Labeling of Hemoglobin with Pyridoxal Phosphate*

(Received for publication, August 4, 1981)

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The reaction of pyridoxal 5'-phosphate (PLP) with deoxyhemoglobin is confined to 2 residues in the β chains, i.e. the α-amino group of valine 1 and the ε-amino group of lysine 82, both of which are located in the polypeptide binding site. The major product is a hemoglobin in which only the two NH₂-terminal amino groups are substituted (symmetric diPLPHb). It is formed by subunit rearrangement of monoPLPHb which is the initial product of the pyridoxylation under anaerobic conditions. TetraPLPHb, with substitutions at lysines 82 and valine 1 of both β chains, is considered as a minor component. It results from subunit exchange of asymmetric diPLPHb consisting of one unmodified αβ dimer and one which is pyridoxylated at both sites. Anaerobic electrophoresis and oxygenation curves show that this reaction is readily reversed by mixing the tetrasubstituted derivative with unmodified hemoglobin.

It was shown previously that the reaction of pyridoxal phosphate with deoxyhemoglobin leads to a specific modification of the 2,3-diphosphoglyceraldehyde binding site between the β chains. A hemoglobin with a PLP residue covalently attached to the α-NH₂ group of each β chain was isolated and found to have the same oxygen affinity as hemoglobin saturated with 2,3-diphosphoglycerate. By analogy with the 1:1 stoichiometry of 2,3-diphosphoglyceraldehyde binding, it was postulated that the initial reaction product is actually monoPLP-hemoglobin which then rearranges to diPLP-hemoglobin by subunit exchange during the isolation (1–4). The details of this rearrangement as well as another one which results in a tetrasubstituted hemoglobin will be described in this paper.

MATERIALS AND METHODS

The hemoglobin used for this investigation was prepared from hemolysates of human blood (†) by chromatography on DEAE-cellulose (Whatman DE52) (5). Hemoglobin concentration was determined from the absorbance at 540 nm after conversion to methemoglobin cyanide. (e = 11.0 X 10⁹/m/heme).

The reaction of HbA with pyridoxyl phosphate (Calbiochem-Behring) was carried out at 10°C in a thermostatted vessel. In a typical preparation 12 ml of hemoglobin solution, which had been dialyzed against 0.1 M Tris-HCl buffer, pH 7.5 (at 10°C), and then diluted to 2.5 X 10⁻⁴ M (as tetramer) with the same buffer, were deoxygenated by bubbling nitrogen through the solution with the addition of 20 μl of cyclohexanol to prevent foaming. After complete deoxygenation (30 to 60 min), 7.5 μmol of PLP in 0.5 ml of Tris buffer was added (2.5 mol/mol of Hb). After 30 min under nitrogen, 75 μmol of NaBH₄ in 0.6 ml of 10⁻³ M NaOH were introduced. This treatment results in the reduction of the imines to secondary amines and also destroys the excess of the reagent. Bubbling with nitrogen was continued for another 30 min after which air was admitted.

The chromatographic isolation of the pyridoxylated fractions on DE52 was based on the method of Abraham et al. (5) using sectional columns (Kontes Co., Vineland, N.J.) at +5°C (6). The reaction mixture was dialyzed against a solution composed of 0.1 M glycine, 5 X 10⁻⁴ M in NaOH, and 0.02 M in NaCl (pH 7.8) and then applied to a column (2.5 X 25 cm) of DE52 equilibrated with 0.2 M glycine, 10⁻³ M in NaOH, 0.02 M in NaCl, pH 7.8 (solution A). The column was eluted at 40 ml/h with an exponential gradient composed of 1 liter of solution A in the mixing bottle and 0.2 M glycine, 10⁻⁴ M in NaOH, 0.04 M in NaCl (solution B) in the reservoir. After 18 h, solution B was replaced by 0.2 M glycine, 10⁻³ M in NaOH, 0.1 M in NaCl. After a further 5 h, three well resolved bands have appeared. The portions of resin containing the three fractions were removed from the relevant sections of the column and the hemoglobin eluted with 0.2 M NaCl (6).

The hemoglobin fractions were analyzed for total phosphate (7) and the hemoglobin was split into α and β chains (8) which were subjected to polyacrylamide gel electrophoresis or separated by column chromatography (8). Mercury was removed from the chains by passage through a column of sulphydryl agarose (Pierce Chemical Co.). Globin was prepared by the method of Teale (9). The lyophilized β-globin was aminomethylated and digested with trypsin as described previously (10).

Separation of the tryptic peptides of the globin chains was carried out by reversed phase high performance liquid chromatography on a unit assembled in the laboratory from commercially available components.² For analysis of the tryptic peptides of pyridoxylated globin chains, the fluorescence of the fractions was measured in a Perkin Elmer MFP-4 fluorescence spectrophotometer. For detection of the pyridoxyl moiety, the fractions were excited at 325 nm and the emission of caprylic alcohol to prevent foaming. After complete deoxygenation (30 to 60 min), 7.5 μmol of PLP in 0.5 ml of Tris buffer was added (2.5 mol/mol of Hb). After 30 min under nitrogen, 75 μmol of NaBH₄ in 0.6 ml of 10⁻³ M NaOH were introduced. This treatment results in the reduction of the imines to secondary amines and also destroys the excess of the reagent. Bubbling with nitrogen was continued for another 30 min after which air was admitted.

For analysis of the tryptic peptides of pyridoxylated globin chains, the fluorescence of the fractions was measured in a Perkin Elmer MFP-4 fluorescence spectrophotometer. For detection of the pyridoxyl moiety, the fractions were excited at 325 nm and the emission was measured at 410 nm. For amino acid analysis, the fractions containing the peptides were dried in a speed vac concentrator (Savant Instruments Inc., Hicksville, NY) (without using heat) and the dried peptide samples were hydrolyzed in 6 N HCl in vacuo at 110°C for 22 h. All amino acid analyses were performed on a Durrum D-500 amino acid analyzer according to standard procedures.

Polyacrylamide gel electrophoresis was carried out aerobically and anaerobically on 7.5% polyacrylamide gels containing 0.2% bisacryl-

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² A. S. Acharya and J. M. Manning, unpublished results.
amide and 0.1% ammonium persulfate using a Tris-Tricine continuous buffer system (upper buffer, 0.033 M Tris, 0.04 M Tricine, pH 8.0; lower buffer, 0.1 M Tris, pH 8.2). For the anaerobic electrophoresis, both buffers were deoxygenated by bubbling argon through the solutions before and during the electrophoresis. Prior to electrophoresis, diethylamine was added to both buffer solutions to a final concentration of 10^{-3} M, and catalase (2 mg/300 ml) to the upper buffer. Before application of the sample, 10 μl of 10^{-3} M dithionite was applied to each gel and incorporated by pre-running at 2 mA/gel for 30 min. The sample containing 10^{-3} M dithionite, under mineral oil, was then applied and electrophoresed for 1 to 3 h at 3 mA/gel. Hybrids between HbA and HbS, produced under aerobic conditions, were used to test the system for anaerobiosis, since such hybrids are stable only under completely anaerobic conditions.

RESULTS

We had shown previously that when deoxyhemoglobin is treated with 2 mol of PLP/mol of Hb, followed by NaBH₄ reduction, four fractions designated in the order of elution as P₀, P₂, P₄, and P₆ are obtained by chromatography on phosphocellulose (Fig. 1) (1). Phosphate analysis showed that they contained 4, 2, 1, and 0 phosphates/mol of hemoglobin, respectively.

The pyridoxal residues in diPLPHb (P₂) were previously shown to be attached to the α-amino group of Val-1 (β) (1). The second substitution in tetrapLPHb (P₄) is also on the β chain since separation into α and β chains of the fractions eluted from a DE52 column showed that both P₂ and P₄ had unsubstituted α chains and that their β chains contained one and two phosphates per chain, respectively, with a corresponding increase in anodic mobility (Table I).

![Figure 1](http://www.jbc.org/)

**Figure 1.** Chromatography of pyridoxylated hemoglobin derivatives on phosphocellulose (1). DeoxyHb was treated with 2 mol of PLP/mol of Hb and reduced with NaBH₄ as described in Ref. 1. After dialysis against 0.02 M phosphate buffer, pH 6.8, the mixture was applied to a column (1.5 × 24 cm) of phosphocellulose (Whatman P-11), equilibrated with the same buffer at 5°C. The column was eluted at 30 ml/h with a 16-h linear gradient of pH 6.8 phosphate buffer from 0.02 to 0.20 M.

![Figure 2](http://www.jbc.org/)

**Figure 2.** High performance liquid chromatography analysis of the tryptic peptides of aminoethylated β chains of P₀, P₂, and P₄. The digest of about 80 μg of the β chain was taken up in 100 μl of 5% acetonitrile containing 0.1% trifluoroacetic acid and loaded on to a Partisil 10 ODS 2 column (4.6 × 250 mm; particle size, 10 mm). The column was equilibrated with 5% acetonitrile containing 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient of 5 to 70% acetonitrile (70% of each), both containing 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 60 ml/h, and 1-ml fractions were collected. The effluent was monitored by the absorbance at 210 nm. The upper panel indicates the position of the fluorescent pyridoxylated peptides of P₀.

### Table I

**Analysis of the fractions in Fig. 1**

| Fraction | P per tetramer | α Chains Rᵣ | β Chains Rᵣ | P per chain |
|----------|----------------|--------------|-------------|-------------|
| P₀       | 4.0            | 0.355        | 0.86        | 1.9         |
| P₂       | 2.0            | 0.350        | 0.78        | 1.0         |
| P₄       | 0              | 0.355        | 0.65        | 0           |

The chromatograms of the tryptic digests of the aminoethylated β chains of P₀, P₂, and P₄ are shown in Fig. 2. It is clear from the tryptic peptide map of P₂, which has been previously identified as pyridoxylated at the α-NH₂ group of Val-1 (β), that one of the normal peptides (eluting at 30 min and having the amino acid composition of β T 1), is replaced by a new fluorescent one designated as a β T 1.46 min (identified as β T 10 by amino acid analysis) and at 56 min (identified as β T 9 by amino acid analysis) have disappeared. Peptide b was isolated and its amino acid composition (Table II) corresponded reasonably well with the combined composition of β T 9 and aminoethylated β T 10 (comprising the region from β 67 to β 95). No other combination of two tryptic peptides could fit the analysis. This pinpoints the ε-NH₂ group of lysine β 82 as the second substitution site in the tetrapyrdoxylated hemoglobin (P₄).

The constitution of the two major products eluted from either P-11 or DE52 columns is therefore αβ(P₄)² and αβ(P₄)², respectively. The formation of these two tetramers during the isolation procedure involves several steps: 1) dissociation into αβ dimers with a dissociation constant of ~10⁻⁶ M; 2) reassociation of the dimers to form an equilibrium mixture of homogeneous and mixed tetramers (see equations below); 3) since the rate of dissociation and associ-
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Amino acid composition of peptide b

|         | Found (Glu = 1) | Expected for β1β2 and α2α1 (sequence 67-85 of β chain) |
|---------|----------------|---------------------------------------------------------|
| Asp     | 3.5            | 4.0                                                     |
| Thr     | 1.4            | 2.0                                                     |
| Ser     | 1.7            | 2.0                                                     |
| Glu     | 1.0            | 1.0                                                     |
| Pro     | 0.3            | 0                                                        |
| Gly     | 2.5            | 3.0                                                     |
| Ala     | 3.0            | 3.0                                                     |
| Val     | 1.4            | 1.0                                                     |
| Met     | 0.1            | 0                                                        |
| Ile     | 0.1            | 0                                                        |
| Leu     | 4.5            | 6.0                                                     |
| Tyr     | 0.1            | 0                                                        |
| Phe     | 1.4            | 2.0                                                     |
| His     | 1.9            | 2.0                                                     |
| Lys + AE-Cys | 1.4 | 2.0*                                                    |
| Arg     | 0.1            | 0                                                        |

a Calculated from the amino acid analysis of a 20-h acid hydrolysate using the value of glutamic acid as 1 residue.
Not corrected for losses during hydrolysis.
Not corrected for incomplete hydrolysis.
Only 1 free Lys would be expected from this peptide if Lys-82 had PLP attached, since pyridoxyllysine elutes at a different position.

Pyridoxylation was therefore performed as usual, but instead of admitting air at the end of the reaction, dithionite was added and the mixture transferred anaerobically to acrylamide gels as described above. Only two products separate with mobilities corresponding to mono- and diPLP-hemoglobin, respectively.

\[
\begin{align*}
\text{Asymmetric diPLPHb} & \rightarrow \text{Homogeneous tetramer} \\
\text{Mixed tetramer} & \rightarrow \text{Homogeneous tetramer} + \text{Homogeneous diPLPHb}
\end{align*}
\]

Since the dissociation constant of deoxyhemoglobin into dimers is only \( \sim 10^{-12} \) M (19), this exchange can be completely suppressed under anaerobic conditions and the original equilibrium distribution is preserved (11, 20).

Comparison of the results of anaerobic and aerobic electrophoresis can therefore be used to distinguish between initial reaction products and those formed by dimer exchange.

The validity of our anaerobic electrophoresis method was first checked with hybridized mixtures of HbS and HbI and the results in Table III show that subunit exchange was essentially prevented under these conditions.

Pyridoxylation was therefore performed as usual, but instead of admitting air at the end of the reaction, dithionite was added and the mixture transferred anaerobically to the acrylamide gels as described above. Only two products separate with mobilities corresponding to mono- and diPLP-hemoglobin, respectively. The data in Table IV show that with the smaller ratio of PLP to hemoglobin, the major product is indeed monoPLP hemoglobin. It is thus clear that P1 and P0 in the aerobic chromatogram shown in Fig. 1, as well as the bulk of P0, arose from subunit exchange. Chromatography on DE52 shows only P0, P5, and P6, the monoPLPHb (P0) having rearranged completely.

Electrophoretic assay of the interconversion of pyridoxylated hemoglobin derivatives

DeoxyHb was treated with PLP and reduced with NaBH4 as described in the text. After completion of the reaction, a sample was withdrawn anaerobically and subjected to anaerobic electrophoresis (oxy) and the remainder was oxygenated and subjected to aerobic electrophoresis (oxy).

The data in Table V show that the initial yield of P2 levels off at 38%, indicating that the initial yield of P2 is about 80%.

The conclusion that P1 is the initial product of the reaction between PLP and Hb is also reflected in the yields obtained with increasing ratios of PLP to Hb (Table V). If P1 is indeed only formed by rearrangement of P0, the proportion of P2 could not exceed 50%. The data in Table V show that the yield of P2 levels off at 38%, indicating that the initial yield of P2 is about 80%.

P1 can react with Val-1 and Lys-82 of the β chains. In theory, therefore, four diPLP hemoglobin species could be formed: two symmetric tetramers with PLP either on both Val-1 residues or on both Lys-82 residues, and two asymmetric tetramers, one with Val-1 and Lys-82 substituted on the same β chain and another with these substitutions on different β chains.
metric one in which Val-1 of both diPLP hemoglobin species is presented in Table IV, a symmetric one in which one give tetraPLP hemoglobin and unmodified hemoglobin (equations 1 and 2).

bic electrophoresis and, since none was present in the absence of monoPLP hemoglobin while the latter rearranges to the other carries both substitutions. It is clear from the data chains. However, evidence consistent with only two kinds of diPLP hemoglobin species is presented in Table IV, a symmetric one in which both a chains are substituted and an asymmetric one in which one a/b dimer is unmodified and the other carries both substitutions. It is clear from the data in Table IV that the symmetric one is produced by dismutation of monoPLP hemoglobin while the latter rearranges to give tetraPLP hemoglobin and unmodified hemoglobin (equations 1 and 2).

The proportions of symmetric and asymmetric diPLP hemoglobin formed during the initial reaction can be estimated by comparing the yield of P2 and P4 (as phosphate) under anaerobic and aerobic conditions (Table IV). Thus, for example, with a ratio of 1.4 PLP to Hb tetramer, 24% of the total phosphate appears as tetraPLP hemoglobin in the aerobic electrophoresis and, since none was present by reaction 2. Therefore, 24% of the phosphate must initially have been asymmetric diPLP hemoglobin and the remainder (11%) was symmetric diPLP hemoglobin. Similarly, since only 65% of the phosphate could have been incorporated into symmetric diPLP hemoglobin by reaction 1, the remaining 11% (76 to 65%) must have been present before subunit exchange occurred.

These subunit exchange equilibria (equations 1 and 2) were also investigated in the reverse direction. TetraPLP hemoglobin (P4) or symmetric diPLP hemoglobin (P2) were each mixed with unmodified hemoglobin in air to allow for complete subunit exchange. The equilibria were then frozen by anaerobic electrophoresis. The results are shown in Fig. 3. It is clear that the equilibrium of reaction 2 is displaced much further to the left than that of reaction 1, since subunit exchange of a mixture of P2 and P4 results in the formation of more than 80% of asymmetric P2.

This conclusion is also borne out by the oxygen equilibrium curves (12) shown in Fig. 4. A comparison of the curve obtained with an equimolar mixture of P4 and P0 (mixed in air) with that of symmetric P2 on the one hand and the calculated curve for the mixture on the other, again shows that the bulk of P4 has changed to asymmetric P2 with the concomitant disappearance of P0. This assumes, of course, that the oxygenation curves of symmetric and asymmetric P2 are very similar, which seems reasonable if one considers the stereochemical similarity of the interaction of the PLP in the two tetramers (Fig. 5).

**DISCUSSION**

The results presented in this paper demonstrate that pyridoxylation of hemoglobin under anaerobic conditions (deoxy conformation) is limited to two amino groups of the Chain, namely the a-amino group of Val-1 and the e-amino group of Lys-82. Pyridoxylation of hemoglobin appears to plateau when about 80% of the deoxyhemoglobin is monopyridoxylated at the a-amino group of Val-1 (\(\beta\)). This results in the elution of 40% of the hemoglobin as P2 when analyzed under aerobic conditions. Phosphate analysis clearly shows that about 80% of the label is present at Val-1 (\(\beta\)), and another 20% at Lys-82 (\(\beta\)). Interestingly, all the reaction product at Lys-82 (\(\beta\)) appears as dipyradoxylated \(\beta\) chain; and none could be detected as monopyridoxylated at Lys-82 (\(\beta\)).

The higher yield of \(\beta\) chains pyridoxylated at the a-amino group is apparently a reflection of the higher reactivity of this a-amino group compared with the e-amino group of Lys-82 (\(\beta\)). The higher reactivity of the a-amino group of the \(\beta\) chain is adequately explained by its low pKa (13). By contrast, the a-amino group of the \(\alpha\) chain remains unsubstituted since its pKa is raised by a system of salt bridges (14).

The unusual reactivity of the e-amino group of Lys-82 compared with most other e-amino groups present on the surface of the molecule probably arises from its location in the binding site with a special affinity for organic phosphates (15). A parallel situation was found in the fact of the dialdehyde, 2-nor-2 formylpyridoxal 5' phosphate, with deox-
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Pyridoxalization of hemoglobin. There, a specific cross-link is formed by the reaction of one of the aldehyde residues with the NH₂-terminal amino group, but the second also forms an imine with lysine 82 of the other β chain (3). It may be recalled here that this ε-amino group is also reactive toward glyceraldehyde (10) and glucose (16).

Stereochemically, the presence of four pyridoxyl phosphates crowded around the central cavity explains why P₄ subunits exchange so readily with unmodified hemoglobin to form asymmetric P₂ (Fig. 3). By the same token, the transformation of asymmetric P₂ to P₄ (equation 2) is rather slow, so that the isolation of pure symmetric P₄ requires sufficiently slow chromatography to allow the dismutation of asymmetric P₂ to go to completion. This clearly was the case by the method described here, since the β chains of P₂ contained none of the lysine substituted peptide b (Fig. 2).

Acknowledgments—We wish to thank Dr. Shigeru Sassa for making the fluorometer available to us and Sheenah Mische for the amino acid analysis. The assistance of Wanda M. Jones and Leslie G. Sussman is gratefully acknowledged.

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Labeling of hemoglobin with pyridoxal phosphate.
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*J. Biol. Chem.* 1982, 257:1320-1324.

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