A Critical Examination of the Reaction of Pyridoxal 5-Phosphate with Human Hemoglobin Ao*

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Synopsis

Pyridoxylated normal adult human hemoglobin (HbAo) has been prepared using both oxygenated and deoxygenated HbAo at pH 6.8 and room temperature without the addition of Tris to produce a mixture with P_{50} of 30 ± 2 torr and a Hill coefficient of 2.3 ± 0.1 similar to that of the isolated adult human hemoglobin from the red blood cell. Reduction of the pyridoxylated HbAo in the oxygen-ligated form by sodium borohydride gives unacceptable levels of methemoglobin (i.e., > 10%). Excessive foaming and methemoglobin formation can be partially avoided using deoxyHbAo. Reduction with sodium cyanoborohydride is much gentler and gives solutions with < 5% methemoglobin. Both reducing agents give products with multiple components as shown by analytical chromatography. Radioautography on the isoelectric focusing gels of HbAo treated with ^{14}C pyridoxal 5-phosphate (PLP) shows three major bands for the cyanoborohydride-reduced derivatives and a much more complex mixture of labeled molecules after the sodium borohydride reduction. When pyridoxylated hemoglobin is prepared without reduction, the preparation, after passage through a mixed-bed resin, contains 0.4 equivalents of PLP per heme, and has a P_{50} of 30 ± 2 torr and an n value of 2.3 similar to the values found after reduction. Upon anion exchange resin chromatography, the PLP is removed, indicating that the reaction forms a reversible Schiff base. On standing at 4°C for one month, this preparation produces a mixture of HbAo and pyridoxylated HbAo with the original P_{50}. Methemoglobin increased to 3% during this incubation. After four months in the cold, the yield of a single chromatographic species is 70% with 20% methemoglobin. This fraction appears to be stable and can be passed through an anion exchange column without release of the PLP. Separation of the individual chains by reverse-phase chromatography indicates that the addition of PLP to HbAo is directed solely to the β-chains. This is also the case for the cyanoborohydride reduced derivatives. When $NaBH_4$ is used for the reduction, radioactively labeled PLP is found on both the α- and β-chains.

INTRODUCTION

Hemoglobin solutions have been studied for many years as possible oxygen-carrying resuscitation fluids.[1,2] After hemoglobin is removed from the red cell it acquires two shortcomings as a "blood substitute." First, its oxygen binding is increased, as reflected by a drop in the P_{50} from 26 ± 2 to 12 ± 2 torr.

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Second, extracellular hemoglobin is quickly lost from the vascular system (vascular half-life ~ 3 h), which is generally considered due to the putative renal clearance of dimers from the tetramer–dimer equilibrium and to the catabolism of the hemoglobin.[3] To overcome these limitations several workers have proposed chemical modification of the hemoglobin. One such effort[4,5] to prepare a modified hemoglobin solution for use as a blood substitute used stroma-free hemoglobin (SFH) and pyridoxylation with pyridoxal 5-phosphate (PLP) proposed by Benesch et al.[6] followed by glutaraldehyde cross-linking. The pyridoxylation step raises the P_{50} from 12 to 20–25 torr. Polymerization with glutaraldehyde forms unspecified intra- and intermolecular hemoglobin cross-links that retard the rate of renal clearance.[4,5]

Polymerization must be preceded by pyridoxylation to obtain an adequate P_{50} in the end product. These preparations were made from hemoglobin obtained by removal of the stroma from lysed red cells. The entire molecular milieu from the glutaraldehyde reaction was used as the final product. These solutions have promising physiological properties,[7-9] even though they contain many protein species. A later study of the procedure developed by DeVenuto and Zegna[4] and made with radioactive PLP was analyzed by isoelectric focusing and high performance liquid chromatography (HPLC).[10] Isoelectric focusing with radioautography of the intermediate pyridoxylated SFH produced 35 bands[11] of labeled protein. The number of PLP bands is not surprising since the starting SFH solution contains all the erythrocytic enzymes and hemoglobin species present in the red blood cell.

In this report the pyridoxylation reactions are reexamined using a purified adult human hemoglobin Ao (HbAo) as the starting material instead of the heterogeneous SFH used previously. With a pure starting material, the reactions might be more readily characterized on a biochemical level. By using new advances in HPLC column technology, we could better determine heterogeneity in the product mixture due solely to the reactions of PLP with HbAo. In addition, the effects of reducing agent(s) on the reaction products of PLP were evaluated. These effects had previously not been characterized. Analysis of the reaction products of pyridoxylated HbAo polymerized with glutaraldehyde will be presented in a subsequent paper.

EXPERIMENTAL

Stroma-Free Hemoglobin

Outdated, packed human red blood cells were washed three times with cold isotonic, sterile saline to remove extracellular protein and the buffy coat. The packed cells were then lysed with 2.5 volumes of cold, sterile distilled water and centrifuged at 20,000 × g for 1 h. To avoid contamination with stroma, only the upper two-thirds of this solution was removed and passed through a mixed-bed ion exchange column (26 × 100 cm, BioRex RGJ01-x8) to obtain solutions of 4–6 g/dl of SFH with conductivities less than 10 μmhos and at a pH of 7.2 ± 0.1. These procedures were conducted at 4°C, and the final deionized solutions were passed through a 0.22-μ filter (Millipore Millistack 40) using gravity flow.

Hemoglobin Ao

Aliquots of SFH were purified by HPLC on the Waters Delta Prep 3000. Samples containing 10–20 g of hemoglobin were placed on a 5.7 × 30 cm stainless steel column, prepacked with Waters QMA-Accell, and equilibrated with Buffer A (50 m*M* Tris HCl, pH 8.5). The column was eluted with a linear gradient of Buffer B (50 m*M* Tris HCl, pH 6.5) at a flow rate of 80 mL/min. The major peak of HbAo was collected in about 30 min. The eluant was concentrated (Amicon Model 2000B, PM10 membrane) to approximately 10 g/dl and again passed through the mixed-bed resin to obtain solutions with conductivity of < 10 μmhos and pH of 7.3 ± 0.05. The solutions were sterile filtered (0.22-μ membrane) into polyvinyl chloride transfer packs and stored at 4°C. The detailed analysis of HbAo prepared in this way has been reported.[12]

PLP was purchased from Sigma, and the ^{14}C-labeled derivative was prepared by Amersham Corporation. Concentrations were estimated by the absorption at 295 nm of solutions in 0.1*M* HCl using a molar absorptivity of 6700.[13] All other reagents used were of the highest purity available.

Pyridoxylation

Deionized solutions of HbAo (1–6 m*M* heme with conductivities < 10 μmhos) were placed into the titration vessel of a Radiometer TTA 80 titration assembly and adjusted to the desired pH with an ABU autoburette. Water-saturated air or nitrogen was then flushed over the surface for periods of up to 4 h at room temperature. Deoxygenation was considered complete when no addition of titrant had occurred in one-half hour. PLP was then added to give the desired concentration and allowed to react for one-half hour, at which time no addition of titrant was needed to maintain the pH. PLP could be added either as a powder or as a water solution adjusted with KOH to the proper pH. Aliquots were removed and assayed for methemoglobin formation and P_{50}. Similar aliquots were passed through the mixed-bed resin (equilibrated with water saturated with air or nitrogen as needed), or reduced directly, collected aseptically, assayed, and stored at 4°C.

Reduction of Pyridoxylated HbAo

Pyridoxylated samples prepared as above were reduced prior to being passed through the mixed-bed ion exchange column using a 3 molar excess per heme of either sodium borohydride or sodium cyanoborohydride (Aldrich). The reduction was generally completed in 1 h. These solutions were then deionized and assayed. Excessive foaming occurred with $NaBH_4$ reduction of the ligated PLP–HbAo, which resulted in an increased formation of methemoglobin as high as 30%. Generally this effect had been moderated using antifoaming agents, but the use of these agents introduced another material that ultimately had to be removed. Reduction in the deoxy state, however, produced lesser amounts of methemoglobin and was the method used for these studies. It was apparent that the reduction in this condition necessarily yielded only the unliganded reaction products.

Analytical Procedures

Hemoglobin concentrations were estimated from the absorbance at 524 nm (Hewlett-Packard 8451A diode array spectrophotometer) using a millimolar absorptivity of 7.8[14] and verified, when needed, by the method of Drabkin.[15] The selection of this wavelength gave a reasonable estimation of the total hemoglobin concentration in a mixture of oxy-, deoxy-, and methemoglobin. Methemoglobin was estimated by the method of Evelyn and Malloy.[16] Phosphate analysis, on the deionized samples only, was conducted as described by Ames and Dubin.[17] Oxygen equilibria were performed at 37°C using a Hemox analyzer and the Hemox buffer (TCS Corp., South Hampton, PA). Values for P_{50} were taken directly from the graphs, and the Hill coefficients were calculated from a plot of the logarithmic values of the fractional saturation at 40–75% against the log of the oxygen tension.

Analytical Chromatography

Analytical chromatograms of the samples (100 μL, 1 g/dL) were performed on a Pharmacia MonoQ resin column (0.5 × 5 cm) using the buffers described for the preparative column. However, a better separation was achieved using 20 m*M* Tris HCl, pH 8.0 (Buffer A) and a linear gradient of Buffer B (0.4*M* NaCl in Buffer A).[18] Normally 1 mg of the PLP–HbAo reaction mixture was injected and eluted at a flow rate of 1 mL/min at room temperature. This same buffer system was used with the MonoQ HR 10/10 column with 500 μL of a 4 g/dL sample for the separation of ^{14}C-labeled derivatives. Fractions (2 mL) were collected, analyzed spectrally, and counted. Reverse-phase HPLC, by the method of Shelton et al.,[19] was used to evaluate the modification of the globin chains. Chain separation was performed on a Waters HPLC using a Wisp injector and detected with a 490 variable wavelength uv detector and an 820 controller. The column was a Vydac C4 (4.6 × 250 mm) with a particle size of 5 μ. A linear gradient of 47–51% solvent B was applied at a flow rate of 1 mL/min for a period of 55 min. After each run, the column was purged with 100% B. Solvent A was aqueous 20% acetonitrile, 0.1% trifluoroacetic acid (TFA) and solvent B was aqueous 60% acetonitrile with 0.1% TFA.

Estimation of Heterogeneity

Samples of the hemoglobin derivatives (0.5 g/dL) were applied to LKB Ampholine Pagplates, pH 3.5–9.5, and processed on 10% cross-linked gels for SDS polyacrylamide gel electrophoresis (PAGE).[20] Approximately 20–30 μg were applied, and the gel was stained with Coomassie blue and destained with 30% methanol: 5% acetic acid.

Radioactivity

Samples from the chromatographic separations (1 mL) were treated with 0.1 mL of 50% H_2O_2 and diluted with 10 mL OPTI-FLUOR. Samples were counted for up to one hour in a Packard Tri-Carb counter. Isoelectric focusing gels containing the ^{14}C-labeled proteins were assayed for radioactivity by applying the air-dried gel to an 8 × 10 inch sheet of Kodak X-omatic-AR x-ray film at room temperature for 1–4 weeks.

TABLE I
Effect of Concentration on the Reaction of PLP with HbAo

PLP/heme added	Deoxyhemoglobin				Oxyhemoglobin			
	P_{50}	n	MetHb	PO_4 M/heme[a]	P_{50}	n	MetHb	PO_4 M/heme[a]
0	12.5	2.3	1.1	0	13.0	2.3	1.8	0
0.25	21.4	1.9	1.2	0.14	19.5	1.9	3.0	0.11
0.50	26.3	2.1	1.5	0.23	25.5	2.1	4.1	0.20
0.75	27.7	2.1	1.7	0.36	25.3	2.2	4.6	0.24
1.00	27.2	2.2	1.9	0.47	29.5	2.3	5.7	0.41
1.25	29.4	2.3	2.0	0.55	28.0	2.3	6.7	0.43
1.50	28.5	2.4	2.1	0.67	30.1	2.3	6.8	0.49
1.75	—	—	—	—	29.9	2.2	8.6	0.54
2.00	30.2	2.4	2.6	0.78	29.5	2.2	9.6	0.54

[a] The concentrations of PLP were determined by the method of Ames and Dubin[17] after passage through a mixed-bed ion exchange column. These samples have not been reduced.

RESULTS

The conditions originally described by DeVenuto and Zegna[4] are those commonly used for the reaction of PLP with HbAo. The general procedure has also been used by McGarrity et al.,[18] Menu et al.,[21] and Marks et al.[22] with only minor modifications. We found that no particular advantage was gained by using deoxyHbAo (Table I) since the P_{50} values were essentially the same for both the oxy and deoxy preparations. Oxygen equilibrium curves are also the same for both preparations with virtually identical Hill coefficients. The oxygen equilibria curves for the reaction of PLP with the ligated HbAo are shown in Fig. 1. These same results may also be obtained without prior

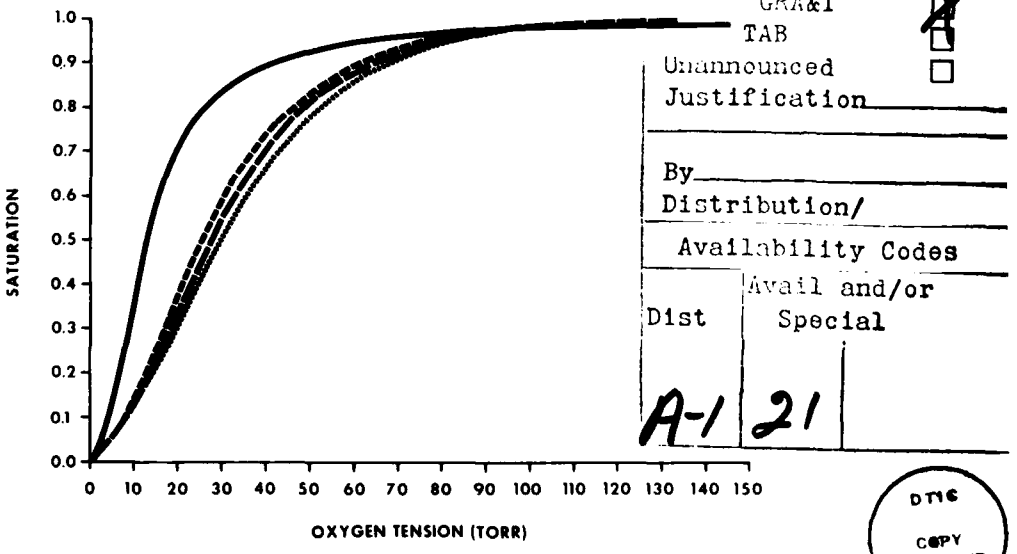

Fig. 1. Oxygen equilibrium curves of HbAo (solid line) and the pyridoxylated derivatives. The P_{50} values are 26, 28, and 30 torr, respectively, for cyanoborohydride reduction (short dash), borohydride reduction (long dash), and the unreduced derivative (points).

TABLE II
Analysis of the Reaction of PLP with HbAo[a]

Treatment	MetHb (%)	P_{50} (torr)	n	PO_4 (M/heme)
Unreduced	1.9	29.5	2.3	0.43
$NaBH_4$	3.9[b]	27.8	2.4	0.78
$NaCNBH_3$	1.3	25.7	2.4	0.43

[a] The reaction was conducted with oxyhemoglobin and an equimolar concentration of PLP:heme. The reductions were conducted with a 3 : 1 molar ratio of reducing agent to heme.
[b] Reduction in the deoxy state.

removal of the excess PLP on the mixed-bed column. Reduction of these reaction products with $NaBH_4$ in the ligated state is unnecessarily harsh and produces excessive amounts of methemoglobin, possibly because of the vigorous foaming that occurs when the pyridoxylated oxyHbAo is used. Reduction using $NaCNBH_3$ is preferred since no foaming occurs and the methemoglobin conversion is not only inhibited but considerably lessened (Table II). Invariably, based on the concept that Schiff base formation is faciliated by prior formation of the imine, the pyridoxylation has been performed in the presence of Tris[6]; however, we find there is no need for this additional component.

Analytical chromatography of the two reduced preparations of pyridoxylated HbAo solutions [Fig. 2(B, C)] gave profiles for the reduced products, which were similar to those found by McGarrity et al.[18] but considerably more detailed than those reported by Menu et al.[21] The amounts of products formed are approximately 70% for the cyanoborohydride reduction and 75% for the borohydride reduction. Heterogeneity shown on the analytical chromatograms is confirmed by the results of reverse-phase chromatography and isoelectric focusing gels (Table III, Figs. 3 and 4).

The unreduced preparation of pyridoxylated HbAo gave a peak identical to that of the original HbAo [Fig. 2(A)]. After chromatography, this fraction has a P_{50} of 12 torr similar to that of the original HbAo and contains little or no phosphate, as indicated by the absence of radioactivity [Fig. 2(A)]. Apparently the initial binding of PLP is of low energy and can be dissociated on the anion exchange column, as may be expected for a Schiff base.

When solutions of unreduced PLP and oxy- or deoxyHbAo were allowed to incubate at 4°C for periods of up to a month, the P_{50} was maintained and analytical chromatography gave a profile of two peaks of roughly equal proportions [Fig. 5(A)]. The second peak contains all the radioactivity and has $P_{50} = 30 \pm 2$ torr. The first peak is located in the position where HbAo is eluted and has $P_{50} = 12 \pm 2$ torr. When the second peak is rechromatographed, the same two peaks are again obtained, indicating the equilibrium nature of this product. Reduction of the material in the second peak gives a chromatographic profile similar to those shown in Fig. 2(B, C). After 4 months, however, the pyridoxylated derivative can be repeatedly chromatographed without reversion to HbAo. The amount of the product formed at this time is about 70% with a 20% concentration of methemoglobin [Fig. 5(B)]. Pyridoxyl Schiff bases exist as a mixture of two tautomers,[23] which may

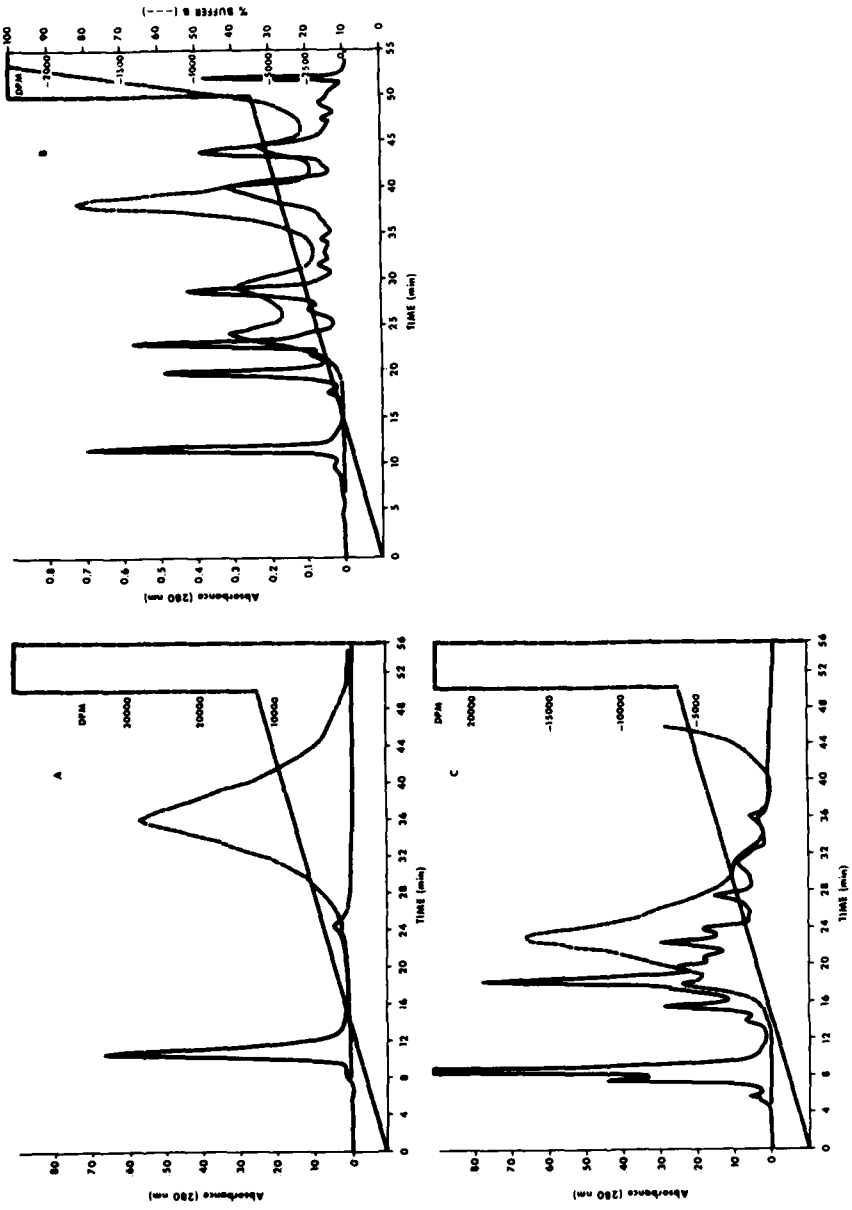

Fig. 2. Analytical chromatograms of the reaction products of HbAo with PLP (1 mole per heme). (A) Unreduced PLP–HbAo, (B) PLP–HbAo reduced in the deoxy form with 4 molar equivalents of sodium borohydride and (C) PLP–HbAo reduced with 4 molar equivalents of sodium cyanoborohydride. The dashed lines are the counts. The chromatographic conditions are described in the text for the analytical column.

TABLE III
Analysis of the Reverse-Phase Chromatograms of Pyridoxylated HbAo

Preparation	Peak (min)	Area (%)	Counts (%)
PLP–HbAo	25.2	1.0	0
	27.7	1.0	3.5
	34.1	10.1	4.7
	36.4	33.9	91.8
	45.6	40.6	0
PLP–HbAo	15.8	1.7	0
($NaBH_4$)	20.3	1.7	0
	24.1	11.7	45.5
	29.0	24.7	19.8
	36.8	2.8	1.8
	42.4	44.4	32.5
	51.2	0.8	0.4
PLP–HbAo	22.2	0.9	0
($NaCNBH_3$)	29.8	21.3	11.9
	32.6	17.9	88.1
	43.6	46.8	0
	51.6	1.4	0
	55.5	0.6	0
HbAo Pre-β	21.0	3.2	—
β	28.5	42.7	—
α	40.8	42.6	—

account for the changes seen upon incubation. The nature of the modification is currently being studied.

DISCUSSION

PLP was shown to react with hemoglobin to produce oxygen-binding effects analogous to that of the allosteric cofactor 2,3-diphosphoglycerate (2,3-DPG).[6] SFH, which had been stripped of 2,3-DPG, was deoxygenated with nitrogen and treated with a solution of PLP in Tris buffer. The PLP forms a Schiff base with the Tris, which "reacted with hemoglobin by a transamination reaction" (Benesch et al., Ref. 6, p. 3577). The new Schiff base formed between PLP and hemoglobin was reduced with sodium borohydride. Separation of this material on a phosphocellulose column produced four species: unreacted hemoglobin (45%), monoreacted PLP/hemoglobin (6%), direacted (45%), and trireacted (4%). The major binding site was identified as the N-terminal valine of the β-chain. Later, a secondary reaction site was found on the ϵ-amino group of lysine β-82.[24] The predominant product is a symmetric diPLP derivative formed by subunit rearrangement of the monoPLP upon exposure to oxygen. Reaction of PLP with liganded (oxy) hemoglobin is reported to occur at the α-chain N-terminal valines, but this was reported to not produce a change in oxygen affinity as seen for the β-chain reactions.[25]

Since PLP is thought to act as a surrogate for 2,3-DPG, which has its effect on the oxygen equilibrium by insertion into the β-cleft, (25, 26), presumably pyridoxylation could occur with the N-terminal valines of either or both of the chains. Upon reduction, both the α- and β-chain valines are found to be

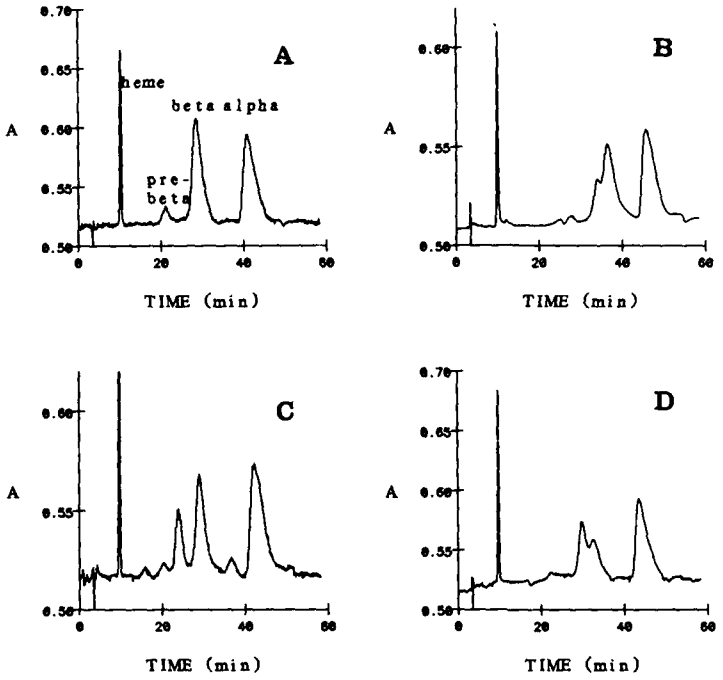

Fig. 3. Reverse-phase chromatograms of unreacted HbAo (A), unreduced PLP–HbAo (B), PLP–HbAo reduced with sodium borohydride (C), and PLP–HbAo reduced with sodium cyanoborohydride (D). The samples were 5 μL of 1 g/dl solutions. The β- and α-chain regions of the native molecule have been labeled. The peaks were detected at 220 nm.

modified as well as β-82 lysine.[18] The proliferation of species may partially result from the dimer–tetramer equilibrium[21] and by randomization, which has been shown to occur with glyceraldehyde-labeled hemoglobin upon reduction.[27] However, the results from reverse-phase chromatography indicate that the unreduced preparation and the derivative reduced with $NaCNBH_3$ appear modified only on the β-chains (Table III). The products of the $NaBH_4$ reduction indicate that the PLP is distributed on both the α- and β-chains, and that a number of additional species are present. As expected, in no instance was there any indication of polymerization on SDS PAGE plates.

Although six major bands are found by chromatography,[18] many more species are actually present [Figs. 2(B, C) and 4]. Pristoupil et al.[28] reported 21–25 species and Moore et al.[11] showed 35 bands, although they both used SFH for their preparations. The result of the isoelectric focusing show that the unreduced and the $NaCNBH_3$-reduced preparations are similar (3 major bands; 7–10 species), but that the $NaBH_4$-reduced material consists of 20–25 species. It is clear that reduction of PLP–HbAo by $NaBH_4$ leads to a proliferation of products that contain variable amounts of PLP (Figures 2, 4, and 6). Use of HbAo, however, certainly results in mixtures that are less complex than those previously reported. The patterns for unreduced and $NaCNBH_3$-reduced HbAo by both IEP and reverse-phase chromatography are remarkably similar and considerably less heterogeneous than the $NaBH_4$-reduced products.

Fig. 4. Isoelectric focusing electrophoresis of the pyridoxylated derivatives of HbAo. The left lane is the unreduced PLP–HbAo, the middle lane is the sample reduced with $NaCNBH_3$, and the right lane is the sample reduced with $NaBH_4$. The gradient is from pH 3.5 to 9.5. Samples (2 g/dL) of 30–40 μL have been applied. The gel is not stained.

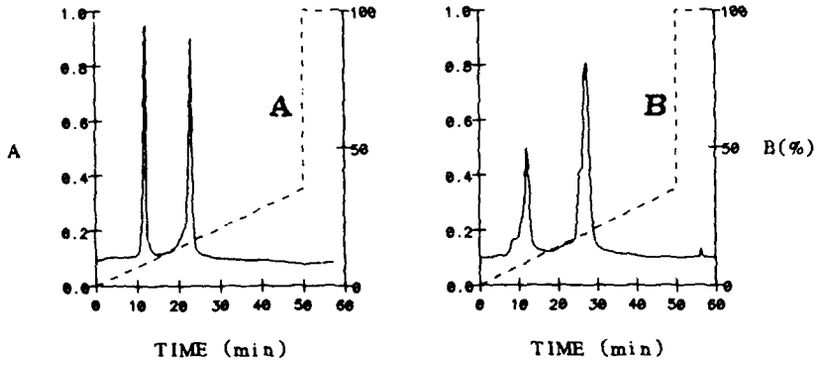

Fig. 5. Effect of aging on the chromatographic pattern of unreduced PLP–HbAo. (A) After three weeks at 4°C using the same conditions as those for Fig. 2. (B) Result of aging for 4 months at 4°C. There is a 70% yield of the derivative with a 19.5% methemoglobin content. The chromatographic conditions are those described for the analytical system. The gradient is shown as the dashed line.

Fig. 6. Autoradiography of the isoelectric gel electrophoresis of the samples shown in Fig. 4.

Unreduced PLP-treated HbAo gives a single phosphorylated product with an elevated P_{50} (Figs. 1 and 5), as shown by chromatography. Pyridoxylation of oxyHbAo also gives a single chromatographically separable peak with an increased P_{50}. On the basis of reverse-phase analysis (Fig. 3), the reaction appears to be directed solely to the β-chains. This result is in disagreement with Schnackerz et al.,[25] who reported that the PLP reacts with the α-chain N-terminal valines in the ligated state, which leads to an increased oxygen affinity. Despite the ease of preparation and the satisfactory P_{50} values obtained with either oxy- or deoxyHbAo, the stability of unreduced PLP-treated HbAo is questionable. It is initially formed as a Schiff base, which has an equilibrium constant near unity and is thermodynamically unstable. At the concentrations to be used for resuscitation therapy, the release of large quantities of PLP would not be desirable since it has been shown that PLP inhibits a variety of enzymes.[29] The stable derivative found after longer incubation times may possibly be useful but the high methemoglobin content is a distinct disadvantage.

Using the Hemox analyzer and the Hemox buffer, the P_{50} values are increased to desirable levels for both the reduced and unreduced derivatives. This finding is at variance with the observation that 2,3-DPG, in equivalent amounts in this system, does not elevate the P_{50}. The same effect was observed with the cross-linking reagent, 2-nor-formylpyridoxal 5-phosphate, which gave a derivative with $P_{50} = 35$ torr, whereas 3 m*M* 2,3-DPG and hemoglobin have a P_{50} of 14 torr.[30] They conducted their oxygenations at pH 7.2 in 0.1*M* Tris or bis-Tris containing 0.1M NaCl.

Conditions for the pyridoxylation of hemoglobin solutions have not been standardized, although this is the objective of two recent reports.[21,22] The optimal conditions recommended by Marks et al.[22] require the use of deoxygenated SFH solutions at pH 6.8 (Tris buffer) and 24°C. The reaction is run with 2.5 molar equivalents/heme and the reduction uses a 1 molar equivalent of $NaBH_4$. They also suggest that the reaction be performed at room temperature, and we also find very little difference in the reactions at 4°C and room temperature. Higher pH values tend to give products with a lower P_{50} and a lower methemoglobin content. Lower pH values tend to increase both the P_{50} and the methemoglobin. There is no need for the Tris buffer if the pH is controlled by pH stat. There is also no need to conduct the reaction in the deoxy state, although the methemoglobin concentrations are marginally lower (Table I). P_{50} values are essentially unchanged at PLP concentrations greater than equimolar, and the higher concentrations of the reagent produce greater heterogeneity and more oxidation. Reduction, if it is to be performed, should be done with $NaCNBH_3$, which gives a product with low methemoglobin and less heterogeneity. Elimination of the reduction step with a one-month incubation should be considered since this will maintain the desired P_{50} values and give an homogeneous and chromatographically pure product in about 50% yield. Considerably less heterogeneity may be also obtained by conducting the reaction on purified HbAo. With current HPLC preparative techniques, this purification is readily accomplished.

Mixtures of the pyridoxylated derivatives such as those previously reported may be suitable for resuscitation therapy if the overall functional properties are appropriate. However, with such complex mixtures, it is difficult to assign

properties of efficacy or toxicity to any of the components in the solution. A well-defined, homogenous preparation with suitable functional properties is preferred and is also desirable for quality control. The simplified procedure given here can be used to attain these goals. Since a resuscitation fluid must necessarily be administered in large quantities, it should be consistently prepared and be as pure and as uniform as possible.

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