, 1971) and the

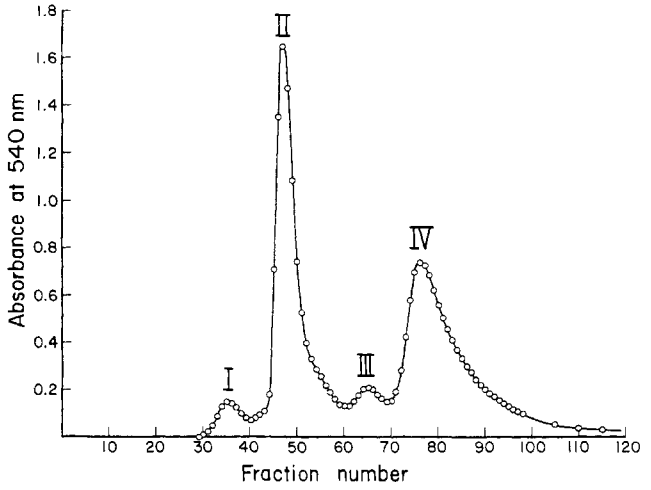

FIGURE 7: Chromatogram of the reaction product of deoxyhemoglobin and pyridoxal-P on phosphocellulose. Deoxyhemoglobin was treated with 2 moles of pyridoxal-P/mole of hemoglobin and reduced with $NaBH_4$ as described in the text. The relative amounts of the components are: I 4.3%; II 44.6%; III 6.2%; IV 44.9%.

minimal effect observed with hemoglobin A_{Ic} (Bunn and Briehl, 1970).

In the case of P_2glycerate, all the salt bridges are broken on full oxygenation (Benesch *et al.*, 1971) and the phosphate ester is set free as the macromolecule changes to the oxy conformation. When pyridoxal-P is attached covalently to the β N-terminal NH_2 groups, on the other hand, these bonds remain intact on oxygenation and only the salt bridge involving the phosphate is broken. This rupture makes it possible for the hemoglobin molecule to assume an oxy conformation, albeit with more difficulty. For this reason $\alpha_2(\beta^{PLP})_2$-hemoglobin has a permanently lowered oxygen affinity.

Finally, it is of interest to compare the reactivity of the α and β N termini of hemoglobin in the deoxy and oxy conformation toward several reagents. Among these, P_2glycerate shows by far the highest selectivity since it reacts only with the β locus in the deoxy conformation and not at all with fully liganded hemoglobin. Pyridoxal-P has a comparable specificity for the β N terminus in the deoxy conformation but acquires considerable affinity for the α N terminus in the liganded state. In deoxyhemoglobin, carbon dioxide competes with P_2glycerate for the terminal β NH_2 groups (Bauer, 1970) but shows a comparable affinity for the α N-terminal sites (Kilmartin and Rossi-Bernardi, 1971). A uniform carbamylation of both sets of end groups has been found with cyanate (Kilmartin and Rossi-Bernardi, 1971) in COHb, although the behavior of this reagent towards deoxyhemoglobin remains to be investigated.

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Added in Proof

The recent results of Arnone (1972) on the location of a single 2,3-diphosphoglycerate molecule in the deoxyhemoglobin tetramer are in excellent agreement with the main conclusions of this paper.

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