Bovine Hemoglobin Cross-Linked through the β Chains

FUNCTIONAL AND STRUCTURAL ASPECTS*

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2-Nor-2-formylypyridoxal (NFPLP) has been synthesized and coupled to bovine Hb according to the procedure described by Benesch and Benesch (1). The reaction of bovine Hb with NFPLP leads to a cross-linkage between the β subunits, which greatly stabilizes the low affinity T state of the molecule and simultaneously abolishes the tendency of the tetramer to dissociate into αβ dimers. The functional properties, examined from both the equilibrium and kinetic points of view, indicate that the chemical modification affects the O2 affinity, abolishes cooperativity, and induces a slight decrease of the Bohr effect. From modeling studies we are confronted with two different structural alternatives; the cross-link of β chains may be formed between lysine 82 of β2 and the N terminus of methionine 2 of β3, or between two lysine 82 residues of both β3 chains. Digestion of modified β globin chains and isolation of the cross-linked peptide have showed that NFPLP cross-links Met-β2 and Lys-β82. This allowed discussion in some detail of the molecular basis of the Bohr effect of the modified bovine hemoglobin. On the whole, NFPLP-modified bovine Hb could be considered as a first step toward the synthesis of a potential blood substitute.

Over the last few decades, studies on structure-function relationships in hemoglobin have been greatly helped by naturally occurring mutants, artificially induced point mutations and specific chemical modifications at the level of some key amino acid residues. As far as the latter are concerned, the reaction of human hemoglobin with NFPLP1 (1) led to a covalent modification of great interest, as it yielded a hemoglobin with lowered oxygen affinity and almost unchanged cooperativity, thereby offering a unique opportunity to study the effect of organic phosphates without the complexity arising from dissociation of the effector. Moreover, hemoglobin modified by NFPLP offers additional biotechnological advantages since it shows some promise as an oxygen-carrying resuscitation fluid. In fact, this chemical modification overcomes two main disadvantages, which critically restrict the usefulness of free human Hb as an oxygen-carrying substitute for red blood cells. The first of these is inherent to the oxygen dissociation curve of free Hb whose P50 (partial pressure of oxygen at which 50% saturation is achieved) indicates a far greater affinity for O2 than normal human blood and therefore a significantly lower oxygen tension at the level of tissues; the second is related to the split of free Hb tetramers into αβ dimers, which are rapidly eliminated through the renal glomeruli and characterized by the absence of cooperativity and a very high oxygen affinity (2, 3). As previously reported by Benesch and Benesch (1), NFPLP, by reacting with valine 1 and lysine 82 of human Hb and forming a covalent bridge located in the 2,3-DPG crevice in between the two β chains, eliminates at a single blow both the problems reported above. In this perspective, since bovine Hb does not appear to cause rapid antibody formation when injected into non-bovine species (4), many authors have investigated the possibility of using bovine Hb as a substitute for human blood (5, 25). Along the same lines we used NFPLP to modify bovine Hb, with the aim of obtaining further information on the structure-function relationships of this hemoglobin, known to display peculiar functional characteristics. In fact, bovine Hb belongs to a group of mammalian hemoglobins that, in the presence of physiological concentrations of chloride, have an oxygen affinity lower than that of human HbA being also insensitive to the presence of 2,3-DPG. The failure of bovine Hb to respond to 2,3-DPG was explained by the peculiar N termini of β subunits, i.e. the deletion of the residue at position β1 and the presence of a hydrophobic residue, usually Met, at position β2. According to Perutz and Imai (6), these structural differences are at the basis of the decreased influence of organic phosphates. On this basis, bovine Hb appears to be modulated in vivo essentially by chloride ions, which may thus be considered its physiologically relevant effector. We were interested in investigating the possibility of improving the functional characteristics that render this protein a potential blood substitute through a specific chemical modification at the level of the anion binding site between the two β chains. To this end we were helped by a recent elegant work on the structural features of bovine hemoglobin (7), which allowed us to go further into the structure-function relationships of the NFPLP-modified bovine hemoglobin.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals employed were obtained in the highest grade commercially available from Aldrich. Bovine blood was provided from the local slaughterhouse.

Preparation of NFPLP—The product was obtained according to the procedure described by Benesch and Benesch (1) and Pocker (8) with minor modifications necessary for synthesis on a gram scale (9).

Protein Preparation—Hb was prepared and purified as described...
previously for human hemoglobin (10); the amount of methemoglobin was checked by measuring the absorption at 630 nm and always found to be less than 1%. Red cells, washed three times with isotonic NaCl solution, were lysed with two volumes of 1 mM Tris-HCl buffer (pH 8.0); stromas were eliminated by centrifugation. Removal of any organic phosphates from bovine Hb was obtained by gel filtration on a Sephadex G-25 column (2 cm × 30 cm), equilibrated with 10 mM Tris-HCl buffer (pH 8.0), containing 0.1 mM NaCl. All columns were kept at 2–4 °C. Cellulose acetate electrophoresis on the hemedysate and purified Hb was performed as described by D'Avino et al. (11).

Preparation of the Hb-NFPLP Adduct—The modified protein was prepared by reducing the imine formed, under anaerobic conditions, by NFPLP and stroma-free bovine Hb with sodium borohydride according to the method of Benesch and Benesch (1). Reaction of Bovine Hb and NFPLP—A 15 mg/ml solution of bovine Hb was extensively dialyzed against 0.1 mM Tris acetate buffer, pH 7.5. Chemical modification of the Hb was done on 12 ml (≈3 μmol) of the dialyzed solution in 0.1 mM Tris acetate buffer, pH 7.5, in the presence of 20 μl of anti-floating (caprylic alcohol) and after 60 min of flushing with nitrogen. The molar Hb tetramer to NFPLP ratio was 1:2.5. Addition of sample solution at 2500 min, and the precipitated globin was isolated by centrifugation of the acid/acetone phase. The sample was allowed to stand at 0°C for 10 min, to be less than 1%. Red cells, washed three times with isotonic NaCl solution, were lysed with two volumes of 1 mM Tris-HCl buffer (pH 8.0), containing 0.1 M NaCl. All columnswere kept at 2–4°C. Cellulose acetate electrophoresis on the hemedysate and purified Hb was performed as described by D'Avino et al. (11).

Preparation of Globin by Ad/d Acetone—Extraction was done basically following the method of Rossi-Fanelli et al. (12). Hb in 10 mM phosphate buffer, pH 7.0, was added slowly by stirring with 10 volumes of cold acetone, 0.2% in HCl cooled at –20 °C according to the method of Ascoli et al. (13). The globin precipitated, and the heme remained in the acid/acetone phase. The sample was allowed to stand at 0°C for 10 min, and the precipitated globin was isolated by centrifugation of the sample solution at 2500 × g at 4°C. The precipitated chains were more than 90% electrophoresed on cellulose acetate.

Reverse Phase HPLC—The α and β subunits of bovine Hb-NFPLP were resolved on a Brownlee C8 column (10 μm) using the gradients developed by Shelton et al. (14).

SDS-Polyacrylamide Gel Electrophoresis—The molecular weight distributions of the separated α and β chains of untreated bovine Hb and NFPLP-reacted hemoglobin was estimated by electrophoresis in sodium dodecyl sulfate-polyacrylamide according to Laemmli (15). An LMW electrophoresis calibration kit (94,000, 67,000, 43,000, 30,000, 20,100, and 14,400 daltons) was purchased from Pharmacia Biotech Inc.

Oxygen-binding Isotherms—Spectrophotometrically (Cary 2300) determinations were done by the tonometric method (16) in the presence and in the absence of the physiological cofactor chloride, at 20°C.

Stopped-flow Kinetics Experiments—Oxygen dissociation kinetics were followed using a HI-TECH stopped-flow SHU apparatus by mixing oxygenated hemoglobin with a solution of sodium dithionite. The kinetics of the dissociation of oxygen from NFPLP-Hb was followed by mixing the oxygenated derivative of both the native and the cross-linked protein with sodium dithionite using a stopped-flow apparatus (17).

NMR Spectra—The spectra were obtained on a Varian Gemini spectrometer operating at 300 MHz for 1H and at 75.5 MHz 13C nuclear magnetic resonance (NMR) spectrum for the same sample. The elution of the peptides was monitored at 220 nm. Amino acid composition was carried out on the purified peptide hydrolyzed in the vapor phase with 6 M HCl at 110°C for 24 h. The amino acid composition of the hydrolyzed peptide was determined using a Pharmacia 4151 Alpha Plus instrument.

RESULTS

Characterization of the Adduct Bovine Hb-NFPLP—Fig. 1 shows the 1H and 13C nuclear magnetic resonance (NMR) spectra of the NFPLP dialdehyde. From the reaction between this latter compound and bovine Hb, a Schiff's base was obtained. The reduction with sodium borohydride of this formed base, yielded the bovine Hb-NFPLP adduct that was purified by DEAE-cellulose column as reported under “Experimental Procedures.”

Reverse Phase HPLC Analytical Resolution—Fig. 2 shows a typical chromatogram obtained from the Hb-NFPLP reaction mixture. The elution profile indicates that the α chains had the same mobility in the two proteins, while the β chains of the Hb-NFPLP were slightly more mobile than the β chains of bovine Hb. These assignations were made by using a mixture of the two hemoglobins.

Nuclear Magnetic Resonance—To obtain further confirmation regarding the synthesis of the Hb adduct, 31P nuclear magnetic resonance of purified chromatographic fractions was also performed (Fig. 3), confirming the presence of the phosphorous atom in the molecule of the cross-linked Hb. The chemical shifts are defined as positive if they are downfield from the reference signal. Under the conditions of our experiments, this phosphate group was able to exist in the complex state with Hb-NFPLP.
Electrophoresis—SDS-polyacrylamide gels of bovine hemoglobin modified with NFPLP showed two bands of equal concentration under deoxygenated conditions, with molecular masses corresponding to 16,000 daltons and 32,000 daltons as established by comparison with marker proteins. Since the latter value is twice the molecular weight of isolated hemoglobin chains, this new band has been taken to represent a dimeric species which, through electrophoresis on cellulose acetate performed after denaturation in acidic acetic acid, has been demonstrated to be the result of a chemical linkage established between the \( \beta \) chains.

Spectrophotometric Properties of Bovine Hb-NFPLP—The nanometer values of the absorption maxima for deoxy- (554 and 430), oxy- (576, 541, and 413), nitrosyl- (572, 540, and 416), and carboxyl-cross-linked Hb (571, 539, and 419), in the range of pH 6.7–8.8, were practically indistinguishable from those of the corresponding maxima of bovine Hb.

Functional Characterization—The oxygen affinity of the purified Hb-NFPLP was determined in the presence of 0.1 \( \text{m} \) chloride at pH values between 6.5–8.0 and 20 °C; under these conditions the Bohr effects of Hb-NFPLP and of normal bovine Hb were superimposable at acidic pH, while above neutrality the curves differentiated and bovine Hb-NFPLP showed a decreased affinity (Fig. 4). Moreover, since the physiological effector of this hemoglobin is chloride, a number of experiments were performed in the complete absence of this ion in order to see whether the introduction of the NFPLP in the \( \beta \) cleft led to the inhibition of the chloride effect. Further experiments have shown that the oxygen affinity of the purified Hb-NFPLP is virtually insensitive to the presence of chloride whose effect, clearly present in the normal protein, has completely vanished.

Moreover, the Bohr effect is largely maintained in Hb-NFPLP, although somewhat reduced with respect to the unmodified protein due to the decrease of oxygen affinity observed at pH values higher than 7.2. However, cooperativity is almost completely abolished, as indicated by the Hill coefficient \( (n_H) \), which is very close to unity \((1.1 \pm 0.1)\) (Fig. 5).

Stopped-flow Experiments—In order to define some kinetic parameters of the reaction with oxygen of the bovine Hb-NFPLP, a number of rapid mixing experiments were performed by mixing the oxygenated derivative of bovine Hb-NFPLP with Na\( _2 \)S\( _2 \)O\( _4 \). Parallel experiments were made with unmodified bovine Hb.

In both cases the kinetics of the reaction is strongly biphasic (fast phase: 75%) as the relative proportions of the two processes are essentially pH-independent; the rate of deoxygenation measured for the two kinetic phases appears strongly dependent on pH, although this dependence is more evident in normal bovine Hb. The value of the velocity constant of both phases is independent of hemoglobin and dithionite concentrations, and therefore the reaction may be described in terms of two independent first order processes.

For both the fast and slow phase, the plot of the value of the rate constant versus pH is a symmetrical curve corresponding to a simple titration. However, the cross-linked Hb presented, with or without chloride, a deoxygenation rate constant \( k_{fast} \) slightly slower than the corresponding constant of bovine Hb; in contrast, \( k_{slow} \) was always higher. The titration of Hb-NFPLP showed that both the \( pK_a \) values shifted to more acidic pH in relation to bovine Hb (Table I).

Molecular Modeling—Determination of the crystal structure of Bovine Hb has shown that the most important structural difference with respect to human hemoglobin is a shift, at the level of that crevice which in human hemoglobin constitutes the organic phosphate binding site, of both the N terminus and A-helix closer to the dyad axis \((7)\). In consideration of these features, we explored the residues that could be involved in the cross-linking of the two \( \beta \) chains of bovine Hb by NFPLP.

In principle, and similarly to human Hb, the cross-link could be established between lysine 82 of \( \beta_2 \) and the N-terminal methionine 2 of \( \beta_1 \), or between the two lysine 82 residues of both \( \beta \) chains \((1)\). As reported in Fig. 6, a cross-link between methionine 2 and lysine 82 can be established by moving the amino group of methionine \( \beta_1 \) and the \( \epsilon \) amino group of \( \beta_2 \) lysine 82 closer to
the central cavity by approximately 3.5 and 1.5 Å, respectively. Only a slight movement was necessary for β1 lysine 82 and the side chain of β1 lysine 82 to form electrostatic interactions with the phenolate anion and the phosphate group, respectively.

Cross-linking lysine 82 of β1 and lysine 82 of β2 did not necessitate a significant movement of the β lysines; however, both the amino groups of methionine 2 and histidine 143 were moved by approximately 3–4 Å in order to form electrostatic interactions with the phosphate of NFPLP.

Identification of the Cross-Linked Residues—Fig. 7 shows the comparison between the chromatographic patterns of the NFPLP-reacted and unreacted β chains of bovine Hb. The two chromatograms are completely superimposable with the exception of the peak eluting at a retention time of 61.5 min, present in the chromatogram of the cross-linked hemoglobin and absent in the other. The amino acid analysis of the purified peak gave an amino acid composition corresponding to the sum of the tryptic fragments 1–7 and 77–95 (Table I) derived from the bovine β chain, thus confirming that NFPLP cross-links Met-β2 and Lys-β82.

**DISCUSSION**

According to our modeling studies, it was not possible to discriminate between two different structural alternatives (Met-2-Lys-82 or Lys-82-Lys-82) for the cross-link; this problem has been solved by digestion of β globin chains and following isolation of the cross-linked peptide. Sequence analysis of this peptide has shown that the reaction with NFPLP occurs between the Met-2 of one β chain and Lys-82 of the other β subunit of the bovine Hb molecule. This suggested that the tetrameric structure was no more able to dissociate into αβ dimers. This is clearly shown by electrophoresis on cellulose acetate of the globins, which shows, in addition to the band relative to the α chains, the presence of a band relative to the cross-linked β chains.

Regarding the functional properties of bovine Hb-NFPLP, these indicate that the chemical modification affects its affinity for oxygen and abolishes homotropic interactions and the effect of chloride, inducing a decrease (−35%) of the Bohr effect. The decrease in oxygen affinity observed in the case of bovine Hb-NFPLP in the absence of chloride, when compared to normal bovine Hb, is of peculiar significance since it indicates that the negative charges of NFPLP may substitute chloride ions in regulating the oxygen affinity acting in much the same way as 2,3-DPG does in human HbA. Given the situation, the oxygen affinity of Hb-NFPLP is no longer affected by Cl− and it is very similar to and even lower than (depending on the pH) the corresponding affinity of the native bovine Hb in the presence of these ions.

The loss of cooperativity and the low oxygen affinity suggest that the reaction with NFPLP, performed under anaerobic conditions, preferentially stabilize the T conformational state of the new molecule. This is also indicated by the oxygen binding curve which corresponds, at all the pH values examined, to the lower asymptote of the untreated bovine Hb (Fig. 4). In this respect, it may be worthwhile recalling that the same chemical modification performed on human HbA results in a considerable drop in the oxygen affinity but has only a small effect on the degree of homotropic interactions, being the Hill coefficient n∞ ~ 1.9, versus 2.7 for the native molecule (18). In the case of human HbA, therefore, NFPLP is not able to freeze the Hb molecule in the low affinity conformational state. The difference seen between human HbA and bovine Hb in their reaction with NFPLP must be ascribed to specific structural characteristics belonging to the bovine molecule. This may well be related to the shift of the A-helix toward the dyad axis of the tetrameric structure observed in the crystal structure of bovine Hb, which has been recently interpreted as the main characteristic responsible for the low intrinsic oxygen affinity of this Hb (7). In this perspective, spectroscopic observations on the nitric oxide derivative may be relevant, as these have clearly demonstrated that the Hb of ruminants populates its T conformational state much more than other mammalian hemoglobins, even in the liganded form (6, 19). As a matter of fact, a similar shift, although less pronounced, has been ob-
served in human HbA upon binding of organic phosphate (20). Moreover, previous studies on the structure of human HbA modified by NFPLP have shown that the cross-linking reaction induces the amino group of Val-β1 to move about 3 Å into the central cavity. As a result the N-terminal peptide and the beginning of the A-helix are pulled closer to the EF corner and the E-helix. All these findings, relative to human HbA, support the hypothesis that the relative position of A-helix does play a significant role in the modulation of the oxygen affinity.

In addition, it would be important to outline that on the basis of stereochemical characteristics of the cavity between the two β chains Arnone et al. (21) would have expected the reaction with NFPLP to "freeze" the tetramer in the T state, thereby producing a low affinity non-cooperative tetramer. As already mentioned, contrary to this expectation HbA-NFPLP modified appeared to be still cooperative (nH ≈ 1.9). The forecast of Arnone et al. is completely fulfilled by bovine Hb, whose NFPLP derivative appears first to be frozen in the T conformational state and second to have fully abolished the cooperative effect.

This differences in behavior may find an explanation on the basis of the following considerations.

(a) In bovine Hb the A-helix is closer to the dyad axis with respect to HbA even in the absence of any organic phosphate effector (7). In some way this intrinsic difference of the molecule may facilitate the molecule to fully populate the T conformational state.

(b) Our modeling studies show that the cross-link between Met-β2 and Lys-β82 can be established by moving the amino group of methionine β1 and the ε-amino group of lysine closer to the central cavity by approximately 3.5 and 1.5 Å, respectively. Upon NFPLP reaction, therefore, the A-helix should be further shifted toward the interior of the molecule thereby strongly stabilizing the T state.

Other interesting observations are related to the Bohr effect of both the native and the NFPLP-reacted protein. The cross-linked Hb displays identical Bohr effects both in the presence and in the absence of Cl⁻. Moreover, this Bohr effect is decreased by 35% with respect to that of the untreated molecule. It is therefore very likely that this 35% of the Bohr effect, which in the native protein appears to be Cl⁻-linked, has to be ascribed to one (or both) of the residues that are involved in the cross-linking of the β chains or to some other residues that, to come into operation, need the switch from the T to the R state of the molecule. As far as the enhancement of the alkaline Bohr effect by chloride ions is concerned, point mutations and chemical modification experiments (22, 23) have indicated that, within the central cavity, the replacement of either two pairs of cationic groups by neutral ones, or of a single cationic group by an anionic one, is sufficient to inhibit the Cl⁻ effect no matter which residues are altered.

On the basis of these findings and of structural results, it has
been suggested (7) the absence of any specific chloride binding sites. Hence, it has been proposed that the effect of Cl⁻ could be due to the widening of the central cavity on transition from the R to the T structure. This would allow Cl⁻ to diffuse in and neutralize the excess of positive charges, lining the central cavity, without being bound to any one of them. Consequently, the enhancement of the Bohr effect by Cl⁻ is due to a rise in pK values (i.e., increased binding of protons) of the cationic groups within the central cavity.

Our results are in agreement with this view, since the cross-linkage abolishes the positive charge of Lys-β82 and introduces in the cavity between the β chains the three potential negative charges of the NFPLP molecule. According to the above reported considerations, the Cl⁻ effect on the O₂ affinity and the chloride-induced Bohr effect are vanished.

In addition, it is necessary to consider that the strong impairment of T → R conformation transition also goes in the same direction. Thus the O₂-linked widening of the central cavity and the following diffusion of Cl⁻ can no more occur in the NFPLP-modified bovine Hb; therefore, the differential “binding” of these ions cannot show up.

However, it should not be forgotten that a significant part of the Bohr effect (i.e., the chloride-independent part) appears to be displayed by a molecule that is frozen in the low affinity T state. This may be taken as an indication that, at least in NFPLP-modified bovine Hb, a significant part of the pH dependence of the O₂ affinity finds its molecular basis at the level of the tertiary structure. The details of this effect remain to be seen.

In any case, concerning a potential blood substitute, the functional properties of the NFPLP-modified bovine Hb are not completely satisfactory. The main disadvantage is the absence of cooperativity, which may not be fully compensated by the very low oxygen affinity of the protein. However, an additional step could be the synthesis of an intertetramer supermolecule in which several modified bovine Hb tetramers are linked together by means of bifunctional reagents such as dialdehydes. Since most of these modifications are known to result in a strong destabilization of the T structure in favor of the high affinity R state, we may have the chance of partially restoring heme-heme interactions through the formation of intermolecular cross-links and supermolecular structures.

Finally, since NFPLP-bovine Hb has lost all its cooperative interactions, according to allosteric models (24), the kinetic parameters obtained may be thought to belong to the low affinity T state of the molecule.

The kinetics of the dissociation of oxygen from NFPLP-Hb was followed by mixing the oxygenated derivative of both the native and the cross-linked protein with sodium dithionite using a stopped-flow apparatus. When the reaction of dithionite with free oxygen is not rate-limiting, the time course of the observed process obeys first-order kinetics, and the measured rate constant represents an overall dissociation of the ligand from saturated Hb. The deoxygenation process is characterized by two phases, one being faster with a relative amplitude corresponding to nearly 75% of the total change; Table I reports the k_{off} constants for oxygen measured by the dithionite method. Moreover, the rate of dissociation is significantly affected by pH (Fig. 8). The titration curve is symmetrical and corresponds to simple process whose pK values are, for k_{fast}: 7.30 ± 0.20 and 6.91 ± 0.52, and for k_{slow}: 7.40 ± 0.31 and 5.93 ± 0.45, respectively, for the native and the modified protein. From these values it appears that in the cross-linked Hb the

![Figure 8](http://www.jbc.org/)

**FIG. 8.** Variation of the first-order rate constant k_{o} with pH for the deoxygenation of NFPLP-oxyHb by Na_{2}SO_{3} in presence of chloride ions 0.1M. The curve is a simple titration with pK_{1} = 6.91 ± 0.52, k_{1} for acid form = (66.5 ± 8.7) s⁻¹, and k_{2} for basic form = (32.9 ± 7.6). Temperature = 20 °C. NFPLP-Hb concentration = 100 mM (in heme).

Bohr effect does not receive any significant contribution from the dissociation velocity constant; in fact, the pK of the transition is only in a region of pH in which the Bohr effect is completely over. In contrast, in the case of native protein it appears very clearly that the pH dependence of the oxygen affinity may find part of its kinetic expression in the rate constant of oxygen dissociation on the basis of the pK values of both the Bohr effect and the transition of the velocity constant. Hence, this difference in behavior seems to be related to the inhibition of the ligand-linked allosteric transition brought about by the chemical modification.

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