Nonenzymatic Glycosylation of Hemoglobin

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The incubation of dialyzed hemoglobin A with a number of phosphorylated glycolytic intermediates leads to the formation of covalent hemoglobin adducts that co-chromatograph with hemoglobin A,

Phosphorylated hexoses (glucose-6-P, fructose-6-P, fructose-1,6-P, glyceraldehyde-3-P, dihydroxyacetone-P) containing a free aldehyde or ketone can glycosylate hemoglobin A nonenzymatically from 7 to 12% of the hemoglobin can be modified by a 72-h incubation of an equimolar mixture of hemoglobin A and the phosphorylated intermediate. No significant formation of adduct was seen with a sugar alone (glucose, fructose) or glycolytic intermediate which had a blocked aldehyde (glucose-1-P, glucose-1,6-P, UDP-glucose). The addition of an equimolar amount of 2,3-diphosphoglycerate reduced adduct formation. Evidently, the phosphate is needed to orient and stabilize the intermediate in the bisphosphoglycerate pocket of hemoglobin so that the addition reaction can proceed. All of the hemoglobin A adducts were indistinguishable from hemoglobin A, by ion exchange chromatography and isoelectric focusing. The hemoglobin A-glucose-6-P adduct and hemoglobin A, had a NaBH, reducible linkage in the β chain. The concentration of hemoglobin A, is elevated in patients with diabetes mellitus. This presumably reflects the increased concentrations of glycolytic intermediates (glucose-6-P, fructose-6-P, fructose-1,6-P, dihydroxyacetone-P) which were found to be significantly elevated in the red cells of diabetic patients as compared with normal controls.

The minor hemoglobins appear to arise as derivatives of hemoglobin A. As described in the accompanying paper, we have assigned the gentic group A, to hemoglobin A, and the sugar attached to the NH-terminal valine of the β chain as 1-deoxy-1-amaaloylfructose (7). In the present communication, we have found that phosphorylated hexoses or trioses containing a free aldehyde or ketone group can glycosylate hemoglobin A nonenzymatically to form an adduct that is indistinguishable from hemoglobin A, by ion exchange chromatography and isoelectric focusing. The addition is specific at the β chain with a linkage analogous to that for hemoglobin A.

MATERIALS AND METHODS

Hemolysates were prepared from blood drawn from normal adult volunteers. The red cells were separated from the plasma and washed twice with 0.14 M NaCl solution prior to being lysed in distilled water (twice their volume). All hemoglobin concentrations were measured by the method of Drabkin (8).

Hemoglobin A was separated from the minor hemoglobins, A, A,, and A,, by the column chromatographic method of Trivelli et al. (4). The hemolysate (75 g of hemoglobin) was applied to a column (15 x 50 cm) of the cation exchange resin Bio-Rex 70 (Bio-Rad Co., Richmond, Ca.) which had previously been equilibrated with Developer 6 (0.05 M sodium phosphate buffer, 0.01 M KCN, pH 6.75). The minor hemoglobin fractions were eluted with Developer 6. The hemoglobin A which remained on the column was eluted with 0.25 M sodium phosphate buffer pH 7.0, and concentrated by ultrafiltration (300 to 350 mg/ml). The hemoglobin was then dialyzed free of organic phosphates in vacuum-expanded tubing with three changes of 1000 volumes of 0.14 M sodium chloride buffered with 0.001 M bis-Tris buffer, pH 7.4, at 5°. The dialyzed hemoglobin solution (2.5 mM) was incubated with an equimolar amount of various phosphorylated glycolytic intermediates or sugars. Glucose 6-phosphate, glucose 1,6-diphosphate, fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, fructose 1-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, 2,3-diphosphoglycerate, glucose, and fructose were obtained from Sigma Chemical Co., St. Louis, Mo. Mixtures of Hb A and one of the glycolytic intermediates or sugars were sterilized by Millipore filtration (0.45 μm filter, Millipore Corp., Bedford, Mass.) and incubated at 37°. At intervals, aliquots (20 μg of hemoglobin) were applied to columns of Bio-Rex 70 (0.6 x 25 cm) equilibrated with Developer 6. The modified hemoglobin was eluted from the column in 1 ml fractions. The elution profile of the hemoglobin was determined by measuring the absorbance of the fractions at 415 nm. The quantification of the hemoglobin was achieved by measuring the area under the peaks with a compensating polar planimeter. Quantities of the adducts were expressed as a percentage of the total amount of hemoglobin applied to the column.

Hemoglobin A, and hemoglobin A, are eluted near the void volume by the chromatographic method of Trivelli et al. (4) and are not always separated from each other. In order to attain better separation, we have modified their procedure. The solution of Hb A, obtained by chromatography on Bio-Rex 70 with Developer 6

1 The abbreviation used is: bis-Tris, (bis(2-hydroxyethyl)amino)trimethylethyl]methane.
was concentrated by ultrafiltration, dialyzed against Developer 6 (0.05 m phosphate, 0.01 m KCl), adjusted to a pH of 6.1 with H3PO4, and applied to a Bio-Rex 70 column (0.85 x 25 cm) previously equilibrated with Developer 6, pH 6.1. The column was then washed with 2 bed volumes of Developer 6 at pH 6.1. This buffer was replaced with a solution of Developer 6 adjusted to pH 6.3 and 2-ml fractions were collected. When the pH of the buffer coming from the column reached 6.3, the buffer was again changed to another solution of Developer 6 which had been adjusted to a pH of 6.6. By this method, complete separation of Hb Aα and Hb Aβ was achieved. The isoelectric points of native Hbα or Hb A adducts were measured by isoelectric focusing in a pH gradient of 6 to 8 (LKB ampholites) in polyacrylamide gels (LKB MultiTome apparatus). The formation and reversibility of glucose-6-P-Hb A adduct were also determined by measuring the incorporation of [14C]glucose-6-phosphate into acid-precipitable protein. Hemoglobin A at different concentrations was incubated with uniformly labeled [14C]glucose-6-P (New England Nuclear, Boston, Mass.). At various intervals 50-μl aliquots were added to 2 ml of distilled water and the protein precipitated by the addition of 2 ml of cold 10% trichloroacetic acid. The precipitate was collected by vacuum filtration on a Millipore filter (5 μ pore) and washed 3 times with 5 ml of cold 5% trichloroacetic acid. The Millipore filters were dried, oxidized in a Tri-Carb sample oxidizer, and the amount of radioactivity determined.

The glucose-6-P-Hb A adduct and natural Hb Aα were examined for the site of glycosylation. The modified hemoglobin was reduced by the method of Bookchin and Gallop (9). A 200-fold excess of NaBH4 (Amersham/Searle, Arlington Heights, Ill.), specific activity 1.4 x 10^6 dpm/mmol, was reacted with the hemoglobin solutions at room temperature for 10 min. After extensive dialysis to remove unreacted NaBH4, the globin was precipitated in 2% HCl in acetone at -20°. The globin was dissolved in 8 m urea and separated on CM-Sephadex (Whatman, Clifton, N. J.) columns (1 x 18 cm) according to the method of Clegg et al. (10). The absorbance of the fractions (3 ml) was measured at 280 nm and fractions were pooled according to the protein elution profile. The pooled samples were brought to dryness by ultrafiltration in dialysis tubing. The tubing with each sample was oxidized in a Tri-Carb sample oxidizer and the amount of radioactivity determined in a Packard Tri-Carb liquid scintillation counter.

The concentrations of glycolytic intermediates in the erythrocytes of patients with diabetes mellitus and normal controls were determined by the method of Minakami et al. (11).

**RESULTS**

The incubation of dialyzed hemoglobin A with a number of glycolytic intermediates was observed to lead with time to the formation of a new hemoglobin that eluted from Bio-Rex 70 with Developer 6 in the region of Hb Aα. Fig. 1 shows the elution profile to the adduct formation at 24-h intervals during the incubation of Hb A with glucose 6-phosphate. The unreacted Hb A was not eluted under these conditions. A small amount of hemoglobin present at zero time presumably reflects adduct formation that occurred during the time between the initial elution of Hb A from the Bio-Rex 70 column and the dialysis of tightly bound organic phosphates from the Hb A. The incubation of dialyzed Hb A alone for up to 144 h did not increase the amount of hemoglobin eluted in the position of the adduct. However, the incubation of undialyzed hemoglobin isolated by column chromatography led to significant adduct formation in the absence of added sugar phosphates. Evidently, the Hb A has a significant amount of organic phosphates tightly associated with it.

The ability of a number of sugar and phosphate sugars to form adducts upon incubation with dialyzed Hb A is recorded in Table I. No significant formation of adduct was detected with any unphosphorylated sugar (glucose, fructose, or glycolytic intermediate that had a blocked aldehyde (glucose-1-P, glucose-1,6-P2, UDP-glucose). All of the sugar phosphates tested which had either an available aldehyde or ketone formed adducts with hemoglobin. Under these conditions from 7% to 12% of the hemoglobin was modified. Using the stepwise pH chromatography method described under "Materials and Methods," it was possible to distinguish between Hb Aα and Hb Aβ (Fig. 2). The chromatographic separation of Hb Aα and Hb Aβ was confirmed by isoelectric focusing. All of the adducts formed with the glycolytic intermediates co-migrated with Hb Aα on isoelectric focusing and column chromatography (Table I). This similarity was surprising since fructose 1,6-diphosphate-Hb A adduct has an additional phosphate that apparently does not affect the overall charge of the protein.

The addition of 2,3-diphosphoglycerate to the incubation mixtures caused a reduction in the amount of Hb A glycosylated (Table II). This inhibition varied among the intermediates tested but was markedly effective with fructose-1,6-P2. In the anaerobic state the ability of 2,3-diphosphoglycerate to inhibit glycosylation was only slightly increased.

The rate of adduct formation was found to be related to both the concentration of glucose-6-P and Hb A. Fig. 3A records the incorporation with time of glucose-6-P into acid-precipitable protein of a Hb A solution (0.2 m) mixed with increasing molar ratios of glucose-6-P to Hb A. The formation of adducts appears to plateau under these conditions after 60 h. A plot of glucose-6-P incorporated versus the ratio of glucose-6-P to Hb A reveals a curve that reaches 0.6 mol of glucose-6-P/mol of Hb tetramer at a ratio of 25 glucose-6-P to 1 Hb A (Fig. 3B).

At a fixed molar ratio of glucose-6-P to Hb A (1:1), the rate of adduct formation was found to be strongly dependent on the
Glycosylation of Hemoglobin

Fig. 4 displays the formation of Hb A-glucose-6-P adduct as a result of incubation equimolar amounts of Hb A and glucose-6-P at different final concentrations. At the lower protein concentrations there appears to be a lag and eventual plateau of adduct formation; whereas at protein concentrations that approach those in the erythrocyte the rate of adduct formation is linear for many hours. We do not have an explanation for this phenomenon other than to propose a protein-protein interaction in adduct formation.

The stability of the glucose-6-P Hb A adduct was studied at 4° and 37°. A solution of the [14C]glucose-6-P-Hb A adduct (0.35 mM, specific activity, 0.065 μCi/μmol) in bis-Tris buffer (0.01 M, pH 7.4) was incubated at 4° and 37°. At 24-h intervals aliquots were removed and acid-precipitated. After 300 h, there was no loss of [14C]glucose-6-P radioactivity at 4° whereas the sample evaluated at 37° showed a slow loss of [14C]glucose-6-P counts which plateaued after 144 h (Fig 5). An estimated half-life of the adduct decomposition was 216 h.

The glycolytic intermediates from the erythrocytes of normal and diabetic subjects were measured. Table III records the values observed as well as values reported in the literature for normal subjects. The intracellular concentration of glycolytic intermediates from erythrocytes of patients with diabetes mellitus were significantly higher than normal controls (p < 0.05).

The structures of Hb Aₐₙ and glucose-6-P-Hb A adduct were compared. The glucose 6-P Hb A adduct and Hb Aₐₙ were separately reduced with tritiated borohydride and the amount of radioactivity incorporated into the α and β chains deter-

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**Fig. 2.** The separation of Hb Aₐₙ and Hb Aₐₙ. A solution of Hb Aₐₙ (10 mg) was applied to a column (0.65 x 25 cm) of Bio-Rex 70 previously equilibrated with Developer 6, pH 6.1. Fractions of 2 ml were collected. The eluting buffer was changed at Fraction 10 to Developer 6, pH 6.3, and at Fraction 120 to Developer 6, pH 6.7. The absorbance (○) and pH (○) of the fractions are indicated.

**Table II**

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>1:1 with Hb A</th>
<th>1:1:1 with Hb A + 2,3-DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>5.9</td>
<td>4.49</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>3.53</td>
<td>3.43</td>
</tr>
<tr>
<td>Fructose-1,6-P</td>
<td>9.30</td>
<td>9.15</td>
</tr>
<tr>
<td>Fructose-1,6-Pₐ</td>
<td>7.87</td>
<td>1.92</td>
</tr>
<tr>
<td>Glyceralddehyde-3-P</td>
<td>10.43</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* 2,3-DPG, 2,3-diphosphoglycerate.

**Fig. 3.** A, the formation of glucose-6-P-Hb A adduct with time at varying molar ratios of glucose-6-P (G6P) and Hb A. Solutions of Hb A (0.2 mM) containing 0.01 M bis-Tris buffer, pH 7.4, were incubated with increasing molar ratios of [14C]glucose-6-P to Hb A (0.5:1 → 25:1). At the indicated times 50-μl aliquots were removed and analyzed for acid-precipitable radioactivity as described under "Materials and Methods." B, the amount of glucose-6-P-Hb A adduct formed with increasing molar ratios of glucose-6-P (G6P) to Hb A. The amounts of adduct formed in A after 60 h of incubation are plotted as a function of glucose-6-P concentration.
HbA-Glucose-6-P found in each chain. The $\beta$ chains of both the glucose-6-P-Hb A control, ranged from 4.8 to 7.3% in the diabetics and 3.5 to 4.1% in the controls at the time blood samples were taken.

The two requirements for adduct formation are the presence of a phosphate and a free aldehyde or ketone (Table I). Free sugars or blocked aldehydes are inactive. Evidently, the phosphate is essential to position the aldehyde or ketone in the 2,3-diphosphoglycerate pocket of the hemoglobin molecule to allow adduct formation. Consistent with this was the observation that 2,3-diphosphoglycerate inhibited adduct formation (Table II). After reduction with sodium borohydride and subsequent chain separation, the adduct was localized on the $\beta$ chain (Table IV). Presumably, the adduct forms as a Schiff base and subsequently undergoes an Amadori rearrangement with the NH$_2$-terminal valine of the $\beta$ chain in analogy with the structure of Hb A$_{$. The attachment of pyridoxal phosphate to hemoglobin in the 2,3-diphosphoglycerate pocket as a Schiff base (12) is an additional example of the steering effect of the phosphate in promoting adduct formation with the NH$_2$-terminal valine of the $\beta$ chain.

The rate of adduct formation was dependent upon the concentrations of hemoglobin A and glucose 6-phosphate. At concentrations of hemoglobin that mimic those found in the erythrocyte, the rate of adduct formation was constant and dependent on the amount of intermediate added. At an equimolar concentration of hemoglobin A and glycolytic intermediate approximately 10% of the hemoglobin was modified. The kinetics of adduct formation at lower hemoglobin concentrations were more complicated, and presumably reflected a dependence upon protein-protein interaction.

The adduct of glucose 6-phosphate and hemoglobin A was not irreversible but was slowly reversible. At 37° the estimated half-life was 216 h. No significant decomposition occurred at 4°. The attainment of a plateau of 70% of glucose 6-phosphate remaining attached to Hb A presumably reflects a steady state in which glucose 6-phosphate is going on and off the hemoglobin.

The isoelectric points and column chromatographic properties of all the glycolytic intermediate-hemoglobin adducts were identical with those of the natural product Hb A$_{6}$. The latter hemoglobin, which is elevated in patients with diabetes mellitus, is presumably produced in vivo by addition of glycolytic intermediates to Hb A. The increased rate of Hb A$_{6}$ synthesis in the erythrocytes of patients with diabetes mellitus reflects the observed increased concentration of glycolytic intermediates (glucose-6-P, fructose-6-P, fructose-1,6-P$_2$, dihydroxyacetone phosphate, Table III). Thus Hb A$_{6}$ may be a collection of adducts with glucose-6-P accounting for the majority because of its higher concentration. In addition, the fact that hemoglobin A$_{6}$ is an adduct of glucose with Hb A, suggests that Hb A$_{6}$ has predominantly 1-deoxy-1-(N-val-lyl)fructose 6-phosphate as the NH$_2$ terminus of the $\beta$ chain. Since glucose does not form a detectable stable adduct with hemoglobin in vitro, it may be that in vivo glucose 6-phosphate reacts with hemoglobin to form Hb A$_{6}$, which is then dephosphorylated to form Hb A$_{6}$. Further work is needed to define the nature of the glyco-portion of Hb A$_{6}$ and its role in the production of Hb A$_{6}$.

Since this paper was submitted, a similar study was communicated by Haney and Bunn (13). They utilized a glucose-6-P substrate of high specific activity to study the glycosylation of hemoglobin at a lower temperature (20°) and a much shorter incubation (5 h) than reported in the present study.

**REFERENCES**


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