Functional Properties of Human Adult Hemoglobin Specifically Modified at the α-Amino Groups of the β Chains with D-Glucose 6-Phosphate†

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ABSTRACT: A covalent adduct of D-glucose 6-phosphate and human adult hemoglobin was prepared by in vitro incubation of the phosphorylated sugar and (carbon monoxy)hemoglobin under physiological conditions at 37 °C. Structural analysis indicated that D-glucose 6-phosphate was specifically attached to the α-amino groups of the β chains by a keto–amine linkage. In the absence of phosphate, stripped G6P Hb possessed a low oxygen affinity (P_{50} = 12 mmHg at pH 7; [Cl^-] = 0.1 M), a slightly decreased value of n (n = 2.3), and a substantially reduced alkaline Bohr effect ($-\Delta \log P_{50}/\Delta pH$ = 0.36) when compared to normal hemoglobin. Although the oxygen binding properties of G6P Hb were dependent upon [Cl^-], the addition of inositol hexaphosphate did not significantly alter the properties of this modified protein. These findings are consistent with the premise that the sugar phosphate groups, which are covalently bound to the hemoglobin, mimic the effect of free organic phosphate in stabilizing the deoxy form and consequently lowering the oxygen affinity of the molecule. The lack of effect of inositol hexaphosphate on the functional properties of G6P Hb indicates that this organic phosphate and the glucose 6-phosphate moiety are competing for the identical binding sites on the hemoglobin molecule. Measurement of the temperature dependence of the oxygenation reaction of G6P Hb revealed a decrease in both the enthalpy and entropy terms, consistent with a more deoxy-like structure than normal stripped hemoglobin. Rapid mixing experiments reflected the equilibrium results, in revealing a slow rate of carbon monoxide combination (0.12 μM^{-1} s^{-1} at pH 7) which was unaltered by the addition of phosphate. Furthermore, a rapid, biphasic oxygen dissociation time course (k_f = 87 s^{-1}; k_s = 27 s^{-1}) was observed, suggesting enhanced α,β chain differences in the liganded form of this protein. A sugar phosphate covalently bound would have an effective concentration that is very large and would significantly bind to the hemoglobin regardless of the state of ligation. Thus, this covalently modified hemoglobin may provide a good model system to examine the role of organic phosphate in regulating the oxygen binding characteristics of liganded as well as unliganded human hemoglobin.

Hemoglobin is the most thoroughly studied of all proteins (Wyman, 1948, 1964; Antonini & Brunori, 1971; Edelstein, 1975; Bunn et al., 1977; Perutz, 1979). Understanding the structure and function of hemoglobin has been enhanced by investigation of selected mutants. Additional attention has focused on factors, including DPG,[1] hydrogen ion concentration, CO_2, anions, and temperature which can modify and regulate the behavior of hemoglobin inside the red cell (Benesch & Benesch, 1974).

Human red blood cell hemolysate contains minor hemoglobin components A_{Ia}, A_{Ib}, and A_{Ic} which were first described by Allen et al. (1958). McDonald et al. (1978) resolved additional minor components using a Bio-Rex 70 cation-exchange chromatography. These components, designated Hb A_{Ia1}, Hb A_{Ia2}, and Hb A_{Ib}, comprise 0.2%, 0.2%, and 0.5% of a normal human hemolysate, respectively. All these components have been shown to contain a ketoamine-linked carbohydrate which is engendered by the slow nonenzymatic condensation of sugars and the NH_2 termini of their β chains (McDonald et al., 1978; Garrick et al., 1980). Under physiological conditions, D-glucose 6-phosphate (G6P) can act as an affinity label, forming a covalent adduct specifically modified at the α-amino groups of β chains of hemoglobin (Haney & Bunn, 1976). The adduct formed by G6P was shown to be chromatographically similar to Hb A_{Ia2}, the naturally occurring glycosylated component found in hemolysate (McDonald et al., 1978). Previous functional studies of the glycosylated minor components indicated that Hb A_{Ia1} and Hb A_{Ia2} were low-affinity hemoglobins with a decreased Bohr effect and heme–heme interaction (McDonald et al., 1979). The presence of very small amounts (0.2–0.5%) of phosphorylated minor components Hb A_{Ia1} and Hb A_{Ia2}, coupled with the fact that a variety of proteins in the red cell hemolysate coeluted with these components in the cation-exchange column, hampers detailed structure–functional studies of these components.

In this study, we isolated a well-defined covalently modified hemoglobin, G6P Hb, by incubating (carbon monoxy)-hemoglobin with D-glucose 6-phosphate under physiological conditions at 37 °C. The structural analysis showed that the phosphorylated sugar specifically modified the amino-terminal valine residues of β chains. Its apparent homogeneity allowed us to perform detailed kinetic and equilibrium studies which may shed light on our understanding of the profound effect of organic phosphate on hemoglobin function.

Materials and Methods

Preparation of G6P Hb. Human blood specimens were obtained from normal individuals and red cell hemolysates were prepared according to the method of Geraci et al. (1969). Synthetic G6P Hb was prepared by incubation of D-glucose 6-phosphate (monosodium salt; Sigma Chemical Co.) with Hb

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[1] Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; G6P Hb, hemoglobin modified with glucose 6-phosphate; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Tris, tris(hydroxymethyl)-aminomethane; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; NMR, nuclear magnetic resonance; T and R, conformations of unliganded and liganded hemoglobin, respectively; t and r, conformations of subunits within the hemoglobin tetramer.

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A purified by chromatography on CM-52 (Whatman). Hb A (6.4 mM in heme) was flushed with pure CO gas for 20 min and converted to (carbon monoxy)-HbA. The (carbon monoxy)-HbA was dialyzed against Krebs–Ringer phosphate buffer, pH 7.4, overnight and then incubated under sterile conditions with 150 mM glucose 6-phosphate in the presence of 0.5 mL of a penicillin–streptomycin solution (5000 units each/mL; Flow Laboratories, Inc., Rockville, MD), at 37 °C for 7 days. At the end of the incubation period, the incubation mixture was dialyzed against 0.05 M potassium phosphate buffer, pH 6.6, and chromatographed according to the procedure of McDonald et al. (1978) on Bio-Rex 70 (Bio-Rad). Polyacrylamide slab gel isoelectric focusing (Drysdale et al., 1971; Bunn & Drysdale, 1971) revealed homogeneity of the isolated G6P Hb. The hemoglobin samples used were found functionally identical whether studied immediately or thawed from samples stored in liquid nitrogen. An ion-exchange column (Dintzis, 1953) was used to remove phosphate from the hemoglobin solutions.

Structural Analysis of G6P Hb. Structural characterization involved the preparation of both ^{14}C- and ^{3}H-labeled G6P Hb. Purified Hb A from Bio-Rex 70 (5–6 mM in heme) was incubated under strictly sterile conditions as described above with uniformly labeled 15 mM D-[^{14}C]glucose-6-P (Amersham) at 37 °C for 3–6 days. After passage through Sephadex G-25 coarse to stop the reaction, the [^{14}C]G6P Hb was purified by chromatography on Bio-Rex 70 as described by McDonald et al. (1978). The ^{3}H-labeled G6P Hb was prepared by treating purified unlabeled G6P Hb preparation with [^{3}H]-$NaBH_4$ (New England Nuclear) as previously described by Bookchin & Gallop (1968) and modified by Bunn et al. (1975). These two radioactively labeled G6P Hb samples were then mixed in appropriate proportions with unlabeled G6P Hb and used in the functional studies reported below. The ^{3}H- and ^{14}C-labeled and unlabeled G6P Hb mixture was converted to globin by acid/acetone precipitation (Garrick et al., 1975) and separated into α and β subunits by the method of Clegg et al. (1966) using carboxymethylcellulose chromatography in 8 M urea. The elution profile is shown in Figure 1. Both the ^{14}C- and ^{3}H-labeled peaks elute in the region of the β globin chain, indicating that only the β chain contained the radioactive sugar moiety as well as borohydride-reducible linkage. There was no ^{14}C radioactivity in the position of the α globin chain. The ^{3}H-labeled peak at the elution position of the α chain was expected since previous studies from our laboratory (Bunn et al., 1979; Shapiro et al., 1980) have shown that 8–10% of Bio-Rex 70 purified Hb A is glycosylated at the α-amino groups of the α chains and at the ϵ-amino groups of lysines. Detailed structural analysis of the radioactive β globin chain was carried out according to procedures of Garrick et al. (1980) which were used in the structural characterization of the β chains of the naturally occurring sugar phosphate hemoglobins. Following tryptic digestion, cation-exchange chromatography of the peptides of the radioactive β chain was performed. This peptide analysis revealed coelution of the ^{14}C and ^{3}H radioactivities at a position identical with that of β Tp1 isolated from ^{3}H-labeled Hb A_{Ia2}. Subsequent amino acid analysis revealed a composition identical with that of βTp1. These structural studies corroborate previous findings that G6P specifically modifies the NH_2-terminal valine residues of β chains independent of the state of hemoglobin ligation (Haney & Bunn, 1976). The greater stability of (carbon monoxy)hemoglobin over oxyhemoglobin allowed preparation of large quantities of G6P Hb for functional studies.

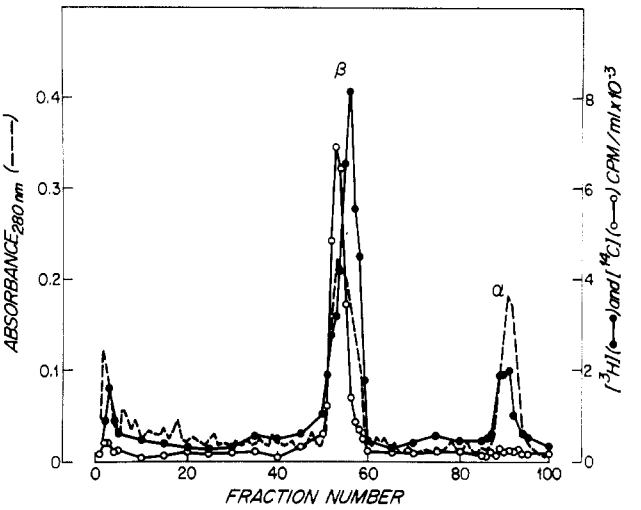

FIGURE 1: Separation of α and β subunits of G6P Hb on a CM-cellulose column (1.2 × 12 cm) in 8 M urea. A mixture of ^{3}H- and ^{14}C-labeled and unlabeled G6P Hb was loaded onto the column, containing 15, 2.0, and 8.5 mg/mL, respectively. Fraction size was 4.6 mL. Column eluents were monitored for optical activity [A_{280} (---)], ^{14}C radioactivity (○), and ^{3}H radioactivity (●). The slight shift of the ^{3}H-labeled peak from the elution position of the ^{14}C-labeled peak in the β-chain region was due to the anomalous elution behavior of $NaBH_4$-reduced peptide which had been noted before (Shapiro et al., 1980).

Oxygen Equilibrium Studies. Manual oxygen equilibrium measurements were performed by a modification of the method of Allen et al. (1950) using a Cary 118 recording spectrophotometer. A tonometer attached to a 2 mm optical path length cuvette was connected to a steady stream of hydrated ultrapure nitrogen gas and filled with a hemoglobin solution which was gently rocked under light for at least 20 min to remove carbon monoxide bound to the hemoglobin. [Hemoglobin samples for kinetic studies (see below) were freed of carbon monoxide in this manner.] Following an appropriate injection of air or purified O_2 into the tonometer, the equilibration time at 20 ± 0.1 °C was determined for each experiment. All measurements were made in the visible region between 500 and 650 nm to monitor percentage saturation as well as methemoglobin formation during each experiment. The value of Hill coefficient (n) and the oxygen pressure at half-saturation (P_{50}) were calculated from the Hill equation by the method of least-squares analysis of the points in the linear region of curves between 20% and 80% saturation. The oxygen equilibrium measurements at the temperatures other than 20 °C were performed exactly the same as described above with each temperature controlled to within ± 0.1 °C.

Kinetic Measurements of Rate Constants with Fast Mixing Technique. Rates of ligand binding and dissociation were measured by the methods of Gibson (1959) in a stopped-flow apparatus temperature regulated at 20 ± 0.1 °C and equipped with a 2 cm path length cell. The overall rate of oxygen dissociation (k) and the rate of oxygen dissociation with carbon monoxide replacement (k_4) were measured by rapidly mixing oxyhemoglobin with a 0.1% dithionite solution in the absence and presence of carbon monoxide. The protein concentrations after mixing were 46 μM in heme. The value of k was measured spectrophotometrically at 577 and 555 nm, whereas that of k_4 was monitored at 578 and 568 nm. The rate of combination of carbon monoxide (l') was measured by mixing a solution of deoxyhemoglobin (6 μM) with an oxygen-free solution of carbon monoxide (94 μM). Dithionite was added to the hemoglobin solution to ensure complete deoxygenation. The reaction was followed in the Soret region at 420 and 431

Table I: Effects of Chloride and IHP on Oxygen Affinities of Hb A and G6P Hb

buffer conditions[a] and effectors	log P_{50}	n[b]	$-\Delta$ log P_{50}/ΔpH[c]
Hb A			
0.01 M Cl^-	0.125	2.3	0.30
0.10 M Cl^-	0.538	2.8	0.60
0.10 M Cl^- + IHP	1.350	2.2	0.92
G6P Hb			
0.01 M Cl^-	0.800	2.1	0.26
0.10 M Cl^-	1.025	2.3	0.36
0.10 M Cl^- + IHP	1.050	2.1	0.52

[a] The basic condition common to all equilibrium studies was that of 100 μM stripped hemoglobins in 0.05 M Bis-Tris-HCl, pH 7.3, at 20 ± 0.1 °C. [b] n is the Hill coefficient obtained from least-squares analysis of the points in the linear region of equilibrium curves between 20% and 80% saturation. [c] The alkaline Bohr effect was estimated from the slope of the plot log P_{50} vs. pH.

nm. All rate constants were obtained from a minimum of three independent determinations. The reactions measured were found to be homogeneous (unless otherwise noted), and the rate constants were obtained from the initial 50% of the reaction by the method of least squares. Fractions of dimeric hemoglobin present in oxyhemoglobin for Hb A and G6P Hb were estimated from the "drift phase" of the time course of oxygen dissociation at 430 nm by rapidly mixing the oxyhemoglobin solution with dithionite (Kellett & Gutfreund, 1970).

Buffers. All buffers were made with Bis-Tris (pH 6.1–7.4) and Tris (pH 7.4–8.8); concentrated HCl was added to adjust the solution to the desired pH. Total chloride concentration was maintained at 0.1 M by the addition of NaCl. Inositol hexaphosphate (IHP) was obtained from Sigma, and its concentration was determined by phosphate analysis (Ames & Dubin, 1960). So that large pH changes during experiments could be prevented, the pH of the IHP solution was adjusted with HCl to 7.0.

Results

Previous studies (Haney & Bunn, 1976; Stevens et al., 1977) indicated that phosphorylated sugars reacted more steadily than glucose to form a Schiff base with hemoglobin. Enhanced reactivity of G6P is probably due to the presence of a higher proportion of the free aldehyde form of G6P in solution than glucose together with the fact that G6P, acting as a DPG analogue, interacts electrostatically at the β-chain binding site. It is likely that G6P behaves similarly to pyridoxal 5′-phosphate (Benesch et al., 1972a) as an affinity label at the NH_2 terminus of the β chain in hemoglobin. Unlike pyridoxal 5′-phosphate, the reaction of G6P with hemoglobin does not require the deoxy conformation. We found that (carbon monoxy)hemoglobin also reacts with G6P specifically at the β chains, albeit at a slower rate than deoxyhemoglobin.

The oxygenation of stripped G6P Hb was studied as a function of pH, chloride ion, and organic phosphate. Four separate preparations of this covalently modified hemoglobin were used for these studies, and all were found to be functionally identical. The oxygen binding properties of stripped Hb A isolated from the same chromatographic procedure are included for comparison.

Effect of pH on the Oxygen Equilibria of G6P Hb. The oxygen affinity of G6P Hb in 0.05 M Bis-Tris buffer, pH 7.0, 0.1 M Cl^-, and 20 °C is approximately 2.5-fold lower than that of Hb A under identical conditions (Figure 2A). A lower affinity at this pH has been previously reported by us for G6P

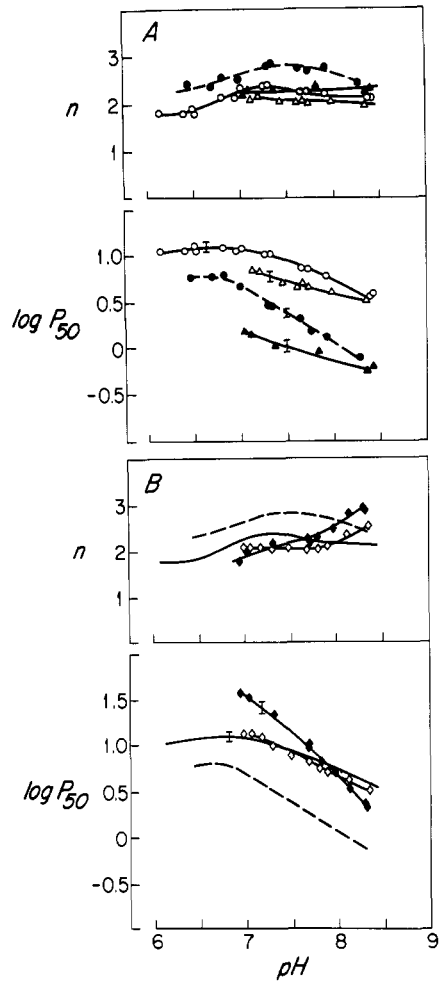

FIGURE 2: pH dependence of the oxygen affinity of Hb A and G6P Hb at 20 °C in the presence of allosteric effectors. (A) The effect of chloride on the oxygen affinity (log P_{50}) and the cooperativity (n value) of Hb A and G6P Hb. 100 μM hemoglobin solutions were prepared in 0.05 M Bis-Tris (pH 6.1–7.4) or 0.05 M Tris (pH 7.4–8.8) titrated with HCl and then adjusted to the desired chloride concentration by the addition of NaCl. Hb A in 0.01 M chloride (▲); Hb A in 0.10 M chloride (●); G6P Hb in 0.01 M chloride (△); G6P in 0.10 M chloride (○). (B) Effect of inositol hexaphosphate (IHP) on the oxygen affinity (log P_{50}) and the cooperativity (n value) of Hb A and G6P Hb. The buffer conditions are the same as in (A). Hb A in 0.10 M chloride without IHP (---); Hb A in 0.10 M chloride plus 1 mM IHP (♦); G6P Hb in 0.10 M chloride without IHP (—); G6P Hb in 0.10 M chloride plus 1 mM IHP (◇). The error bars give an estimation of the mean ± SD determined at the indicated pH.

Hb as well as two naturally occurring sugar phosphate hemoglobins, Hb A_{Ia1} and Hb A_{Ia2}. The sugar phosphate groups, which are covalently bound to these hemoglobins, presumably mimic the effect of free organic phosphate in lowering their oxygen affinities (McDonald et al., 1979).

Over a pH range of 6.2–8.5, the oxygen affinity of Hb A increased about 9-fold whereas that for G6P Hb increased only 2.8-fold (Figure 2A) in 0.05 M Bis-Tris or Tris buffer, 0.1 M NaCl, and 20 °C. The values of the alkaline Bohr effect ($-\Delta$ log P_{50}/ΔpH) obtained for Hb A and G6P Hb were 0.60 and 0.36, respectively (Table I). The former value agrees with that reported by Benesch & Benesch (1974). The decreased alkaline Bohr effect of G6P Hb is noteworthy since previous studies with mutant or chemically modified hemoglobin possessing either low-affinity and/or blocked β-chain NH_2 termini seem to exclude the role of 1β Val in the alkaline Bohr effect (see Discussion).

Effect of Chloride and Phosphate on Oxygenation of G6P Hb. The oxygen equilibria of G6P Hb over a pH range from

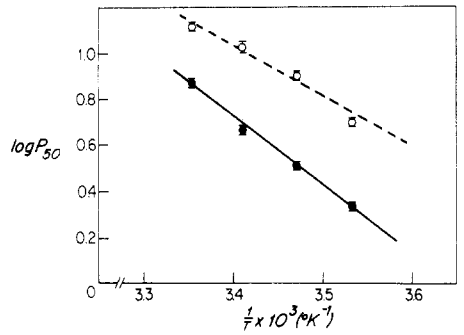

FIGURE 3: van't Hoff plots of the oxygen affinity (log P_{50}) vs. the reciprocal of the absolute temperature for Hb A and G6P Hb. The buffer concentrations were 0.05 M Bis-Tris and 0.1 M NaCl, pH 7.0. The temperatures were controlled to within ±0.1 °C. The lines were drawn from the least-squares analysis of the data points of Hb A (●) and G6P Hb (○). The error bars indicate the mean ± SD estimated from the triplicate determinations of each temperature.

7.3 to 8.5 were measured at 0.01 M Cl^- (Figure 2A). In the presence of low chloride, the oxygen affinity of G6P Hb was always several-fold lower than that of Hb A; however, the oxygen affinities of both G6P Hb and Hb A were increased. An increase in oxygen affinity of Hb A with decreased anion concentration has been well documented in the literature (Rossi-Fanelli et al., 1961; Antonini et al., 1962; Bunn & Guidotti, 1972; Rollema et al., 1975). Both G6P Hb and Hb A exhibited a decreased Bohr effect under conditions of low [Cl^-] (Figure 2A and Table I) as compared with 0.1 M Cl^-. The magnitude of the decrease was 2-fold for Hb A and only 1.4-fold for G6P Hb. As a result, the Bohr effect for these two hemoglobins in 0.01 M Cl^- was very similar.

The log P_{50} of G6P Hb in the absence and presence of 1 mM IHP over the alkaline pH range at 20 °C is shown in Figure 2B and presented in Table I. This allosteric anion had little effect on the oxygen binding properties of G6P Hb. In contrast, a substantial decrease in oxygen affinity as well as an enhanced alkaline Bohr effect was observed for purified Hb A, in agreement with the findings of Benesch et al. (1977). The lack of effect of this potent modifier on the functional properties of G6P Hb is compatible with the premise that IHP and G6P are competing for the identical binding sites on the hemoglobin molecule.

The n value for G6P Hb under all conditions was consistently and significantly lower than that of Hb A with the exception of Hb A, a value which may indicate an altered T → R transition in G6P Hb. However, this lowering of n is most probably the result of heterogeneity introduced by α,β chain differences (see the kinetic studies described below). It is probable that a sugar phosphate covalently attached to the β-chain NH_2 termini would enhance the functional differences between α and β chains.

Effect of Temperature on Equilibrium Properties of G6P Hb. Oxygen equilibria in 0.05 M Bis-Tris buffer, pH 7.0, and 0.1 M Cl^- were measured at four temperatures. The results of these experiments are presented in a van't Hoff plot (Figure 3). The $\Delta H°$ was determined from the slope of this plot and corrected for the heat of solution of oxygen. A summary of the thermodynamic parameters for G6P Hb and Hb A is presented in Table II. Although there was only a 2 kcal/mol energy difference of $\Delta G°$ between G6P Hb and the Hb A control, the difference in $\Delta H°$ was estimated to be about 12.5 kcal/mol. The reactions of oxygenation were exothermic for both these hemoglobins. The $\Delta H°$ determined from the van't Hoff relation should represent the sum of the intrinsic heat resulting from the binding of oxygen plus other contributions

Table II: Thermodynamic Parameters of the Oxygenation Reactions for Hb A and G6P Hb[a]

conditions	$\Delta G°$[b] (kcal/mol)	$\Delta H°$[c] (kcal/mol)	$\Delta S°$[d] (cal deg^{-1} mol^{-1})
Hb A	−27.0	−42.4	−52.6
G6P Hb	−25.0	−29.9	−16.7

[a] The buffer employed was 0.05 M Bis-Tris, pH 7.3, and 0.1 M Cl^-. Benesch et al. (1969) and Chien & Mayo (1980) showed that this buffer had little effect on the P_{50} value of hemoglobin and that the difference between P_{50} and P_m (the medium ligand activity) was very small, thus allowing the extraction of thermodynamic data directly from P_{50} without introducing substantial error (Wyman, 1964). [b] $\Delta G°$ was determined from log P_{50} at 20 °C by using the equation $\Delta G° = 2.3RT(4 \log P_{50})$. P_{50} was converted into oxygen solubility in M. At 20 °C a pO_2 of 1 mM Hg corresponds to 1.8×10^{-6} M dissolved oxygen. [c] $\Delta H°$ obtained from the slope of the van't Hoff plot log P_{50} vs. $1/T$ and corrected for the heat of solution of oxygen (−3.04 kcal/mol at 20 °C). [d] $\Delta S°$ was calculated from $\Delta S° = (\Delta H° - \Delta G°)/T$; T = 293 K.

due to alteration in conformation and heterotropic interactions induced by oxygenation. The most prominent of these ligand-linked heterotropic contributions is the enthalpy change due to the release or uptake of protons, which represents the heat associated with the Bohr effect (Wyman, 1964). This amounts to 5.7 kcal absorbed/mol of oxygen bound for the heats of Bohr proton release (Antonini et al., 1962). When this is taken into account, the intrinsic enthalpy change for Hb A resulting from oxygenation is calculated to be −16.3 kcal/mol of O_2 bound, which is in reasonable agreement with that of Mills et al. (1979) (−15.6 ± 1 kcal/mol of O_2). The corresponding $\Delta S°$ values indicate that the contribution of entropy to the free energy of oxygenation is negative. Benesch et al. (1969) measured the temperature dependence of the oxygenation reaction for Hb A in the absence and presence of DPG and had results qualitatively similar to those here. They found that in the presence of phosphate the heat of reaction and the corresponding entropy term were both decreased. This decrease in enthalpy and entropy is consistent with stabilization of the deoxy structure (Ackers, 1980).

Ligand Binding Kinetics of G6P Hb. The kinetic profiles of the overall oxygen dissociation (k) at pH 7.3 and 20 °C in the presence of 0.05 M Bis-Tris-buffer, 0.1 M Cl^- for G6P Hb and Hb A are shown in Figure 4A. Hb A showed a homogeneous reaction time course with a first-order rate constant of 25 s^{-1}. G6P exhibited a biphasic reaction with rate constants of 87 s^{-1} and 27 s^{-1} for the fast and slow phases, respectively. The biphasic oxygen dissociation kinetic profile of G6P Hb may best be explained by different reactivities of the α and β chains, since the fast and slow reactions appear to both contribute 50% of the overall time course. At pH 8.5 this kinetic heterogeneity is absent and both hemoglobins exhibit monophasic kinetics (Figure 4B). Nonetheless, the rate of ligand dissociation is still 2-fold faster for G6P Hb over Hb A and is consistent with a hemoglobin of low oxygen affinity (Table III).

The time course of oxygen dissociation with carbon monoxide replacement (k_4) for Hb A and G6P Hb at pH 7.3 is shown in Figure 5. This kinetic experiment measures the dissociation of the first oxygen molecule from fully liganded hemoglobin. Since the oxy or liganded state of hemoglobin possesses a higher affinity for ligand than the deoxy or unliganded state, the value of k_4 will always be lower than that of k for a cooperative hemoglobin (see Table III). The value of k_4 for G6P Hb at pH 7.3 and 8.5 is greater than that for

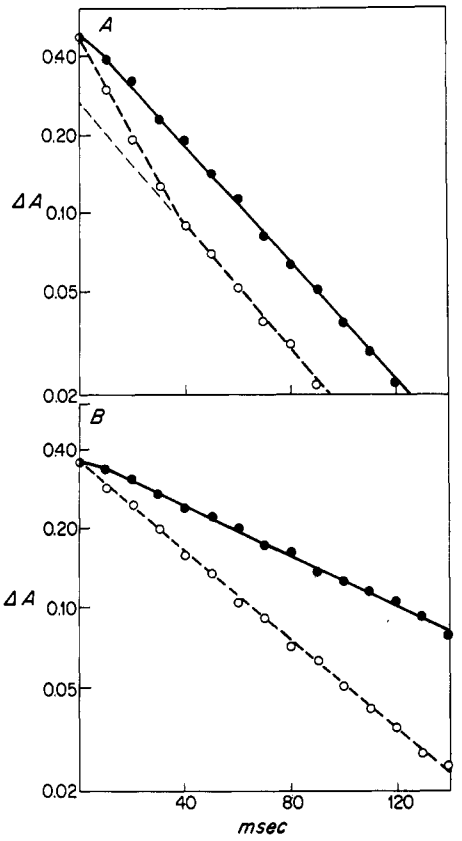

FIGURE 4: Time course of oxygen dissociation (k) at 20 °C. (A) Oxygen dissociation kinetics of Hb A (●) and G6P Hb at pH 7.3 (○). (B) Oxygen dissociation kinetics of Hb A (●) and G6P Hb (○) at pH 8.5. The hemoglobin concentration after mixing was 46 μM in heme for either hemoglobin. The buffer conditions were 0.05 M Bis-Tris (pH 7.3) or 0.05 M Tris (pH 8.5), and the total chloride concentration was 0.10 M (after mixing). These reactions were followed at 577 nm.

Table III: Kinetic Data[a] of Hb A and G6P Hb

conditions	k (s^{-1})	k_4 (s^{-1})	l' (μM^{-1} s^{-1})	% dimer
Hb A				
pH 7.3	25	10	0.19	54
pH 7.3 + IHP			0.085	
pH 8.5	10	8	0.29	
G6P Hb				
pH 7.3	87, 27[b]	20	0.12	27
pH 7.3 + IHP			0.12	
pH 8.5	20	12	0.19	

[a] All rate constants were determined by the stopped-flow technique as described under Materials and Methods. The ratio of the affinity of hemoglobin for carbon monoxide and oxygen (the partition coefficient, M) has been found by Benesch et al. (1972b) to be unaffected by DPG. Thus, the difference in functional properties of Hb A and G6P Hb presented here are most probably due to distinct kinetic properties of the respective hemoglobins and not a consequence of the specific ligand employed in the experiment. [b] Biphasic.

Hb A, demonstrating that the liganded forms of G6P Hb and Hb A are not the same.

The extent of dissociation of the liganded state of G6P Hb appears to be less than that of liganded Hb A. The fraction of oxygenated $\alpha\beta$ dimers presented at pH 7.3 for these two hemoglobins was estimated from the slow absorbance changes following rapid deoxygenation of oxyhemoglobin by sodium dithionite (Kellett & Gutfreund, 1970). The results for Hb A and G6P Hb at 5.4 μM in heme before mixing are given in Table III.[2] The decreased dissociation of G6P Hb in its

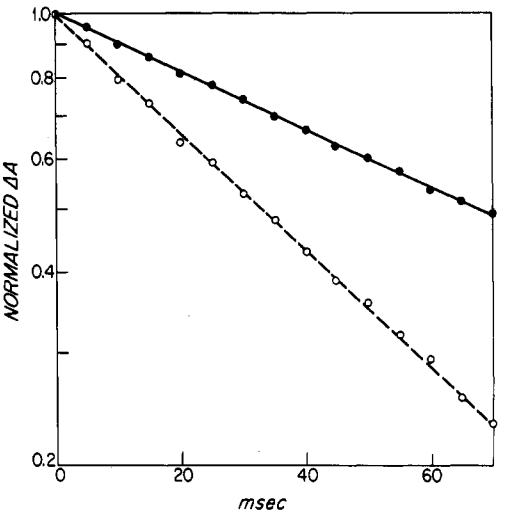

FIGURE 5: Time course of oxygen dissociation with carbon monoxide replacement (k_4) at 20 °C and pH 7.3. The protein concentration after mixing was 45 μM in heme for Hb A (●) and G6P Hb (○). The concentration of carbon monoxide was 480 μM after mixing. The buffer conditions were 0.05 M Bis-Tris and 0.10 M total chloride concentration after mixing. The wavelength was 578 nm.

liganded or R state is consistent with the results of Gray (1974) which indicated that organic phosphate inhibited the dissociation of liganded Hb A into $\alpha\beta$ dimers.

At both pH 7.3 and 8.5, G6P Hb bound carbon monoxide more slowly than Hb A, indicative of a low-affinity hemoglobin (Table III). The addition of IHP had little effect on the rate of combination of G6P Hb as compared with Hb A, in keeping with the oxygen equilibrium findings.

Evidence for α,β-Chain Differences. Gray & Gibson (1971) showed that IHP induced kinetic as well as spectral heterogeneity in the reaction of deoxyhemoglobin with carbon monoxide. G6P, covalently linked to the β chain cleft, might be expected to mimic this effect of IHP. However, no difference in the rate of carbon monoxide binding to G6P Hb near the Soret isosbestic region of the deoxy- and (carbon monoxy)hemoglobin spectra was observed (see Figure 6). The rates of carbon monoxide combination at 424 and 426 nm for G6P Hb were found to be identical (0.12 μM^{-1} s^{-1}). The only difference observed for G6P Hb compared to that of Hb A (Figure 6) other than the absolute rate of ligand binding was that the 425-nm isosbestic line of Hb A was not evident in the kinetic profile of G6P Hb.

Evidence for chain heterogeneity in G6P Hb could be found, however, in the oxygen dissociation reaction. Gibson (1973) described differences in the ligand dissociation rates of the α and β chains of Hb A by demonstrating a wavelength dependence of oxygen dissociation rates in the neighborhood of the oxy–deoxy isosbestic points. We applied this approach to G6P Hb and observed different rates of oxygen dissociation at 584 and 587 nm, wavelengths employed by Gibson (1973). As shown in Figure 7, the rate of oxygen dissociation at these two wavelengths differed by several-fold, being 80 s^{-1} and 30 s^{-1} at 584 and 587 nm, respectively. These rates agree rather well with the rates of the fast and slow phases of oxygen dissociation seen at the oxyhemoglobin spectral peak (Figure 4 and Table III).

Discussion

Previous functional studies of glycosylated components by McDonald et al. (1979) did not show any drastic changes in

[2] Additional kinetic measurements of the $K_{4,2}^{oxy}$ for G6P Hb are currently under way in our laboratory.

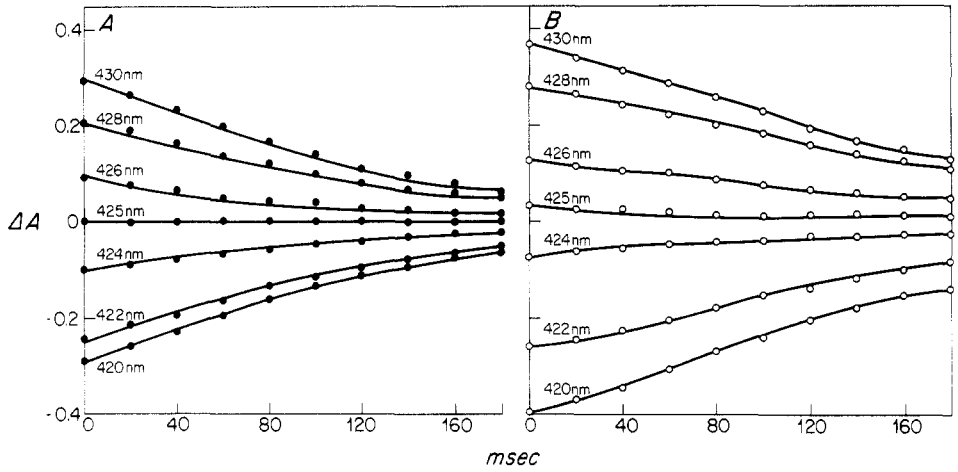

FIGURE 6: Wavelength dependence of carbon monoxide binding to deoxyhemoglobins at 20 °C and pH 7.3. The protein concentrations after mixing were 2.60 and 3.20 μM in heme for Hb A and G6P Hb, respectively. The carbon monoxide concentration was 47 μM after mixing. (A) Hb A (●); (B) G6P Hb (○). The buffer conditions after mixing were 0.05 M Bis-Tris and 0.1 M total Cl, pH 7.3.

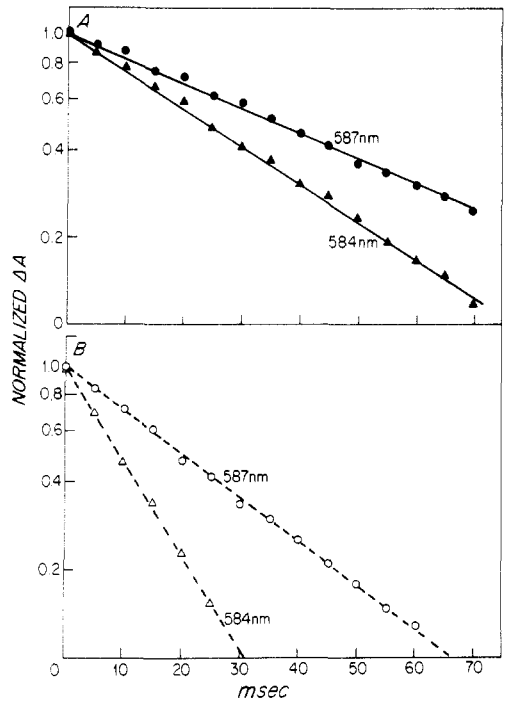

FIGURE 7: Time course of oxygen dissociation of two different wave lengths. (A) Hb A at 587 (●) and 584 nm (▲). Hb A gives the rate constants of 23 s^{-1} and 32 s^{-1} at 587 and 584 nm, respectively. (B) G6P Hb at 587 (○) and 584 nm (△). G6P Hb gives the rate constants of 30 s^{-1} and 80 s^{-1} at 587 and 584 nm, respectively. The hemoglobin concentration was 46 μM, and the final buffer conditions were 0.05 M Bis-Tris and 0.1 M total Cl, pH 7.3, after mixing.

oxygen affinity and alkaline Bohr effect of Hb A_{Ic} when compared to those properties of Hb A. On the other hand, the striking functional properties of Hb A_{Ia1} and Hb A_{Ia2} prompted us to investigate G6P Hb, a synthetic adduct which shares many properties with Hb A_{Ia2}. G6P Hb prepared here by incubating Hb A in carbon monoxy form under physiological conditions was shown to be specifically modified at the α-amino groups of β chains similar to that formed by incubating Hb A in the deoxy form with G6P (Haney & Bunn, 1976). It is noteworthy that, unlike pyridoxal phosphate (Benesch et al., 1972a) and cyanate (Kilmartin & Rossi-Bernardi, 1969), the reaction of G6P with (carbon monoxy)hemoglobin did not produce any modification at α-amino groups of α chains despite the fact that in liganded hemoglobin the pK of the NH_2-terminal groups of α chains is about the same as that of β chains (Garner et al., 1975). The results of structural analysis suggests that G6P behaves as a DPG analogue and can fit the β-chain crevice through the interaction of phosphate groups of G6P with specific cationic groups on the β chain (Arnone, 1972). This interaction permits the formation of a Schiff base linkage at 1β Val residues. The Amadori rearrangement following the Schiff base formation (Bunn et al., 1975) probably contributes to the greater stability of G6P Hb compared to that of the pyridoxal phosphate adduct which required reduction of the Schiff base with $NaBH_4$ in order to form a stable secondary amine (Benesch et al., 1972a).

Structure–Function Relationships of G6P Hb. Interpretation of the functional properties of normal hemoglobin modified by a chemical agent (as well as mutant hemoglobins) is often complicated by the fact that a structural alteration at one site can have distant effects. Most mutant and chemically modified hemoglobins have higher affinities than normal hemoglobin. Among the few exceptions, Hb Kansas ($\alpha_2\beta_2^{102Asn\rightarrow Thr}$) was shown to have a low affinity for oxygen, a low value of the Hill coefficient (n = 1.3), and a normal alkaline Bohr effect (Bonaventura & Riggs, 1968). In contrast the Hb Kansas, G6P Hb has an n value of 2.3 (pH 7.0) and a decreased alkaline Bohr effect. G6P Hb, like Hb Raleigh ($\alpha_2\beta_2^{1Val\rightarrow Acetyl\text{-}Ala}$) (Moo-Penn et al., 1977) and carbamylated Hb with modified β-chain NH_2 termini (Nigen et al., 1974; Nigen & Mmanning, 1975) has low oxygen affinity. The phosphate group of G6P Hb would be expected to stabilize the T quaternary structure, similar to organic phosphate free in solution. In addition, the decrease of oxygen affinity in G6P Hb might be due to blocking of the NH_2 termini of β chains. According to Bonaventura & Bonaventura (1978), the blocking of positively charged residues of the DPG binding site could decrease oxygen affinity by stabilizing the deoxy conformation.

G6P Hb had an oxygen affinity 2.5-fold lower than that of stripped Hb A at pH 7.0. This lowered affinity is much less in magnitude than that seen for Hb A upon the addition of 1 mM IHP. Similarly, the rate of carbon monoxide combination (l') of Hb A in the presence of IHP was 1.4-fold slower than that of G6P Hb. Chain heterogeneity induced by the addition of IHP to Hb A (Gray & Gibson, 1971) could not be detected in G6P Hb. It appears that the structural changes accompanied by the covalent modification of Hb A with G6P are very different from those displayed by hemoglobin modified with IHP. G6P Hb is much more similar to Hb A containing

concentrated DPG. In fact, an addition of 100 mM DPG is required to lower the affinity of Hb A by 2.5-fold (Benesch & Benesch, 1974). The concentration of covalently bound phosphate in the microenvironment of the DPG binding site must be quite large and, hence, very effective in lowering oxygen affinity. This decreased affinity of G6P Hb is reflected in the slower ligand combination rate (l'). Interestingly enough, only the addition of 1 mM DPG (Pennelly & Noble, 1978) is needed to obtain this rate for stripped Hb A. Thus, the G6P moiety does more than solely stabilize the deoxy form of G6P Hb.

In fact, the properties of liganded G6P Hb and Hb A are intriguingly different. Measurement of oxygen dissociation with carbon monoxide replacement revealed a faster rate for G6P Hb than for Hb A at neutral pH. Even at pH 8.5, where DPG no longer has any effect (Benesch et al., 1969), G6P Hb releases ligand more rapidly than Hb A. The differences in overall oxygen dissociation are even more striking. G6P Hb exhibits a biphasic kinetic profile which is resolved into two homogeneous rates at 587 and 584 nm, respectively. These are the precise wavelengths employed by Gibson (1973) to monitor α,β chain differences in normal hemoglobin. Presumably the rate at 587 nm represents the α-chain dissociation and the rate at 584 nm, the β-chain dissociation. The value of k for the α chain of G6P Hb and Hb A differ by 1.3-fold whereas the β-chain rate differs by 2.5-fold. It might be expected that the β chain, having a molecule covalently bound to its NH_2 termini, would exhibit a greater change in reactivity than an unmodified α chain irrespective of the quaternary constraints. Liganded or oxy-Hb A binds DPG, as does deoxy-Hb A but much more weakly (Riggs, 1971; Benesch & Benesch, 1974). Gray (1974) showed that DPG stabilizes the tetrameric state of liganded hemoglobin. Our kinetic results indicate that G6P Hb is less dissociated than Hb A.

Alkaline Bohr Effect of G6P Hb. G6P Hb differs from other low-affinity hemoglobins and hemoglobins with blocked β NH_2 termini in having a marked (40%) decrease in the alkaline Bohr effect. Since the addition of organic phosphate generally increases the alkaline Bohr effect (Benesch et al., 1969), an enhancement might be expected for G6P Hb. There is considerable evidence, however, that the addition of anion first results in an increase in the Bohr effect, followed by a *decrease* at high anion concentration (Benesch et al., 1969; Riggs, 1971; Van Beek & DeBruin, 1980). When the concentration of DPG was raised from 0 to 250 mM (0.05 M Bis-Tris buffer; 0.1 M Cl^-; 20 °C; pH 7–7.6; [Hb] = 60 μM), the Bohr effect was first enhanced but then decreased, falling below that of stripped hemoglobin when DPG exceeded 150 mM (Benesch et al., 1969). Likewise, Van Beek & De Bruin (1980) have recently shown that as the anion concentration (Cl^-, Br^-, and I^-) is increased there is a corresponding increase in the Bohr effect, followed by a decrease at high salt concentrations. This decrease in the effect of pH on the oxygen affinity of Hb A at high concentrations of salt has been attributed to the binding of these anions to oxyhemoglobin as well as deoxyhemoglobin (Riggs, 1971; Van Beek & DeBruin, 1980).

The Bohr effect has been coupled with the quaternary change in the hemoglobin molecule upon binding of ligand (Perutz et al., 1969; Perutz, 1970). In the presence of organic phosphate, this Bohr effect is made up of the "true" Bohr protons as well as an enhanced effect due to stronger binding of phosphate at low pH (Benesch & Benesch, 1974). Under conditions of 0.1 M NaCl and 2 mM DPG, residues Val 1α, His 122α, Val 1β, His 2β, His 143β, and His 146β contribute to the alkaline Bohr effect (Perutz et al., 1980, and references cited therein). Three of these residues, Val 1β, His 2β, and His 143β, also participate in binding organic phosphate (Arnone, 1972) and as such probably do not contribute to the "true" Bohr effect (Benesch & Benesch, 1974; Perutz et al., 1980). Of the remaining residues, Val 1α, is strongly dependent upon [Cl^-] (O'Donnell et al., 1979; Van Beek & DeBruin, 1980). At very low [Cl^-], G6P Hb and HbA exhibit a Bohr effect that is similar. This is the condition under which Kilmartin et al. ((1980) and references therein) have evidence that the contribution of His 146β becomes dominant. Russu et al. (1980), however, report little participation of this residue under these conditions. Because of the controversy concerning the contribution of this residue to the alkaline Bohr effect (Russu et al., 1980; Ohe & Kajita, 1980; Perutz et al., 1980), an investigation of G6P Hb by proton NMR[3] and by hydrogen ion titration would be of considerable interest.

Proposed Model. The functional and structural results support the following model. It is unlikely that both G6P molecules can be accommodated in the central cavity at the same time, even in the unliganded state. The phosphate group of one G6P molecule probably occupies the DPG binding site in the deoxy state while the other G6P is "free" in solution. The binding of this former phosphate contributes to the low oxygen affinity by mimicking the effect of DPG. It is uncertain whether the phosphate moiety is expelled from the binding site upon oxygenation. Our functional studies indicate that even after full oxygenation the β chains of G6P Hb may remain partially in the t structure and therefore the phosphate binding might persist. Irrespective of the position of the phosphate groups, the covalent attachment of two G6P molecules to the β NH_2 termini of liganded hemoglobin results in a very high effective concentration of phosphate, and this is mirrored in the unique functional and structural properties of liganded G6P Hb. Future investigations of G6P Hb by X-ray analysis[4] and by proton and phosphorus NMR should shed light on the understanding of the conformational changes of hemoglobin in response to organic phosphate binding.

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[3] Drs. I. M. Russu and C. Ho (unpublished data) have performed proton NMR studies on G6P Hb in a Bis-Tris buffer system and have observed a broadening of the 2β His peak as well as perturbations of other histidines, consistent with an altered Bohr effect in this protein.

[4] Further verification of the structural changes proposed in the report should await the results of X-ray diffraction study of this modified hemoglobin, which is currently under way in the laboratory of Dr. Arthur Arnone.

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