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BIS-PYRIDOXAL POLYPHOSPHATES: A NEW CLASS OF SPECIFIC INTRAMOLECULAR CROSSLINKING AGENTS FOR HEMOGLOBIN

Ruth E. Benesch and Suzanna Kwong

Department of Biochemistry and Molecular Biophysics
Columbia University, 630 W. 168 St., New York, N.Y. 10032

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A series of compounds related to bis-pyridoxal phosphate has been synthesized and used to crosslink deoxyhemoglobin. The yield of crosslinked hemoglobin increased dramatically from about 15% for the di- or triphosphate to about 70% for the tetraphosphate. The site of attachment of the intramolecular crossbridge was found to be from the N-terminal amino group of one β chain to lysine 82 of the other. Since the distance between these residues is only 11Å, the bis-pyridoxal tetraphosphates probably have a "stacked" conformation. The crosslinked hemoglobins bind oxygen cooperatively but with a greatly decreased affinity. The increased ability to unload oxygen together with the stabilization of the tetramer qualifies them as promising cell-free blood substitutes.

Hemoglobin crosslinked covalently between the αβ dimers is of interest from two points of view. One is to see to what extent dissociation into αβ dimers and especially subunit exchange between these dimers and intact tetramers (1, 2) play a role in the conformational change of the oxygen carrier from the T state to the R state. The second is that in order to use hemoglobin as an emergency blood replacement, dissociation into αβ dimers must be prevented, since these are filtered by the glomerulus and therefore rapidly excreted.

A number of crosslinking agents for this purpose have been described, including diaspirins (3), a dialdehyde derivative of pyridoxal phosphate (4) and most recently 4,4'-diisothiocyanostilbene-2,2'-disulfonate (5).

In this communication we describe a series of new crosslinking reagents consisting of two pyridoxal residues joined covalently by a bridge containing phosphates.

Materials and Methods

These bis-pyridoxal phosphate (bis-PLP) derivatives are listed in Table I. Only No. 1, (bis-PL) P_2 has been described previously (6). All the compounds were synthesized by the anion exchange method of Michelson (7) as modified for pyridoxal derivatives by Fukui et al. (6, 8, 9).

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The syntheses involved the following steps:

(1) The reactants were converted into their tributylammonium salts by standard methods.

(2) Pyridoxal 5' diphosphate β-diphenyl ester was prepared from tributylammonium pyridoxal phosphate by reaction with diphenylchlorophosphate at room temperature for 3 hours followed by extraction of the excess reagent with ether as described (6,7). It was used in the anion exchange reaction for all the compounds except no. 2, which required pyridoxal-5'-triphosphate γ diphenyl ester. The latter was prepared in the identical manner from pyridoxal diphosphate, synthesized as described by Fukui et al. (6).

(3) Anion exchange was allowed to proceed at room temperature for 1-1½ hours in dry pyridine (~ 1 ml per millimole of the diphenylester). The anions used, as their tributylammonium salts, were pyridoxal phosphate for compounds 1 and 2, pyrophosphate for compound 3, methylene diphosphonate for no. 4, fructose 1,6 diphosphate for no. 5 and 2,3 diphosphoglycerate for no. 6. The ratio of diphenylester to anion in the exchange reaction was 0.6/1 for compounds 1 and 2, and 4/1 for the others.

(4) The products were purified by chromatography on a 1.5 x 17 cm column of Dowex 1-X2 using a gradient of LiCl from 0 to 0.2 M in 0.01N HCl (6,7). After concentration to a small volume and precipitation with acetone, the lithium salts of the bis-pyridoxal polyphosphates were further purified by chromatography on silica gel, using MeOH/$CHCl_3$ (6:4) as the solvent.

Solutions of the bis-pyridoxal polyphosphates were standardized spectrophotometrically using the molar extinction coefficient $\varepsilon_{392} = 9.400$ reported for (bis-PL) P_2 (6). Normal adult human hemoglobin (HbA) was prepared from fresh blood and stored in liquid N_2 as described previously (10).

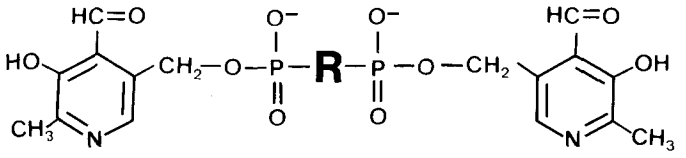
The reaction with deoxyhemoglobin was carried out in an atmosphere of argon at 10°C exactly as described previously for 2-nor-2-formylpyridoxal 5'-phosphate (4,11). After reduction with $NaBH_4$, the argon was replaced by CO. The yield of crosslinked hemoglobin was determined by passage of the reaction mixture through a 2.5 x 35 cm column of Sephadex G-100 "superfine" in 1.0M $MgCl_2$. Under these conditions uncrosslinked hemoglobin is completely dissociated into αβ dimers (12) and excellent separation between dimers and tetramers is achieved. The methods used to determine the site of substitution on the hemoglobin molecule have been described (10).

Results and Discussion

The bis-pyridoxal polyphosphates used for this study are listed in Table I together with their extinction coefficients in the U.V. It is clear that the introduction of a fourth phosphate between the two pyridoxal rings is accompanied by a decrease in the extinction coefficient of the 392 nm absorption band while the shoulder at 324 nm is replaced by another absorption maximum. These changes in spectrum would be expected if "stacking" of the two pyridine rings occurs in compounds 3-6 but not in compound 1 or 2 (13). In that case there should be a substantial change in the distance between the two aldehyde residues on going from (bis-PL)P_3 to (bisPL) P_4 and this is completely borne out by the results on their reaction with hemoglobin. As can be seen from Table II, there is a dramatic increase in the amount of intramolecular crosslinking with the introduction of a fourth phosphate between the two pyridoxal rings.

TABLE I

Bis-Pyridoxal Polyphosphates



No.	Abbreviation	R	εM	
			392 nm	324 nm
1.	(bis-PL) P_2	Oxygen	8.800	3.900 (sh)
2.	(bis-PL) P_3	Orthophosphate	9.100	4.400 (sh)
3.	(bis-PL) P_4	Pyrophosphate	6.700	4.700
4.	CH_2 (bis-PL) P_4	Methylene diphosphonate	6.800	4.500
5.	Fructose (bis-PL) P_4	Fructose 1,6-diphosphate	7.100	5.300
6.	PLP-DPG-PLP	2,3-diphosphoglycerate	7.600	5.700

The effect of different ratios of crosslinking agent to hemoglobin on the reaction is shown for two of the compounds in Fig. 1. It is clear that these compounds are remarkably efficient and specific in their reaction with deoxy hemoglobin, since the yield of intramolecularly cross-linked hemoglobin is over 60% when only stoichiometric amounts are used. By contrast, using diaspirins the yield is about 15% (14) and with 4,4'-diisothiocyanostilbene-2,2'disulfonate it is only 5% (5). Furthermore, it should be noted that the tetramer fraction isolated from the reaction

TABLE II

Covalent Intramolecular Crosslinking of Deoxy Hemoglobin by the Bis-Pyridoxal Polyphosphates

Compound	% Tetramer
(bis-PL) P_2	16
(bis-PL) P_3	18
(bis-PL) P_4	68
CH_2 (bis-PL) P_4	53
Fructose (bis-PL) P_4	41
PLP-DPG-PLP	70

The reactions were performed as described (4, 11) using 1.4 moles of bis-pyridoxal compound per mole of deoxy hemoglobin. The proportion of tetramer was determined by gel filtration as described in the text.

of hemoglobin with 1 mole/mole (or less) of these compounds was entirely homogeneous by acrylamide gel electrophoresis (data not shown) whereas higher ratios gave rise to a minor component with a larger anionic mobility.

The site of attachment of the crosslink on the hemoglobin molecule was determined as follows: - The modified hemoglobin was dissociated into α and β chains by chromatography on CM cellulose in urea as described (15). The α chains were normal as judged by their elution volume, U.V. spectrum and the absence of phosphate. The β chains eluted earlier than normal ones, contained all the phosphate and had a U.V. absorption with a maximum at 325 nm, typical of pyridoxylamino acids. The label was further localized by chromatography of a pronase hydrolysate of the globin on a sulfonic acid resin (UR-30) as described previously (10). Equal amounts of pyridoxyl-lysine and pyridoxyl-valylhistidine were found together with a trace of pyridoxyl valine. These results suggest strongly that the bis-pyridoxal polyphosphates crosslink deoxyhemoglobin tetramers at the same site as 2-nor-2-formylpyridoxal 5'-phosphate, i.e. between the N-terminal amino group of one β chain and lysine 82 of the other one.

The distance between these two amino groups is about 11Å in deoxyhemoglobin (16). This is much smaller than the distance between the two aldehyde residues in the bis-pyridoxal tetraphosphate compounds in their extended form. The very high affinity of these compounds for this particular site therefore strongly confirms the idea that their pyridine rings interact. It will be of great interest to see whether the conformational change with ligand binding, which involves a large increase

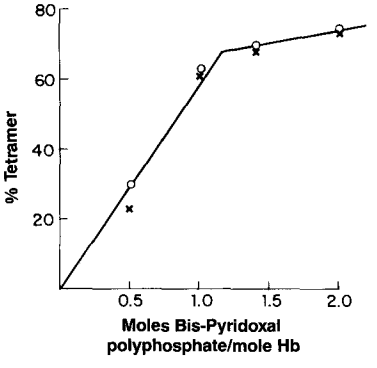


Figure 1. Effect of pyridoxal polyphosphate concentration on the yield of crosslinked hemoglobin.

The reactions were carried out with 2.5×10^{-4}M deoxyhemoglobin as described (4, 11), using the indicated concentrations of pyridoxal polyphosphate.

Crosses = $(bis\text{-}PL)P_4$
Open Circles = PLP-DPG-PLP

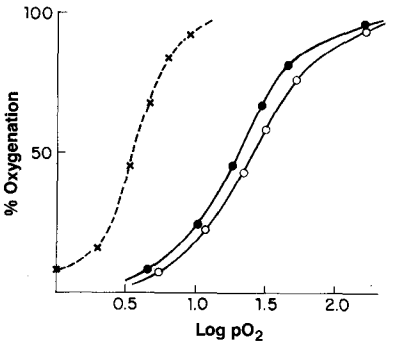


Figure 2. Oxygen Equilibrium Curves of Native and Crosslinked Hemoglobin.
Crosses = native HbA
Filled Circles = tetramer crosslinked with compound 3, Table I
Open Circles = tetramer crosslinked with compound 6, Table I
All measurements at 20°C in 0.05M bisTris pH 7.3, 0.1M Cl^-, 0.1mM EDTA.

in the distance between the two amino groups to which the crosslink is attached, destroys the interaction of the pyridine rings within the crosslinked hemoglobin.

The location of the crosslink in the central cavity between the β chains is also borne out by the oxygenation curves in Fig. 2. The crosslinked hemoglobins have a greatly decreased affinity for oxygen but retain cooperativity. In fact, both the position and the shape of their oxygen binding curves is very similar to that of unmodified hemoglobin in the presence of the most powerful allosteric regulator, inositol hexaphosphate.

In conclusion, the bis-pyridoxal polyphosphates appear to have a number of advantages as intramolecular crosslinking reagents for hemoglobin:

(1) The synthesis by the anion exchange method involves only two steps and can easily be completed in three days including purification of the product.

(2) Other functional groups can be readily incorporated between the two pyridoxal rings. Thus, for example, the fructose compound, no. 5, was invaluable for following the purification by monitoring the fructose/pyridoxal and fructose/phosphate ratio.

(3) The reaction of the compounds with deoxyhemoglobin shows an exceptional degree of specificity and a very high yield of crosslinked tetramer (Fig.1).

(4) Under the conditions used for the reaction with hemoglobin there is no intermolecular crosslinking since no hemoglobin species with molecular weight higher than the tetramer were found.

(5) Crosslinking with bis-pyridoxal polyphosphates simultaneously overcomes the two major disadvantages of hemoglobin as a blood substitute

since it stabilizes the tetramer and at the same time lowers the oxygen affinity and therefore dramatically facilitates oxygen unloading (Fig. 2).

Acknowledgements

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