Glycerated Hemoglobin, \( \alpha_2^A \beta_282 \) (EF6) \( N^\circ \)-Glyceryllysine

A NEW POST-TRANSLATIONAL MODIFICATION OCCURRING IN ERYTHROCYTE BISPHEROSGLYCEROEROMUTASE DEFICIENCY

(Received for publication, October 30, 1986)

Yves Blouquit‡, Marie-Dominique Rhoda‡, José Delanoe-Garin‡, Raymonde Rosa‡, Jean-Claude Pronej, Claude Poyart‡, Georges Puzo§, Jean Marie Bernassau||, and Jean Rosa‡

From the ‡Institut National de la Santé et de la Recherche Médicale, U. 27 Hôpital Foch, Suresnes, France, the §Centre de Recherche de Biochimie et de Génétique Cellulaire du Centre National de la Recherche Scientifique, Toulouse, France, the ||Institut National de la Santé et de la Recherche Médicale, U. 27 Hôpital Foch, Suresnes, France, and the \| Laboratoire de Synthèse Organique, École Polytechnique, Palaiseau, France

A new minor Hb fraction initially designated Hb\(_c\), has been found in the hemolysate of an erythremic patient that we have previously described with a complete erythrocyte bisphosphoglycerate mutase (EC 5.4.2.4) deficiency. Hb\(_c\), (3.5% of the total) was detected by isoelectric focusing and exhibited electrophoretic and chromatographic properties similar to those of several variants of the Hb central cavity. By density fractionation of red cells, it was demonstrated that Hb\(_c\) was an aging hemoglobin as in the case of glycated Hb A\(_1\). Functional studies revealed a low oxygen affinity and almost complete inhibition of the allosteric effect of the organic phosphate effectors. Structural studies demonstrated an absence of tryptic cleavage between the peptides \( \beta_\theta T9 \) and \( \beta_\theta T10 \) suggesting the presence of an adduct on Lys \( 82 \) or on a neighboring residue. Fast atom bombardment mass spectrometry and a specific enzymatic assay with glyoxylate reductase demonstrated that the \( 82 \) adduct was a glycerate moiety. It was concluded that Hb\(_c\), was a glycercated Hb, \( \alpha_2^A \beta_282 \) (EF6) \( N^\circ \)-glyceryllsine, to our knowledge the first example of glycercated protein. The mechanism of formation of glyceryl Hb, which was found in the four studied subjects with a bisphosphoglyceromutase deficiency, remains to be determined.

As opposed to most eukaryotic proteins the human hemoglobin chains remain unmodified after translation. Only a small proportion of human globin chains undergo a post-translational modification: 10% of the N-terminal amino groups of fetal \( \gamma \) chains are acetylated in cord blood (1) and 4% of the N-terminal valine residues of the \( \beta \) chain are glycylated in adult Hb A\(_1\). In addition some \( \alpha \)- or \( \epsilon \-)NH\(_2\) groups of Hb A are also glycylated during red cell aging (3). A low percentage of hemoglobin is also pyridoxylated in normal red blood cells (4). In individuals with diabetes mellitus, Hb glycation increases markedly (5) and the level of Hb A\(_1\), the main glycated Hb, is a good index of diabetes severity (6). A new minor Hb fraction initially designated Hb\(_c\), has been found in the hemolysate of an erythremic patient that we have previously described with a complete erythrocyte bisphosphoglycerate mutase (EC 5.4.2.4) deficiency. Hb\(_c\), (3.5% of the total) was detected by isoelectric focusing and exhibited electrophoretic and chromatographic properties similar to those of several variants of the Hb central cavity. By density fractionation of red cells, it was demonstrated that Hb\(_c\) was an aging hemoglobin as in the case of glycated Hb A\(_1\). Functional studies revealed a low oxygen affinity and almost complete inhibition of the allosteric effect of the organic phosphate effectors. Structural studies demonstrated an absence of tryptic cleavage between the peptides \( \beta_\theta T9 \) and \( \beta_\theta T10 \) suggesting the presence of an adduct on Lys \( 82 \) or on a neighboring residue. Fast atom bombardment mass spectrometry and a specific enzymatic assay with glyoxylate reductase demonstrated that the \( 82 \) adduct was a glycerate moiety. It was concluded that Hb\(_c\), was a glycercated Hb, \( \alpha_2^A \beta_282 \) (EF6) \( N^\circ \)-glyceryllsine, to our knowledge the first example of glycercated protein. The mechanism of formation of glyceryl Hb, which was found in the four studied subjects with a bisphosphoglyceromutase deficiency, remains to be determined.

EXPERIMENTAL PROCEDURES

Reagents

With the exception of some reagents whose source is specified in the text, all substrates and commercial enzymes were purchased from Boehringer Biochemicals, Mannheim. Buffer salts were obtained from Merck Chemical Division, Darmstadt. Bio-Rex 70 resin and Bio-Gel P\(_2\) were purchased from Bio-Rad, and acetonitrile was from Pierce Chemical Co. Percoll was obtained from Pharmacia, Uppsala, Sweden, and DEAE 52 cellulose and CM-cellulose 23 from Whatman. Glyco-Gel B boronate affinity resin was purchased from Pierce Chemical Co.

Methods

Electrophoresis, isoelectric focusing, and chromatography were performed on propositus or normal donor hemolysates prepared by the method of Geraci (12) from freshly heparinized venous blood. Electrophoreses at alkaline pH (Tris-EDTA-borate buffer) on cellulose acetate strips (Titan III, Helena Laboratories, Beaumont, TX), citrate agar electrophoresis (pH 6.2) on plates Titan IV from Helena, and globin chain electrophoresis in 6 M urea, 1% \( \beta \)-mercaptoethanol (pH 6 and 9) on cellulose acetate strips (Sepharose X, Gelman Instrument Co.) were performed as described in Ref. 13. The following procedures were also used: isoelectric focusing (IEF)\(^*\) on thin layer plates initially designated Hb\(_c\), has been found in the hemolysate of an erythremic patient that we have previously described with a complete erythrocyte bisphosphoglycerate mutase (EC 5.4.2.4) deficiency. Hb\(_c\), (3.5% of the total) was detected by isoelectric focusing and exhibited electrophoretic and chromatographic properties similar to those of several variants of the Hb central cavity. By density fractionation of red cells, it was demonstrated that Hb\(_c\) was an aging hemoglobin as in the case of glycated Hb A\(_1\). Functional studies revealed a low oxygen affinity and almost complete inhibition of the allosteric effect of the organic phosphate effectors. Structural studies demonstrated an absence of tryptic cleavage between the peptides \( \beta_\theta T9 \) and \( \beta_\theta T10 \) suggesting the presence of an adduct on Lys \( 82 \) or on a neighboring residue. Fast atom bombardment mass spectrometry and a specific enzymatic assay with glyoxylate reductase demonstrated that the \( 82 \) adduct was a glycerate moiety. It was concluded that Hb\(_c\), was a glycercated Hb, \( \alpha_2^A \beta_282 \) (EF6) \( N^\circ \)-glyceryllsine, to our knowledge the first example of glycercated protein. The mechanism of formation of glyceryl Hb, which was found in the four studied subjects with a bisphosphoglyceromutase deficiency, remains to be determined.

EXPERIMENTAL PROCEDURES

Reagents

With the exception of some reagents whose source is specified in the text, all substrates and commercial enzymes were purchased from Boehringer Biochemicals, Mannheim. Buffer salts were obtained from Merck Chemical Division, Darmstadt. Bio-Rex 70 resin and Bio-Gel P\(_2\) were purchased from Bio-Rad, and acetonitrile was from Pierce Chemical Co. Percoll was obtained from Pharmacia, Uppsala, Sweden, and DEAE 52 cellulose and CM-cellulose 23 from Whatman. Glyco-Gel B boronate affinity resin was purchased from Pierce Chemical Co.

Methods

Electrophoresis, isoelectric focusing, and chromatography were performed on propositus or normal donor hemolysates prepared by the method of Geraci (12) from freshly heparinized venous blood. Electrophoreses at alkaline pH (Tris-EDTA-borate buffer) on cellulose acetate strips (Titan III, Helena Laboratories, Beaumont, TX), citrate agar electrophoresis (pH 6.2) on plates Titan IV from Helena, and globin chain electrophoresis in 6 M urea, 1% \( \beta \)-mercaptoethanol (pH 6 and 9) on cellulose acetate strips (Sepharose X, Gelman Instrument Co.) were performed as described in Ref. 13. The following procedures were also used: isoelectric focusing (IEF)\(^*\) on thin layer plates.

\(*\) This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, from the Centre National de la Recherche Scientifique, and from the Ministère de la Recherche et de la Technologie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: IEF, isoelectric focusing; CID/MIKE, collision-induced dissociation/mass analysis ion kinetic energy; HPLC, high performance liquid chromatography; glycercate-2,3-P\(_2\), diphosphoglycerate; PAB, fast atom bombardment; Glc-6-P, glucose 6-phosphate; bis-Tris, 2-(bis[2-hydroxyethyl]aminio)-2-(hydroxymethyl)-propane-1,3-diol.
polyacrylamide gel (pH 6-9) (14), globin chain analysis on acid-urea- Triton acrylamide gels (X), chromatography on aminoephil benzylated agaroar, in order to retain glycated hemoglobin using Glyco- Gel B resin (16), thiobibarbaric acid (17), and alkali denaturation (18) tests. Hb A$_2$ and the novel abnormal form, designated Hb G, were separated from Hb A, by Bio-Rex 70 chromatography according to McDonald (19). Approximately 8 g of HbCO were applied to a 2.5 x 40-cm column which was developed with a linear gradient of sodium produced with 5 liters of a 0.048 M starting sodium phosphate buffer, pH 6.75, and 5 liters of the same buffer containing 0.3 M NaCl. The flow rate was 1.6 liters/h. Fractions containing Hb A, and Hb G, were pooled and concentrated in a Sartorius SM 16566 ultrafiltration apparatus (Sartorius, Gottingen). Hb A, and Hb L, were separated by DEAE 52 chromatography according to Abraham (20). A solution containing 250 mg of HbCO was applied to a 2.5 x 40-cm column which was developed with a linear sodium gradient obtained with 250 ml of a 0.2 M starting glycine buffer, pH 7.85, and 250 ml of the same buffer containing 0.1 M NaCl. The flow rate was 100 ml/h. Purity of the hemoglobin fractions was assessed by IEF.

Functional Studies

Oxygen Equilibrium Studies—Oxygen-binding studies in red cell suspensions were performed at 37 °C in 0.14 M NaCl, 50 mm bis-Tris buffer with an automatic continuous method (Hemox Analyzer, TCS, Southampton, PA) as previously described (21, 22). Oxygenation measured for the purified HbG, isolated from patients' samples were carried out at 25 °C. The buffer solutions were made of 50 mm bis-Tris or Tris buffer (for pH values higher than 7.5), 100 mm NaCl, 0.5 mm EDTA, and 20 mg/ml catalase to avoid oxidation of the hemes during the runs. The oxygen-binding curves were recorded in solutions containing 50-200 M HbG, on a hemoclip. A spiker was introduced into the optical cuvette to reduce the light path from 1.2 to 0.2 cm. The methemoglobin formed during the 45-min duration of each deoxygenation curve never exceeded 4%. Experiments were performed at different pH values or after addition of glycercate-2,3-P$_2$ (Na salt), inositol hexaphosphate, or NaCl as indicated in Tables I and II. For the oxygen-binding experiments, the recording system has been interfaced to an HP 85 microcomputer which was programmed to store on tape up to 500 values of absorbance and PO$_2$. The P50 and n50 values representing, respectively, the partial pressure of oxygen and the Hill coefficient at half-saturation were computed from the experimental points in the range of 40-60% saturation by linear regression analysis. The amount of protons released by HbG upon oxygenation in the range of pH 6.5-9 (Bohr effect) was calculated from the linkage equation relating log P50 to pH (23), using a nonlinear least squares fitting procedure (24, 25).

In D-Glycerate Fractionation of Red Cells—Fractionation of the red cells was performed according to the method described in Ref. 26 with a discontinuous “Percoll” gradient which allows the separation of the cells according to their density. The relationship between density and age was ascertained by measuring, in hemolysates of each fraction, the glutamine-glutamate transaminase activity or the hemolysates corresponding to each fraction (F1 to F6 in order of increasing density) were further analyzed for their Hb content by thin plate isoelectric focusing.

Structural Studies

After desheminization by acid-acetone precipitation, the abnormal chain was prepared by CM-cellulose 8 m chromatography (28), aminomethylated, stripped of urea on a Bio-Gel P$_2$ column, and hydrolyzed in 250 ml of 1 N-tetraethyl methyl chloroiridate (ketone, Worthington) according to Ref. 29. Tryptic peptides were characterized by HPLC using a Beckman system 343 with a Kratos (Kratos Analytical Instruments, Ramsey, NJ) wavelength detector set at 214 nm. The column system was a Waters MBondapak C18 (10 mm, inner diameter 3.9 mm, 30 cm) (Waters Associates, Milford, MA). The solvent system was made according to Ref. 29 with some modifications using a solvent A, 0.02 m ammonium acetate (pH 5.7) and a solvent B, 0.01 m ammonium acetate (pH 5.7) mixed 60:50 with acetonitrile. The gradient was linear from 0 to 25% in 30 min, from 25 to 50% in 10 min, from 50 to 100% in 15 min, and from 50% to 100% in 5 min. Amino acid composition was determined with a Biotronik 6000 IE (Biotronik, Munich) after 20 h of hydrochloric acid hydrolysis of the separated peptides. Pepsin hydrolysis of β$_2$-T10 was performed according to the method described in Ref. 30 using Worthington peptic in 0.5 ml of 0.1 N HCl and an enzyme/substrate ratio 1:25 for 2 h at room temperature. The characterization of peptic peptides was performed as described for the trypptic peptides with the following gradient from 0 to 50% in 50 min and from 60 to 100% in 5 min.

NMR Analysis—NMR analysis of Hb G, was performed in order to detect a possible phosphorylated adduct. The NMR measurements have been performed with an XL-100 (Varian Associates, Inc., Palo Alto, CA) operating in the Fourier transform mode at 40 MHz. The spectra width was 5000 Hz and the flip angle 45°. The number of transients that were accumulated for the hemoglobin samples ranged from 30,000 to 130,000. During these experiments the probe temperature was regulated to 4 °C. Glucose-6-P (Glc-6-P) was dissolved in D$_2$O (200 mM). Glc-6-P Hb was obtained according to the method described in Ref. 31. Glc-6-P Hb and Hb G, were deonized by chromatography on a column of AG-501-X8 (Bio-Rad) and concentrated before use.

Mass Spectrometry—The mass spectrometer was a MM-ZAB 2F instrument (VG Instruments) equipped with a FAB source. A xenon atom beam of 8 keV energy was focused on the target loaded with 3-10 μg of the peptide dissolved into 2 ml of a 1:1 mixture of glycerol and thioglycerol. The spectra were recorded on sensitive UV paper at a speed of 1 mm unit/s. For CID/MIKE measurements, helium was introduced into the gas cell in order to reduce the main beam to about 30% of its original value. The electric sector was scanned within 150 s in the energy range 8-1 keV. In order to identify a putative adduct and its binding to Hb G, high resolution FAB mass spectrometry associated with CID/MIKE measurements was performed with a Finnigan 8820-25 analyzer described in Ref. 32. Precise mass measurements were made at V.G. Analytical Ltd. (Manchester) on a V.G. 7070E instrument fitted with an alternating FAB probe connected to a data system. A mixture of alkaline iodides was used as reference compounds. The accurate mass determination was improved by interpolation between the cluster ions $\text{Rb}^+_m$, $\text{Rb}^+_m \cdot \text{D}_2_0 \cdot \text{Rb}^+_m$ (m/z 510.5417) and cesium $\text{Rb}^+_m$ (m/z 556.5380) using the computer averaging system.

Assay of Glycerate

Glycerate was measured by the specific enzymatic method using the glycerate dehydrogenase activity displayed by the glycolate reductase from spinach leaves (33, 34). The method used was an adaptation of the technique described by Sallach (35) for the determination of the glycerate dehydrogenase activity of the enzyme according to the following equation.

$$\text{d-Glycerate + NAD}^+ \rightarrow \text{hydroxyproprate} + \text{NADH} + \text{H}^+$$

The oxidative reaction is shifted to the right by carrying out the reaction at pH 9.2 and in the presence of a carboxyl-trapping reagent, i.e. hydrazine sulfate. The assay medium contained 0.1 M Tris-glycine, pH 9.2, 300 μl of hydrazine sulfate (pH 9.2) in an adequate sample volume. The reactive solution was started at 37 °C by adding 7.5 μg of glycolate reductase (Sigma) and was allowed to reach completion. The resulting values were compared to those of a reference curve of absorbances versus increasing glycerate concentrations. The reaction was sensitive enough to detect 15 nmol of d-glycerate in the cuvets. This reaction is specific and does not occur with glyceraldehyde, dihydroxyacetone, glyceraldehyde 3-phosphate, 3-phosphoglycerate, or 2-phosphoglycerate.

RESULTS

A minor abnormal hemoglobin was detected during the reevaluation of a previously described subject presenting a complete red cell bisphosphoglyceromutase deficiency (11). In brief, the main features of the patient were: a well tolerated erythrocytosis nevertheless requiring episodic venipunctures, the absence of detectable red cell glyceraldehyde-2,3-P$_2$ and of bisphosphoglyceromutase activity leading to large modifications of the glycolytic intermediate concentrations and particularly a large increase in fructose 1,6-P$_2$, dihydroxyacetone phosphate, and glyceralde-3-P. The propositus hemoglobin pattern previously studied by cellulose acetate electrophoresis and by IEF did not exhibit a major abnormal component. More recent analyses by Bio-Rex 70 chromatography revealed a high level of Hb A$_2$ of 9.6% (control, 5.4 ± 0.4). This result
contrasted with the absence of an abnormal level of Hb F, as estimated by alkali denaturation, and of diabetes mellitus. This prompted us to study more carefully the minor Hbs of the propositus. The isofocusing pattern of the propositus hemolysate (Fig. 1, samples 2 and 7) and of the Bio-Rex 70 fraction eluting with the Hb Alc retention time (Fig. 1, sample 6) demonstrated the presence of a normal amount of Hb Alc and of an abnormal minor component (Hb,) representing 3.5% of the total Hb and 40% of the Bio-Rex fraction. The pI of Hb, was intermediate between those of Hbs Alc and . At this pI a faint band is present in normal control (Fig. 1, sample 1) and is sometimes increased in stored red blood cell samples (not shown). The propositus Hb, level remained constant over a period of several months. Hb, was also found at the same level in the three propositus sisters who also displayed complete bisphosphoglyceromutase deficiency. A lower amount of Hb, was detected among the members of this family as well as of another unrelated family presenting a partial bisphosphoglyceromutase deficiency (36). Large quantities of Hb, mixed with Hb Alc were isolated from the propositus hemolysate by chromatography on Bio-Rex 70. Hb, was separated from Hb Alc by chromatography on a DEAE-52 column, and the properties of the purified abnormal hemoglobin were studied. On citrate agar electrophoresis Hb, mobility was similar to that of Hb Alc and Hb F, a property which together with the behavior of these Hbs on Bio-Rex is observed in several abnormal hemoglobins with a substitution of one of the residues lining the central cavity between the 2 $\beta$ chains (37, 38). In urea buffer, the migration of the $\beta$ chains in cellulose acetate electrophoresis was more anodal than $\beta^{A}$ and $\beta^{Hb}$ chains. The $\beta$ chains were separated from $\alpha$, $\beta$, $\beta^{Hb}$, $\gamma^{A}$, and $\gamma^{G}$ chains by acrylamide electrophoresis in urea-Triton buffer. Hb, was not retained on affinity chromatography column Glyco-Gel.B, indicating that in contrast to Hb Alc it did not contain a cis-diol adduct (19). No reaction was obtained with the thio-barbituric test (17) indicating that Hb, was not a glycated Hb. Since several minor Hb fractions have been suspected to contain phosphorylated adducts (19) we submitted Hb, to NMR analysis. Compared to control Glc-6-P Hb, the NMR spectrum for Hb, did not exhibit any peak corresponding to a phosphorylated derivative (Fig. 2).

**Kinetics of Hb, Formation**—To explain the presence of Hb, in the propositus family it could be postulated that the abnormal component was the product of a $\beta^{Hb}$ gene not yet detected or a post-translationally modified normal hemoglobin. To test this latter possibility, the kinetics of formation of Hb, were studied. A density gradient separated the propositus red blood cells into six different fractions (Fig. 3). The glutamo-oxalo-transaminase red blood cell activity decreased from the lightest fraction (F1) to the densest (F6), indicating that the cells have been separated according to age, the oldest being the densest as expected. The concentrations of Hb Alc, and Hb, showed a similar increase in parallel with red cell aging. The results support the hypothesis of a post-translational origin for Hb, probably slowly synthesized by a nonenzymatic reaction as observed for Hb Alc (39).

**Functional Studies**—Oxygen binding by the propositus red cells showed an increase in oxygen affinity with a $P_{50}$ value of 16 mm Hg (normal value, 26 ± 1 mm Hg) previously described and explained by the absence of glycerate-2,3-P$_2$ (11). The oxygen-binding parameters for purified Hb, solutions are compared to control Hb A in Table I. In standard conditions of temperature and chloride concentration, Hb, exhibits approximately a 50% decrease in oxygen affinity and slightly reduced $n_{50}$ values. Table I shows also that Hb, reacts very weakly with heterotrophic cofactors and particularly with glycerate-2,3-P$_2$. This indicates that the abnormality in Hb, is likely to involve one of the cofactor cationic binding sites.
Oxygen-binding parameters for Hb and Hb A solutions

| Effectors       | Hb       | Hb A
|-----------------|----------|--------|
|                 | $P_{50}$ | $n_{so}$ | $P_{50}$ | $n_{so}$ |
| NaCl 0.1 M     | 9.0     | 2.4     | 5.8     | 2.9     |
| NaCl 0.4 M     | 13.3    | 2.5     | 11.8    | 2.8     |
| NaCl 0.6 M     | 14.4    | 2.5     | 12.3    | 2.7     |
| NaCl 0.1 M, 5 mM glycerate-2,3-P$_2$ | 11.0 | 2.7 | 18.7 | 2.7 |
| NaCl 0.1 M, 1 mM inositol hexaphosphate | 14.6 | 2.2 | 69.0 | 2.2 |

* $P_{50}$ and $n_{so}$, the partial pressure of oxygen and Hill coefficient at half-saturation, were computed for oxygen saturation levels between 40–60%.

** Glycerate-2,3-P$_2$ and inositol hexaphosphate were sodium salts.

TABLE II

Heterotropic effects for Hb, and Hb A at 25 °C

| Heterotropic effects       | Hb       | Hb A |
|---------------------------|----------|------|
| Alkaline Bohr effect      | −0.475   | −0.525 |
| Chloride effect           | 0.27     | 0.47  |
| Glycerate-2,3-P$_2$       | 0.09     | 0.51  |
| Inositol hexaphosphate effect | 0.21 | 1.08  |

* Calculated as $d$ log $P_{50}/d$ log(Cl$^-$) between 0.1 and 0.6 M NaCl.

** $\Delta$ log $P_{50} \pm 5$ mM glycerate-2,3-P$_2$ (sodium salt).

*** $\Delta$ log $P_{50} \pm 1$ mM inositol hexaphosphate.

that line the entrance to the $\beta_8\alpha_2$ cleft in normal Hb A (40). The effects of heterotropic cofactors for Hb$_A$ and Hb A are summarized in Table II. Whereas, the effects of glycerate-2,3-P$_2$ and inositol hexaphosphate on the functional properties of Hb$_A$ are nearly abolished, the chloride effect is only halved. The latter result is in agreement with the existence of two classes of binding sites for chloride in Hb A, one with a high affinity at the $\alpha_1\alpha_2$ N-C interface and the second, with low affinity, at the $\epsilon$-amino group of Lys (EF6) 882 (41, 42). This result suggested that a residue of the central cavity might be involved in the abnormal function of Hb$_A$ and focused attention on lysine (EF6) 882. The oxygen Bohr effect was measured in the range of pH values between 6.5 and 9. Compared to Hb A the maximum proton release upon oxygenation of Hb, was slightly diminished (Table II and Fig. 4).

**Structural Studies**—The structural study of the Hb$_A$ was performed on a sample isolated by the chromatographic steps already described. The globin chains were separated by CM-cellulose chromatography in urea buffer. The elution profile was not strikingly different from that produced by globin chains from Hb A. The chains were aminoethylated, submitted to a tryptic hydrolysis, their peptides separated by HPLC on a uBondapak C18 column, and their amino acid composition determined. Fig. 5 presents the HPLC profile of the tryptic peptides of the aminoethylated $\beta$ chains. It is similar to that of $\beta^e$ aminoethylated chains (not shown) except the presence of a new peak eluted at the end of the chromatography and presented an amino acid composition identical to that of the sum of the amino acids present in T9 and T10 (Table III). The small quantities of T9 and T10 present most probably arose from a slight contamination by $\beta^a$ peptides as indicated by the presence of T1. The absence of tryptic hydrolysis in $\beta^e$ of the bound between Lys 82 and Gly 83 suggested that an adduct was bond to Lys 82 or to an amino acid in its immediate vicinity.

**Mass Spectrometry Analysis**—To assess the precise location of the putative adduct and identify its structure it was decided to use mass spectrum analysis. Since the size of the 29-residue $\beta^a$T9-T10 was too large for suitable results with this method, 30 nmol of the $\beta^e$T9-T10 were hydrolyzed by pepsin, an enzyme...
TABLE III
Amino acid composition of the βT9-10 peptide from Hb, obtained by HPLC

| Amino acids | Found | Expected for βT9 and βT10 (sequence 67-95 of the β chain) |
|-------------|-------|----------------------------------------------------------|
| Asp         | 3.90  | 4.0                                                      |
| Thr         | 2.0   | 2.0                                                      |
| Ser         | 2.0   | 2.0                                                      |
| Glu         | 1.2   | 1.0                                                      |
| Pro         | 0     | 0                                                        |
| Gly         | 3.1   | 3.0                                                      |
| Ala         | 3.0   | 3.0                                                      |
| Val         | 1.1   | 1.0                                                      |
| Met         | 0     | 0                                                        |
| Ile         | 0     | 0                                                        |
| Leu         | 5.7   | 0.0                                                      |
| Tyr         | 0     | 0                                                        |
| Phe         | 2.1   | 2.0                                                      |
| His         | 1.7   | 2.0                                                      |
| AE-Cys*     | 0.88  | 1.0                                                      |
| Lys         | 1.8   | 2.0                                                      |
| Arg         | 0     | 0                                                        |

* Aminoethyl cysteine.

Fig. 6. HPLC elution pattern of pepsic hydrolysate of βT9- T10 peptide. The darkened peak 5 contains the 82-85 sequence.

TABLE IV
Amino acid composition of the β pepsic 82-85 peptide (peak 5 of Fig. 6)

| Amino acids | Found | Expected |
|-------------|-------|----------|
| Thr         | 0.96  | 1        |
| Gly         | 1.16  | 1        |
| Phe         | 0.82  | 1        |
| Lys         | 1.06  | 1        |

which releases the tetrapeptide 82-85: Lys-Gly-Thr-Phe (30). Fig. 6 shows the pattern of the βT9-T10 pepsic hydrolysate obtained after HPLC. Among the 12 peaks obtained, the amino acid composition of the peak 5 (Table IV) corresponded to the 82-85 sequence. This peak was three times lyophilized and subjected to mass spectrum analysis. The positive FAB mass spectrum, using thioglycerol as matrix, is presented in Fig. 7. An intense peak was observed at m/z 540. It could be assigned to the (M + H)+ ion of the peptide since a signal of lower intensity was detected at 22 mass units above, corresponding to the ion (M + Na)+. Accurate measurements made on m/z 540 by means of an alternating FAB probe controlled by a data system indicated a mass of 540.2660 (averaged value of two successive determinations which gave, respectively, the values 540.2659 and 540.2661 for the MH+ ion of the peptide).

Since the nominal m/z value for MH+ was an even number, the molecule should contain an odd number of nitrogen atoms. Thus, a computational examination of the possible elemental compositions indicated that the best fitting was C24H38O9N5 (theoretical 540.26695). The error was less than 2 ppm. It was already established that acidic hydrolysis of the modified peptide gave the same amino acid composition as the unmodified one (Table IV). Thus, the modified peptide 82-85 included, in addition to amino acids lysine, glycine, threonine, and phenylalanine, a new moiety corresponding to C3H4O3.

In order to detect the position of the modification on the sequence a CID/MIKE spectra was measured on m/z 540. It was shown that a partial N-terminal sequence could be obtained (Fig. 8). Two main ions were observed: the former corresponded to the loss of a phenylalanine residue at m/z 375; the latter to the loss of both phenylalanine and threonine residues at m/z 274. Thus, the modification was associated with the remaining dipeptide, i.e. Lys-Gly, but no further characteristic fragmentation could be detected. The most likely hypothesis to explain these findings is a modification of the lysine residue on its ε-amino group by amidation with a molecule of glyceric acid (Scheme 1). This proposal takes into account the recovery of unmodified lysine and glycine upon acid hydrolysis of the β82-85 pepsic peptide (Table IV). To confirm this "glycerate hypothesis" we developed a specific enzymatic assay described under "Methods" and looked at the presence of glycerate on the βT9-10 peptide.

Final Evidence for Glycerate as the Adduct in Hb.
metric amounts of nonsubstituted amino acids, the adduct, if not destroyed, should be found free in the hydrolysate. In a preliminary control we observed that glycerate added to a mixture of amino acids was quantitatively recovered after 22 h of hydrolysis. Subsequently, sufficient quantities of βT9-T10 were isolated by HPLC and subjected to 6 N HCl hydrolysis for 22 h at 110 °C. The hydrolysate was lyophilized three times and divided into two equal aliquots for amino acid analysis and glycerate determination. In two separate experiments glycerate was present in stoichiometric quantities compared to the peptide, confirming the hypothesis formulated according to the mass spectrometry analysis. According to these results we concluded that the structure of Hb was αNβ82 (EP8) N'-glyceryllysine, and we propose to name it glyceryl Hb.

**DISCUSSION**

This report describes a new post-translational modified hemoglobin: the glyceryl hemoglobin. This novel minor hemoglobin fraction is progressively formed in the red blood cells of patients with total bisphosphoglyceromutase deficiency. The formation kinetics of this hemoglobin are close to those of patients with total bisphosphoglyceromutase deficiency. This Hb was visible in stored red cells.

The presumptive localization of the adduct on lysine β82 was relatively straightforward because the peptidic bond between βT9 and βT10 normally hydrolyzed by trypsin with a high yield in β chain was not cleaved in case of glyceryl Hb. Such an absence of action of trypsin has been previously described in another example of ε-NH2 lysine β82 in vitro modification involving adduction with glyceraldehyde (8). Before mass spectrometry analyses had been performed on the abnormal peptide it was not possible to identify the chemical structure of the adduct, and only partial information indicated that the adduct was not a compound bound to the Lys β82 by a Schiff base, since it was not reduced by tritated borohydride. By contrast, the high resolution FAB mass spectrometry associated with CID/MKE measurements was extremely efficient in identifying the adduct and its binding to the lysine on small quantities of purified material. It is a new example of the efficiency of the FAB mass spectrometry which has been used already successfully for fast characterization of genetically determined abnormal hemoglobins on micromolar quantities of material (43). Such a method would be very useful for future determinations of other adducts of hemoglobin, particularly if, as in the present case, a specific enzymatic determination can be used together with the mass spectrometry analysis for the final identification of the adduct.

Precise oxygen-binding measurements revealed that the glyceryl Hb has a low oxygen affinity, slightly reduced Hill coefficient, and an almost complete inhibition of the allosteric effect of the organic phosphate effectors; these results are expected from the disappearance of the positive ε-NH2 of lysine β82, one of the positive groups involved in the binding of glyceraldehyde-2,3-P2 to deoxy Hb (49). Similar results including the observed halved effect of chloride on oxygen affinity were obtained with Hb Providence (44) in which Lys β82 is absent. Moreover, the decreasing order of oxygen affinities for various hemoglobins allelic at the β82 locus, Hb Rahere (Lys→Asn) which is neutral and of Providence I (Lys→Asn) > glyceryl Hb (Lys→N'-glyceryllysine) > Hb Providence II (Lys→Asp) demonstrated the marked effect on the oxygen affinity of the nature of the side chain of the residue β82. The polarity of the lateral chain of glyceryl Hb is effectively intermediate between that of Hb Providence I which is neutral and of Providence II which is acidic. Indeed, precise information concerning the location of the adduct, the nature of its linkage, and even its structure could be deduced from careful analysis of some electrophoretic, chromatographic, and functional properties of glyceryl Hb.

The mechanism of formation of glyceryl Hb remains to be determined, and it would probably be difficult. The absence of bisphosphoglyceromutase activity in the propositus erythrocytes produces large modifications of the glycolytic intermediates involving increases of fructose-1,6-P2, of triose phosphates, and of glyceraldehyde-3-P. Preliminary results indicated that these perturbations did not produce an elevation of glycerate which was undetectable in the propositus erythrocytes as in the controls. A good candidate, as a primer, could be the glyceraldehyde-3-P. The concentration of glyceraldehyde-3-P cannot usually be determined in the red cells because of its extreme instability, but it is reasonable to assume that its level may be raised in the absence of bisphosphoglyceromutase. This ester phosphate could bind to positive groups lining the central cavity between the two β chains. One may postulate that its C1 carboxyl group will amideify ε-NH2 of lysine

\[
\begin{align*}
\text{HN} & - \text{CH} - \text{CO} - \\
\text{CH}_2\text{CH}_2 & \text{O} \\
\text{NH} & \\
\text{CH} & \text{= O} \\
\text{CHOH} & \\
\text{CH}_2\text{OH} & \\
\text{LYSINE} & \beta 82 \\
\text{GLYCERIC ACID} & 
\end{align*}
\]

**Scheme 1**
glycerol  in Bisphosphoglyceromutase  Deficiency

Acknowledgments—We are indebted to Dr. S. Edelstein, to Dr. F. Galacteros, and to Dr. J. P. Rosas for stimulating discussion and revision of this manuscript, to M. C. Calvin, B. Bohn, and L. Leclerc for their technical help, and to A. M. Dulac and M. Segear for preparation of this manuscript. We gratefully acknowledge V-G. Analytical Ltd. (Manchester) for the accurate mass measurements in the FAB ionization mode.

REFERENCES

1. Schroeder, W. A., Cua, J. T., Matsuda, G. & Fenninger, W. D. (1962) Biochim. Biophys. Acta 63, 532–534
2. Garlick, R. L., Mazer, J. S., Higgins, P. J. & Bunn, H. F. (1963) J. Clin. Invest. 71, 1062–1072
3. Shapiro, R., McManus, M. J., Zalut, C. & Bunn, H. F. (1980) J. Biol. Chem. 255, 3120–3127
4. Srivastava, S. K., Van Looln, C. & Beutler, E. (1972) Biochim. Biophys. Acta 278, 617–621
5. Rahbar, S., Blumenfeld, O. & Ranney, H. M. (1969) Biochem. Biophys. Res. Commun. 36, 838–843
6. Koenig, R. J., Peterson, C. M., Jones, R. L., Saudek, C., Lehrmax, M. & Cerami, A. (1976) N. Engl. J. Med. 295, 417–420
7. Stevens, V. J., Fantl, W. J., Newman, C. B., Sima, R. V., Cerami, A. & Peterson, C. M. (1981) J. Clin. Invest. 67, 361–369
8. Zaugg, R. H., Walder, J. A., Walder, R. Y., Steele, J. M. & Klotz, I. M. (1980) J. Biol. Chem. 255, 2816–2821
9. Maugh, T. H. (1981) Science 211, 468–470
10. Moo-Pen, W. F., Jue, D. L., Bechtel, K. C., Johnson, M. H., Schmidt, R. M., McCurdy, P. R., Fox, J., Bonaventura, J., Sullivan, B. & Bonaventura, C. (1976) J. Biol. Chem. 251, 7557–7562
11. Rosa, R., Prehu, M. O., Beuzard, Y. & Rosa, J. (1978) J. Clin. Invest. 62, 907–915
12. Geraci, O., Parkhurst, L. J. & Gibson, Q. H. (1989) J. Biol. Chem. 264, 4661–4667
13. Bloquith, Y., Delaneo-Garin, J., Lacombe, C., Arous, N., Cayre, Y., Peduzzi, J., Bracconier, F. & Galacteros, F. (1984) FEBS Lett. 172, 155–158
14. Basset, P., Beuzard, Y., Garel, M. C. & Rosa, J. (1978) Blood 51, 971–982
15. Lacombe, C., Galacteros, F., Blibeuch, R., Beuzard, Y., Basset, P., Bracconier, F., Arous, N., Bloquith, Y. & Rosa, J. (1984) in Proteins of the Biological Fluids (Peeters, L. H. ed.) Vol. 32, pp. 1001–1005, Pergamon Press, Oxford
16. Malia, A. K., Hermanson, G. T. & Kohrn, R. I. (1981) Anal. Chem. 53, 649–661
17. Fluckiger, R. & Winterhalter, K. H. (1976) FEBS Lett. 71, 356–360
18. Beke, K., Marti, H. R. & Schlicht, I. (1989) Nature 334, 1077–1088
19. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J. & Bunn, H. F. (1978) J. Biol. Chem. 253, 2327–2332
20. Abraham, E. C., Reese, A., Stallings, M. & Huisman, T. H. J. (1976–77) Hemoglobin 1, 27–44
21. Asakura, T. (1979) Crit. Care Med. 7, 391–395
22. Poyart, C., Martin, J. L., Galacteros, F., Leclerc, L., Beuzard, Y., Magne, F. & Bohn, B. (1984) in Hemoglobin (Schneek, A. G. & Paul, C., eds) pp. 137–149, Université Libre de Bruxelles, Bruxelles
23. Wymann, J. (1964) Adv. Protein Chem. 19, 223–286
24. Bevington, R. R. (1989) in Data Reduction and Error Analysis for the Physical Sciences, Chapter II, pp. 153–182, McGraw-Hill Publications, New York
25. Giel, S. T., Gand, H. T., Wymann, J. & Barisas, B. G. (1978) Biophys. Chem. 8, 53–59
26. Salvo, G., Caprari, F., Sammugia, P., Mariani, G. & Salvati, A. M. (1982) Clin. Chim. Acta 122, 293–300
27. Beutler, E., Blume, K. G., Kaplan, J. C., Lohr, G. W., Ramot, B. & Valentine, W. N. (1977) Br. J. Haematol. 35, 331–340
28. Clegg, J. B., Naughton, M. A. & Weatherhall, D. J. (1966) J. Mol. Biol. 19, 91–108
29. Schroeder, W. A., Shelton, J. B., Shelton, J. R. & Powars, D. (1979) Hemoglobin 3, 145–159
30. Konigsberg, W., Goldstein, J. & Hill, R. J. (1963) J. Biol. Chem. 238, 2028–2033
31. Chiou, S.-H., Garrick, L. M. & McDonald, M. J. (1982) Biochemistry 21, 13–20
32. Barber, M., Bordali, R. S., Sedgwick, R. D. & Tyler, A. N. (1982) Biomed. Mass Spectrom. 4, 208–214
33. Kohn, L. D., Warren, W. A. & Carroll, W. R. (1970) J. Biol. Chem. 245, 3821–3830
34. Kohn, L. D. & Warren, W. A. (1970) J. Biol. Chem. 245, 3831–3839
35. Sallach, H. J. (1966) Methods Enzymol. 9, 221–228
36. Galacteros, F., Rosa, R., Prehu, M. O., Najeany, Y. & Calvin, M. C. (1984) Novo. Rev. Fr. Hematol. 26, 69–74
37. Barrick, R. C. & Schneider, R. G. (1981) Biochem. Biophys. Acta 668, 485–489
38. Winter, W. P. & Yodh, J. (1983) Science 221, 175–178
39. Bunn, H. F., Haney, D. N., Kamin, S., Gabbay, K. H. & Gallop, P. M. (1976) J. Clin. Invest. 57, 1652–1659
40. Arnone, A. (1972) Nature 249, 34–36
41. Chiancone, E., Norne, J. E., Bonaventura, J., Bonaventura, C. & Forsten, S. (1974) Biochim. Biophys. Acta 336, 403–406
42. de Bruin, S. L., Rollema, H. S., Janssen, L. H. M. & van Os, G. A. J. (1974) Biochem. Biophys. Res. Commun. 58, 210–215
43. Wada, Y., Hayashi, A., Masanori, F., Katakuse, I., Ichihara, T., NakaHashi, H., Matsuo, T., Sakurai, T. & Matsuda, H. (1983) Biochim. Biophys. Acta 749, 244–248
44. Bonaventura, J., Bonaventura, C., Sullivan, B., Ferruzi, G., McCurdy, P. R., Fox, J. & Moo Penn, W. F. (1976) J. Biol. Chem. 251, 7663–7671
45. Larkin, P. A., Stephens, A. D., Bechtel, K. C., Johnson, M. H., Adams, L. & Lehmann, H. (1975) Br. Med. J., 290–292
46. Ikeda, E., Kosaka, J., Pikkarainen, P., Rahila, E. L., El Mazni, M. A. F., Nagai, K., Lang, A. & Lehmann, H. (1976) Acta Haematol. (Basel) 56, 257–275
47. Elbaum, D., Wiedemann, B. & Nagel, R. L. (1982) J. Biol. Chem. 257, 8464–8468