HEMOGLOBIN COVALENTLY BRIDGED ACROSS THE POLYPHOSPHATE BINDING SITE

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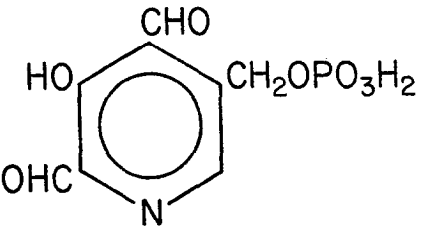
Summary Hemoglobin has been cross-linked covalently by reaction with 2-nor-2-formylpyridoxal 5'-phosphate followed by reduction with sodium borohydride. It is shown that a bridge is formed across the polyphosphate binding site between the two β chains. The modified hemoglobin binds oxygen cooperatively with a greatly decreased affinity demonstrating that the cross-link stabilizes the deoxy conformation but does not prevent the conformational change associated with oxygenation.

We have previously shown that pyridoxal phosphate (PLP) has analogous effects to 2, 3 diphosphoglycerate (DPG) on the oxygen affinity of hemoglobin (1). Furthermore, PLP can be attached covalently to the N-terminal amino groups of the β chains by reduction of the initially formed Schiff's base with sodium borohydride, leading to a hemoglobin with a permanently lowered oxygen affinity (2, 3).

The synthesis of a bifunctional analog of PLP, i.e. 2-nor-2-formylpyridoxal 5'-phosphate



has been described recently (4). This compound was designed to introduce

[1] Abbreviations: PLP, pyridoxal phosphate; DPG, 2,3 diphosphoglycerate; NFPLP, 2-nor-2-formylpyridoxal 5'-phosphate; SDS, sodium dodecyl sulfate.

stable cross-links into enzymes by reduction of the imines formed between the two aldehyde groups and suitably situated amino groups on the protein (4). This paper deals with the reaction of hemoglobin with this compound and the preparation and properties of the cross-linked derivative.

The effect of increasing concentrations of NFPLP on the oxygen affinity of hemoglobin is shown in Figure 1, together with PLP for com-

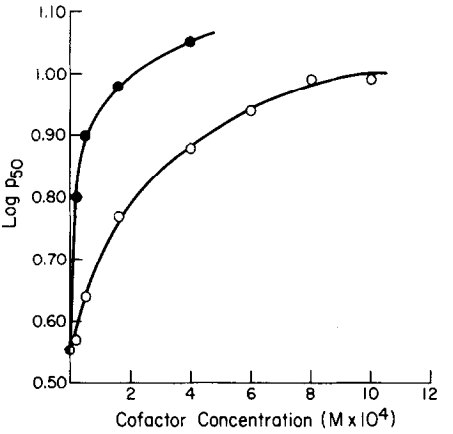


Figure 1. Effect of NFPLP and PLP on the Oxygen Affinity of Hemoglobin

Filled circles NFPLP
Open circles PLP

Hemoglobin concentration 1 x 10^{-5} M (as tetramer) in 0.1 M Tris buffer pH 7.30, total chloride 0.1 M, temperature 20°C. p50 is the oxygen pressure at 50% oxygenation. Oxygen equilibria were measured by the method of Benesch, et al. (15).

parison. The results clearly show that the bifunctional reagent produces a much larger decrease in the oxygen affinity, especially at low concentrations, indicating tighter interaction with deoxyhemoglobin.

Covalent attachment of NFPLP to deoxyhemoglobin was brought about by reduction with $NaBH_4$ as described previously for PLP (3). The product was isolated by preparative polyacrylamide gel electrofocussing

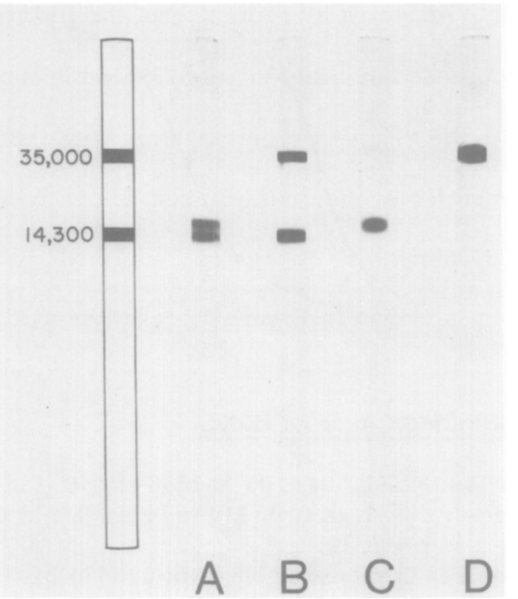


Figure 2. SDS Acrylamide Gel Electrophoresis

A - Unmodified HbA
B - HbXL
C - α chains prepared from HbXL
D - β chains prepared from HbXL

The marker proteins used were lysozyme (14,300 daltons) and pepsin (35,000 daltons).

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in a pH 6 - 8 gradient (5). It appeared as a sharp band representing 50 - 60% of the total hemoglobin and with a lower isoelectric point than the unmodified protein. The isolated derivative (HbXL) was found to contain one phosphate per hemoglobin tetramer. Separation into α and β subunits (6) gave only normal α chains and β subunits with a faster mobility than normal. Only the β fraction contained phosphate which amounted to one-half a phosphate per β chain.

The existence of a cross-link between the β chains, suggested by these results, was confirmed by SDS acrylamide gel electrophoresis (7) of the globin prepared from HbXL and its constituent α and β chains. Under

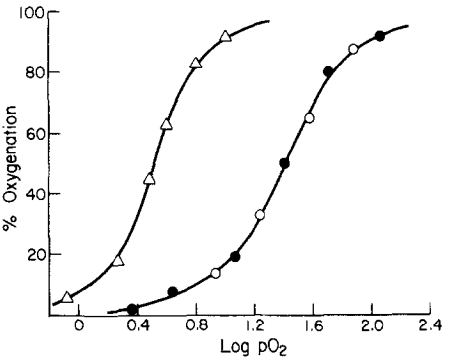

Figure 3. Oxygen Binding Curve of HbXL

Filled circles: HbXL in 0.05 M bisTris, 0.1 M Cl^-, pH 7.30.
Open circles : HbXL in 0.05 M bisTris, 0.1 M Cl^-, 2 x 10^{-4} DPG. pH 7.30.
Open triangles: Unmodified Hb A in 0.05 M bisTris, 0.1 M Cl^-, pH 7.30.
Hemoglobin concentration 5 x 10^{-5} M (as tetramer), temperature 20°C.

Table I

Oxygenation Parameters of HbXL

	log p50 (a)		n (b)	
	0.1 M Cl^-	0.015 M Cl^-	0.1 M Cl^-	0.015 M Cl^-
HbXL	1.40	1.21	1.9	2.2
HbA	0.55	0.15	2.7	2.7

(a) p50 is the oxygen pressure at 50% oxygenation
(b) n is the exponent in the Hill equation

$$\frac{Y}{1-Y} = K(pO_2)^n$$

where Y is the fractional oxygenation.

The hemoglobin concentration was 1 x 10^{-5} M (as tetramer) in 0.05 M bisTris buffer pH 7.3, temperature 20°C.

these conditions, unmodified hemoglobin shows a split band corresponding to a molecular weight of about 17,000 daltons (Fig. 2A). HbXL, on the other hand, appears as two bands, of equal concentration, with molecular weights

corresponding to monomers and dimers respectively (17,000 daltons and 34,000 daltons)(Fig. 2B). Since, on separation into subunits, only monomeric α chains and dimeric β chains are obtained (Fig. 2C and D) the cross-link is clearly located between the two β chains.

HbXL binds oxygen with a dramatically reduced affinity (Fig. 3) comparable to that of unmodified hemoglobin in the presence of millimolar concentrations of inositol hexaphosphate (8). The new bridge between the β chains therefore greatly stabilizes the deoxy as against the oxy conformation of the molecule. The oxygenation curve of the cross-linked hemoglobin is completely unaffected by DPG (Fig. 3), indicating that the polyphosphate binding site is blocked. Nevertheless, ligand binding is still influenced by chloride (Table I), although this effect is smaller than in the case of Hb A. The sigmoid character of the oxygen binding curve of the cross-linked hemoglobin is particularly noteworthy and clearly demonstrates that a significant conformational change can still take place in this protein (Fig. 3 and Table I).

The exact location of the cross-link remains to be determined. However, the above evidence that NFPLP must connect the two β chains, together with our previous work on PLP and the failure of HbXL to respond to DPG, all point to the polyphosphate binding site as a likely locus. There are four free amino groups in this region, the two α amino groups of the β-1 valines and the two ϵ amino groups of the β-82 lysines. Although the former were previously shown to be the site of attachment of PLP to deoxyhemoglobin (3), the N-termini are too far apart (18Å) to be connected by NFPLP. The distance in deoxyhemoglobin between the ϵ amino groups of the β-82 lysines, i.e. 8.1 Å, on the other hand, is very

close to the distance between 2 N atoms attached to the 2' and 4' positions of the reagent, i.e. 7.5 Å.[2] There remains the possibility of a cross-link between the N-terminal amino group of one β chain and the 82 lysine of the other, but this seems less likely since the distance is 11 Å.[3]

The available evidence therefore points to a cross-link between the β-82 lysines. The flexibility of lysine side chains would create a cross-link with minimum rigidity which would allow the considerable degree of conformational change observed on ligand binding. The introduction of this cross-link also demonstrates that a hemoglobin tetramer can bind ligand cooperatively without subunit exchange (10).

HbXL appears to be the first cross-linked hemoglobin with significant retention of allosteric properties. Previous attempts to cross-link hemoglobin with bifunctional reagents (11 - 14) have resulted in derivatives which had lost the linked functions associated with conformational change.

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[2] Reaction with lysine side chains is quite plausible, since this is, in fact, the site of attachment of PLP to numerous enzymes (9).

[3] These distances were calculated from the atomic coordinates of human deoxyhemoglobin, kindly supplied by Dr. Arthur Arnone.

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