The Nicotinamide Adenine Dinucleotides as Allosteric Effectors of Human Hemoglobin*

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Robert Cashont†, Celia Bonaventura, and Joseph Bonaventura§
From the Marine Biomedical Center and the Duke University Marine Laboratory, Beaufort, North Carolina 28516

Aldo Focesi†
From the Department of Bioqui, Instituto de Biologia Unicamp, C P 1170 13.100 Campinas, S Paulo, Brazil

The oxygen binding properties of human hemoglobin are appreciably altered by the nicotinamide dinucleotides NADH, NADP*, and NADPH. These cofactors are important in the control of many metabolic pathways and in providing reductive potential for a number of enzymatic reactions, including in vitro reduction of methemoglobin. Specific binding of these cofactors to hemoglobin and their potential for acting as allosteric modifiers of hemoglobin function have not been previously recognized. Detailed oxygen binding studies utilizing a thin-layer method suggest that the nicotinamide dinucleotides bind with high affinity to the deoxyhemoglobin tetramer at the β chain anion-binding site and stabilize the low affinity “T-state” conformation. Stripped Hb A in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 6.5, at 20 °C is half-saturated at a P0, of 1.6 mm Hg. In the presence of 0.5 mM NADH, NADP*, or NADPH, the P0, is raised to 3.8, 7.1, and 12.5 mm Hg, respectively. The Bohr factor for stripped Hb A in 0.05 M HEPES buffer is sensitive to these effectors and is raised from 0.25 to about 0.65 by the addition of NADPH. The data suggest that routine use of these effectors in studies of human hemoglobin variants or the allosteric mechanism of Hb A be considered carefully. The relatively low intraerythrocytic levels of the nicotinamide dinucleotides in relation to hemoglobin dictate that these cofactors cannot significantly affect in vivo oxygen delivery. However, the converse is theoretically possible. The binding of the cofactors to hemoglobin and the preferential binding of their reduced forms may affect cofactor-dependent metabolic processes in red blood cells.

In early studies of hemoglobin function, it was not understood why hemolysates prepared by water lysis had oxygen affinities significantly higher than that of the intact red cell. In fact, dialyzed hemolysates had oxygen affinities nearly as high as those of myoglobin (Fig. 1 in Benesch and Benesch, 1969). Although 2,3-diphosphoglycerate was reported to be present in high concentrations in human red blood cells as early as 1941 (Rapoport and Guest, 1941), it was not until 1967 that Chanutin and Curnish (1965a, 1965b, 1967) and Benesch and Benesch (1967a, 1967b) discovered that 2,3-diphosphoglycerate was an important allosteric effector of hemoglobin function. It was apparent why earlier investigators were puzzled by the experiments done with lysed red cells: dilution of the hemoglobin caused the ratio of 2,3-diphosphoglycerate to hemoglobin to decrease and dialysis led to still further decreases in 2,3-diphosphoglycerate. The physiological significance of the allosteric interaction of 2,3-diphosphoglycerate with human Hb A is that its preferential binding with the deoxy form of the molecule leads to a decrease in oxygen affinity, thereby facilitating oxygen delivery to the respiring tissues surrounding the capillary beds. In subsequent years, a wide variety of anionic effectors of human and non-human tetrameric hemoglobin function have been discovered, as reviewed elsewhere (Bonaventura and Bonaventura, 1980). The best known of these anionic effectors are organic and inorganic phosphates, chloride, and lactate. Carbon dioxide also lowers hemoglobin’s oxygen affinity, not only by carbamino formation at the amino-terminal residues of the α and β chains, but also by its action, in the form of bicarbonate, as an anionic effector of hemoglobin function (see Antonini and Brunori, 1971). To this list should be added another class of metabolically important compounds which are found within the red cell: the nicotinamide dinucleotides NADPH, NADP*, and NADH. As will be shown in this paper, these compounds act as heterotropic allosteric effectors of hemoglobin function and bind to the human hemoglobin tetramer with high affinity.

In order to investigate whether the nicotinamide dinucleotides exert their effect on oxygen binding to Hb A by interacting with the β chain anion-binding site, we made use of a human hemoglobin variant whose amino acid substitution is in the positively charged cluster of β chain residues in the major anion-binding site. This variant, hemoglobin Providence-Asp, has aspartate residues at the β-82 positions of the two β chains in the human hemoglobin tetramer instead of lysine residues (Moo-Penn et al., 1976) and has been shown to have a marked decrease in affinity for 2,3-diphosphoglycerate (Bonaventura et al., 1976).

Knowledge of the binding affinity of the nicotinamide dinucleotides and their in vivo molar ratios to hemoglobin suggest that a different type of molecular control may occur in connection with these compounds. In contrast to 2,3-diphosphoglycerate and similar effectors that act to alter the oxygen transport function, the preferential binding of the...
reduced forms of the nicotinamide dinucleotides by hemoglobin allows for possible control or modulation of metabolism.

MATERIALS AND METHODS

Hb A, Hb Providence-Asn, and Hb Providence-Asp were prepared and stripped of anionic cofactors as previously described (Bonaventura et al., 1976). Samples were stored immersed in liquid nitrogen until use.

β-NADPH, β-NADP⁺, β-NADH, and β-NAD⁺ were obtained from Sigma. Some comparative studies were carried out using α-NADH and α-NADPH, also purchased from Sigma. Unless otherwise stated, all references to nicotinamide dinucleotide cofactors in this paper refer to the β forms.

Routine oxygen equilibrium measurements were performed either by the spectrophotometric method of Riggs and Wolbach (1956) or using a modified Hemoscan oxygen dissociation analyzer in a stepwise mode. When used in parallel experiments, good agreement was obtained between the two methods. Extended oxygen binding curves were determined using a thin-layer binding cell (Dolman and Gill, 1978) obtained from the laboratory of Dr. Stanley Gill at the University of Colorado at Boulder. These precisely determined curves were analyzed as described by Johnson (1984) in conjunction with a nonlinear least squares curve fitting program (Johnson et al., 1981). These procedures allow estimation of the parameters associated with the oxygen binding model of Monod et al., (1965). Experiments were conducted in 0.05 M HEPES¹ buffer with pH adjusted with NaOH unless otherwise specified. By this means, it was possible to evaluate the effector binding over a wide pH range without chloride present.

RESULTS

Nicotinamide Dinucleotides as Heterotropic Effectors of Hemoglobin Function—A thin-layer cell as described by Dolman and Gill (1978) was used to obtain precise oxygen binding curves for analysis of cofactor binding to hemoglobin (see "Materials and Methods"). Fig. 1 shows representative Hill plots of oxygen binding to stripped Hb A and Hb A in the presence of NADPH and in the presence of 2,3-diphosphoglycerate. It is evident from the figure that NADPH acts in an analogous fashion to 2,3-diphosphoglycerate in altering the oxygen binding properties of hemoglobin. Alterations in the shape of the ligand binding curves are reflected in the slopes of the Hill plots at half-saturation, with n values of 2.9, 2.0, and 3.2 for stripped Hb A, Hb A with NADPH, and Hb A with 2,3-diphosphoglycerate, respectively. The depressed value of n in the presence of NADPH is due to a more asymmetrical binding curve rather than to decreased cooperativity since Hill coefficients as high as those seen with 2,3-diphosphoglycerate are observed at higher levels of oxygen saturation. Like 2,3-diphosphoglycerate, the nicotinamide dinucleotides exert the major part of their effect on the T-state of the hemoglobin tetramer while leaving the R-state virtually unaffected. The P₅₀ for the T state, calculated from the linear extension of the binding curves at low saturation where the Hill plots have slopes of unity, is 0.7 in the completely stripped condition and increases to 1.18 and 1.84 in the presence of NADPH and 2,3-diphosphoglycerate, respectively. At high (saturating) concentrations of NADPH, the shift in oxygen affinity at half-saturation is somewhat less than that seen with saturating concentrations of 2,3-diphosphoglycerate (log P₅₀ at pH 7.3 of 0.8 and 1.2, respectively). These results were independent of the buffer used in that equivalent data were obtained when Hb A was in 0.05 M Tris, a condition that results in a lowered oxygen affinity due to the Cl⁻ added as HCl required for pH adjustment.

¹The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; P₅₀, partial pressure of oxygen when half of the heme groups are saturated; P₁ₐ, median partial pressure of oxygen in an oxygen binding experiment (Wyman, 1964; Brouwer et al., 1982).

FIG. 1. Hill plots of O₂ binding to Hb A in the presence of allosteric effectors. Δ, 1.5 mM 2,3-diphosphoglycerate, pH 7.3; ○, 0.5 mM NADPH, pH 7.0; □, stripped Hb A, pH 7.0. Heme concentration was 1 mM, and measurements were made in HEPES buffer at 20°C using the thin-layer binding cell described under "Materials and Methods." Y, fractional saturation of heme sites with oxygen.

FIG. 2. Titration of Hb A with the nicotinamide dinucleotide cofactors. Measurements were made by tonometry using 50 mM HEPES buffer, pH 7.4, at 20°C. Heme concentration was 60 μM. ○, NADPH; □, NADP⁺, Δ, NADH; ●, NADH + inositol hexaphosphate (100-fold over Hb tetramers).

Studies of the α and β forms of the reduced nicotinamide dinucleotide cofactors showed that for both NADPH and NADH there are no significant differences between the effects of the α and β isomers on the P₅₀ values for human hemoglobin.

Throughout the pH range studied, the reduced nicotinamide dinucleotides are more effective at lowering hemoglobin’s oxygen affinity than are the oxidized forms of the cofactors. This phenomenon is linked to the greater affinity of the reduced forms for deoxyhemoglobin, as illustrated by the titrations shown in Fig. 2. The titrations were analyzed using an iterative curve-fitting technique in conjunction with the equation of Szabo and Karplus (1976) to estimate the relative affinities of these compounds for Hb A. As described by these
The amino acid substitution of Hb Providence has a significant effect on the affinity for oxygen. It is notable that the affinity for oxygen is lowered by NADPH, which supports the fact that NADPH binds to the hemoglobin binding site. As shown in Table I, deoxy-Hb A binds these forms (except NADP+) with high affinity. A striking difference between the effectors is the stronger binding of 2,3-diphosphoglycerate to oxy-Hb A. The upper trace shown in Fig. 2 is a NADPH titration carried out with Hb A in the presence of 1.5 mM inositol hexaphosphate. Under these conditions, the oxygen affinity is initially low, due to the strong allosteric influence of inositol hexaphosphate. It remains so until the NADPH concentration is 5 mM or higher where the cofactor binding is significantly competitive with that of inositol hexaphosphate. The titration suggests a common binding site for the two classes of effectors and supports the fact that NADPH binds to the hemoglobin tetramer with high affinity.

Fig. 3 shows the pH dependence of Hb A in the presence of the four nicotinamide dinucleotides studied. It is notable that not only is the affinity for oxygen lowered by NADPH, NADP+, and NADH, but also that the magnitude of the Bohr effect is increased from 0.25 in stripped Hb A to approximately 0.65 in the presence of 0.5 mM NADPH, NADP+, or NADH. This indicates a linkage between proton binding and cofactor binding that is reminiscent of that seen with the binding of other organic phosphates to Hb A.

Table II compares the effects of the nicotinamide dinucleotide cofactors on oxygen binding by Hb A and Hb Providence-Asp at pH 6.5 and 7.3. It is relevant that Hb Providence-Asp at pH 6.5 shows little response to 2,3-diphosphoglycerate and is essentially unaffected by 2,3-diphosphoglycerate at pH 7.3 (Bonaventura et al. 1976). The same pattern is seen for the nicotinamide dinucleotide effectors, as is evident from the data of Table II. The amino acid substitution of Hb Providence-Asp in the β chain anion-binding site decreases the ability of the nicotinamide dinucleotide cofactors to change the log \( P_{50} \) values of Hb Providence-Asp as compared to Hb A, indicating a decreased association of cofactor with the β chain anion-binding site.

**Cofactor-induced Asymmetry in Equilibrium Oxygen Binding Curves**—Evidence of cofactor-induced asymmetry is sometimes evident in equilibrium oxygen binding studies. Fig. 4 is a plot of \( \log P_n \) versus \( \log P_{50} \) for Hb A and Hb Providence-Asp with varying cofactor concentrations. In a symmetrical oxygen binding curve with no differential association of cofactor between the different chains, one would expect to find \( P_n = P_{50} \) and a plot of \( \log P_n \) versus \( \log P_{50} \) to have a slope of approximately 1. It is apparent from Fig. 4 that for Hb A in the presence of low levels of 2,3-diphosphoglycerate (left half of curves), a large amount of asymmetry is introduced by the cofactor. This is not the case as the ratio of 2,3-diphosphoglycerate to hemoglobin rises or in the presence of NADPH, NADP+, or NADH. In these latter cases, the points all fall on a line with slope approximately 1. This same line contains all points for the two Hb Providence variants (in the presence of 2,3-diphosphoglycerate) which (as discussed earlier) have a greatly reduced association with 2,3-diphosphoglycerate due to the amino acid substitution in the anion-binding site.
their binding interactions. Both the 2'-phosphate of NADPH and NADP+, pH 7.3; □, Hb Providence-Asp + 2,3-diphosphoglycerate, pH 6.2; ○, Hb Providence-Asp + 2,3-diphosphoglycerate, pH 6.2; +, Hb A + NADPH, pH 7.3; ×, Hb A + NADP+, pH 7.3.

**DISCUSSION**

**Stereochemistry of Hb A Nicotinamide Dinucleotide Interactions**—It is clear from hemoglobin oxygen binding studies done in the presence of the nicotinamide dinucleotides NADPH, NADP+, and NADH that these cofactors act as heterotrophic allosteric effectors of hemoglobin function. These effects appear to be exerted by interaction of the cofactors with the β chain anion-binding site, a situation analogous to that seen with 2,3-diphosphoglycerate, inositol hexaphosphate, and ATP. In particular, we have shown that both α and β forms of these cofactors bind with high affinity to Hb A, with binding constants like those of the above-mentioned organic phosphates. Greatly decreased effects on the oxygen affinity of Hb Providence-Asp suggest that β-82 lysine is at least a contributor to the binding site of the nicotinamide dinucleotides. The competition between NADPH and inositol hexaphosphate effects (Fig. 2) also supports the hypothesis that the nicotinamide dinucleotides alter oxygen affinity by interactions with residues that contribute to the β chain anion-binding site.

Fig. 5 presents a schematic representation of the open form of NADP+ (a conformation it assumes upon binding to lactate dehydrogenase) and indicates the regions of the molecule that appear to be of importance in its interactions with Hb A. The two central phosphates are reasonable candidates in light of the studies of Arnone (1972). The calculated binding constants suggest that the conformational differences between deoxy and oxy forms of Hb A more strictly exclude the nicotinamide dinucleotide cofactors interact with groups adjacent to, but not actually a part of, the β chain anion-binding site as defined by the studies of Arnone (1972). The calculated binding constants suggest that the conformational differences between deoxy and oxy forms of Hb A more strictly exclude the nicotinamide dinucleotide interactions with the oxy form than occurs for either 2,3-diphosphoglycerate or inositol hexaphosphate. Further clarification of the stereospecificity of these interactions will require x-ray structural analysis.

**Effect of Nicotinamide Dinucleotides on In Vivo Oxygen Transport**—The discovery that the reduced and oxidized nicotinamide dinucleotides NADPH, NADP+, and NADH can act as heterotrophic allosteric effectors of hemoglobin function suggests the possibility that these interactions may have physiological importance. If such a physiological role does exist, it may be related more to the metabolism of the red blood cell than to hemoglobin's ability to bind oxygen at the air-lung interface and deliver it to respiring tissues. Although the affinity of NADPH binding to hemoglobin (micromolar range) is "high," the concentration of the nicotinamide dinucleotides in erythrocytes, compared to the hemoglobin concentration, dictates that the quantitative role of these molecules in lowering the oxygen affinity of hemoglobin can only be very small. Hemoglobin tetramer is present at a concentration of approximately 5 mM in the erythrocyte (Wintrobe, 1967), and 2,3-diphosphoglycerate is present in approximately equimolar amounts (Guest, 1942). The nicotinamide dinucleotides, however, are present at molar ratios of less than 1:50 important in the interaction with the binding site. A comparison of the size of 2,3-diphosphoglycerate with those of the extended forms of NADPH, NADP+, and NADH reveals a significant difference in both size and the distribution of charged groups which are thought to take part in binding. Although for many NADP+- and NAD+-dependent enzymes the cofactor binds in its extended conformation (for discussion, see Fita and Rossman, 1985), in the case of catalase the bound NADP+ has been shown to be folded into a right-handed helix (Fita and Rossman, 1985). It is possible that a similar folding process could bring the charged groups of the nicotinamide dinucleotides into a conformation which facilitates its binding to hemoglobin. It is likewise possible that at least some of the charged groups on the nicotinamide dinucleotide cofactors interact with groups adjacent to, but not actually a part of, the β chain anion-binding site as defined by the studies of Arnone (1972). The calculated binding constants suggest that the conformational differences between deoxy and oxy forms of Hb A more strictly exclude the nicotinamide dinucleotide interactions with the oxy form than occurs for either 2,3-diphosphoglycerate or inositol hexaphosphate. Further clarification of the stereospecificity of these interactions will require x-ray structural analysis.
compared to hemoglobin tetramer (Gross et al., 1966). This is clearly not consistent with an intracellular role for the nicotinamide dinucleotide cofactors as major modulators of hemoglobin oxygen affinity.

Possible Metabolic Effect of Hemoglobin-Nicotinamide Dinucleotide Interactions—Another possibility to be considered is that physiologically important interactions would occur if binding of the nicotinamide dinucleotide cofactors to hemoglobin reduces their effective concentrations within erythrocytes. These cofactors are important in control of many metabolic pathways and provide the reducing potential for a number of enzymatic reactions. Among the latter are enzymatic reactions responsible for in vivo reduction of methemoglobin. Moreover, our binding data (Table I) suggest that the modulation of intracellular levels of nicotinamide dinucleotide cofactors could potentially operate on two levels. First, we find a stronger interaction between hemoglobin and the reduced forms of the cofactors, suggesting that intracellular binding could affect the redox potential within the cell by lowering the effective concentrations of NADH and NADPH compared to the oxidized forms. Second, our data show that the nicotinamide dinucleotide cofactors bind almost exclusively to the deoxy form of the Hb A tetramer. This suggests that the binding of the cofactors to hemoglobin in vivo would be greatest in the relatively deoxygenated blood leaving the capillary beds in the tissues and much less in the oxygenated blood leaving the lungs. Thus, one might predict that in oxygenated blood, the absolute levels of free nicotinamide dinucleotide cofactors would be high with respect to deoxygenated blood. One would also predict relatively low values for the ratio of oxidized to reduced cofactor forms free in oxygenated blood (i.e. oxygenated blood would be reduced with respect to deoxygenated blood to the extent that the redox potential is affected by shifts in the nicotinamide cofactor ratios). These two sets of intracellular conditions might then be expected to favor different subsets of enzymatic activity as the red cells course through the bloodstream. Inherent in this possible scenario of events is the realization that physiologically differences may be attributable to mutations in the human hemoglobin tetramer or evolutionary differences between species that affect the β chain anion-binding site and its ability to bind 2,3-diphosphoglycerate and the nicotinamide dinucleotide cofactors. Significant metabolic consequences with regard to NAD- and NADP-linked enzymatic processes may result that are not classically considered to be affected by functional changes in the hemoglobin tetramer.

Evidence exists that a significant portion of nicotinamide dinucleotide cofactors within human red blood cells are bound within the protein fraction. Studies by Jacobasch et al. (1974) based on measured total NAD levels in the cell and the NAD+/NADH ratio calculated on the basis of the measured pyruvate/lactate ratio suggest that a large proportion of intracellular NADH is bound and is not part of the free pool. Recent studies (Kirkman, et al., 1986) strongly suggest that the availability of intracellular NADP+ to glucose-6-phosphate dehydrogenase is reduced by the binding of the cofactor to other proteins within the cell. Although the data cited did not implicate hemoglobin as a major effector of this intracellular NADP+ binding, it should be noted that the experiments reported were carried out under oxygen conditions where our data imply that the binding interactions would be minimal or nonexistent.

The presence within the erythrocytes of a large number of other anions that can bind to hemoglobin could reduce the influence of these interactions. Since we suggest that the nicotinamide dinucleotides interact with the β chain anion-binding site, the dominant competing anion is 2,3-diphosphoglycerate. Red cells contain a relatively large amount of this effector, and it binds to hemoglobin with high affinity. Calculations2 based on the relevant affinity constants and the reported concentrations of hemoglobin and its effectors within erythrocytes suggest that alterations of red cell metabolism may indeed result from the differential binding of the reduced forms of the nicotinamide dinucleotide cofactors. Specifically, at the estimated value of Ka for NADPH (10-6) and at a 1:1 ratio of Hb A to 2,3-diphosphoglycerate, 93% of the reduced dinucleotide could exist in the form of a hemoglobin-dinucleotide complex in deoxygenated solutions. The possibility exists, therefore, that hemoglobin may serve as a "sink" for a significant quantity of the intracellular nicotinamide dinucleotides.

The results presented here demonstrate in vitro interactions between hemoglobin and the nicotinamide nucleotides. The in vivo condition is undoubtedly complex, but these interactions are of potential significance in any of the redox interactions carried out by the circulating erythrocytes. The in vitro results here serve to clarify yet another class of molecules that can act as modulators of oxygen binding by hemoglobin and will hopefully also stimulate studies of the physiological consequences of these interactions.

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2 A simple competitive binding equation to illustrate this point can be derived as follows: HK = total concentration of hemoglobin in the cell; KF = total concentration of nicotinamide cofactor; D = total concentration of 2,3-diphosphoglycerate; K = dissociation constant for nicotinamide cofactor; Ke = dissociation constant for 2,3-diphosphoglycerate; [H] = free hemoglobin concentration at equilibrium; [HN] = concentration of Hb-nicotinamide cofactor complex; [HD] = concentration of Hb-2,3-diphosphoglycerate complex.

Equations 1-5 below describe competitive binding of NADH and 2,3-diphosphoglycerate to a common site (since the first linking equation does not contain a [HDN] term which would represent the simultaneous binding of a 2,3-diphosphoglycerate and a nicotinamide nucleotide to the same hemoglobin tetramer).

\[ H_K = [H] + [HD] + [HN] \]  
\[ N_K = [HN] + [N] \]  
\[ D_K = [HD] + [D] \]  
\[ K_{[HD]} = [H]/[D] \]  
\[ K_{[HN]} = [HN]/[N] \]  

Appropriate substitutions yield the following binding equation.

\[ H_K = \frac{K_{[HN]}}{N_K} + \frac{K_{[HD]}}{K_{[HN]} N_K} + \frac{K_{[HN]}}{N_K} + \frac{K_{[HN]}}{N_K} \]

Solution of this equation for [HN]/[N], the proportion of cofactor bound, involves a cubic solution and will not be shown here. However, our analysis reveals the following approximations. Conservatively estimating \[ H_K = 2 \times 10^{-5} \text{ M}, \]
\[ D_K = 2 \times 10^{-5} \text{ M}, \]
\[ N_K = 2 \times 10^{-5} \text{ M}, \]
and \[ K_{[HN]} = 1 \times 10^{-5} \text{ M}, \] one can estimate the following values of [HN]/[N]: as a function of the estimated nicotinamide dinucleotide cofactor dissociation constant (K): for K = 1 \times 10^{-5}, 1 \times 10^{-6}, 1 \times 10^{-7}, and 1 \times 10^{-8}, [HN]/[N] = 0.99, 0.93, 0.57, and 0.12.
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