Hemoglobin Providence

A HUMAN HEMOGLOBIN VARIANT OCCURRING IN TWO FORMS IN VIVO*

(Received for publication, April 19, 1976)

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Hemoglobin Providence Asn and Hemoglobin Providence Asp are two abnormal hemoglobins which apparently arise from a single genetic change that substitutes asparagine for lysine at position \( \beta 82 \) (EF6) in the \( \beta \) chain of human hemoglobin. The second form appears to be the result of a partial in vivo deamidation of the asparagine situated at position \( \beta 82 \). Cellulose acetate and citrate agar electrophoresis of hemolysates from patients with this abnormality shows three bands. Globin chain electrophoresis at acid and alkaline pH shows three \( \beta \) chains. These three chains correspond to the normal \( \beta ^{+} \) chain and two abnormal \( \beta \) chains. Sequence analysis indicates that the two abnormal chains differ from \( \beta ^{+} \) at only position \( \beta 82 \). In the two abnormal chains, the residue which is normally lysine is substituted either by asparagine or by aspartic acid. These substitutions are notable because \( \beta 82 \) lysine is one of the residues involved in 2,3-diphosphoglycerate binding. Additionally, \( \beta 82 \) lysine is typically invariant in hemoglobin \( \beta \) chain sequences. Sequence data on the two forms of Hemoglobin Providence are given in this paper. The functional properties of these two forms are described in the next paper.

The relationship between the structure and function of hemoglobin has been considerably advanced by investigations of the sequence and functional properties of naturally occurring hemoglobin variants. These investigations have provided information on the mechanism of both homotropic and heterotropic allosteric effects and on the nature of the heme pocket and the subunit contact areas (1-3). Some abnormal hemoglobins have few functional differences; others, with substitutions at "key" positions, have provided investigators with high resolution probes for studying structure-function relationships. In Hb Providence a key residue is altered. We present evidence that in Hb Providence there is a substitution at a heretofore invariant residue, \( \beta 82 \) (EF6) lysine, one of the amino acids involved in the binding of 2,3-diphosphoglycerate and other anionic allosteric effectors (1). The diphosphoglycerate binding site consists of a cluster of eight cationic groups located on the two \( \beta \) chains of a hemoglobin tetramer. Our data indicate that in Hb Providence, a point mutation reduces the number of cationic groups to six by a substitution of asparagine for lysine at position \( \beta 82 \). There appears to be a subsequent epigenetic deamidation of the \( \beta 82 \) Asn in some of the mutant molecules. This results in a second form of Hb Providence, one in which position \( \beta 82 \) is occupied by an aspartic acid. The microenvironment around \( \beta 82 \) may contribute specificity to the observed deamidation. In Hb Providence Asp the net positive charge of the diphosphoglycerate binding site is further reduced.

MATERIALS AND METHODS

Blood was obtained from the propositus, a 22-year-old black female, and other members of her family. Hemolysate preparation and

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standard electrophoretic procedures used have been previously
described (4). The mutant hemoglobins were isolated by DEAE-Sepha-
dex chromatography according to the method of Huisman and Dozy (5).
Globins were prepared by adding the hemoglobin to 1% HCl in
acetone. The α and β chains were prepared either from purified
hemoglobin fractions after DEAE-chromatography or directly from
hemolysates. The chain separation procedure was that of Clegg et al.
(6). The abnormal β chains were reduced, aminoethylated (7), and
digested with L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone-
treated trypsin for 4 h at 37°, or digested with cyanogen bromide in 70% formic acid (8). The tryptic peptides were separated by ion
exchange chromatography (9). Cyanogen bromide peptides were sep-
harated by gel filtration on Sephadex G-50 fine (10).

Amino acids were analyzed with a Beckman model 121 Amino
Acid Analyzer according to the method of Speckman et al. (11). Peptides were sequenced with a Beckman 890C Sequencer by the
programs provided by the manufacturer (12). Phenylthiohydantoin
derivatives of amino acids (PTH-amino acid derivatives) were identi-
cified with a Beckman GC-65 Gas Chromatograph (13) and with a
Waters ALC/GPC 202 High Pressure Liquid Chromatograph with
two tandem columns (0.4 × 30 cm) of µBondapak C₁₈. High pressure
liquid chromatography allowed for quantitative determination of the
PTH-derivatives of arginine, histidine, lysine, asparagine, glutama-
mine, aspartic acid, glutamic acid, and glycine (derivatives which
were previously identified by qualitative methods or which gave poor
results with gas chromatography). In the high pressure liquid chroma-
tography a flow rate of 2 ml/min and buffers of the following
compositions were used: 95% 0.01 M sodium acetate and 5% CH₃CN, pH
4.0 (Buffer A), and 95% 0.01 M sodium acetate and 95% CH₃CN, pH
6.0 (Buffer B). For all derivatives except arginine and histidine,
the starting buffer was a mixture of 90% Buffer A and 10% Buffer B, and the limiting buffer was a mixture of 90% Buffer A and 10% Buffer B. For citrate agar electrophoresis, samples containing approximately 1 g/dl of hemoglobin were applied
to glass plates (7.5 × 5 cm) coated with 1% agar made with 0.05 M
sodium citrate buffer, pH 6.2. A current of 5 mA/slide at 50 to 90 V
was used for 2 h. The plates were stained with a solution containing
0.1% benzidine, 5% acetic acid, and 0.6% hydrogen peroxide. The
zones corresponding to “Std CSFAJ” show the separation of an
artificial mixture of hemoglobins C, S, F, A, and J, respectively.

RESULTS

Normal Hb A and the two forms of Hb Providence were
observed in the propositus and one of her sisters. These two
individuals are clinically normal. Other siblings do not have
the mutant hemoglobin. Neither the mother nor the grand-
mother possesses the abnormal hemoglobin. The father and
grandfather are dead. The sister who has Hb Providence has
children who also have the hemoglobin variant (see Fig.
1). Individuals with Hb Providence tend to have mild polycy-
thenia. Their values for red cell mass are in the upper part of
the normal range.

Fig. 2 shows electrophoretic results of a fresh hemolysate on
cellulose acetate (pH 8.4, upper panel) and citrate agar (pH
6.2, lower panel). Immediately after blood was drawn, a hemolysate
was prepared, and samples were applied at a hemoglobin concentra-
tion of 4 to 6 g/dl to cellulose acetate strips (7.5 × 6 cm) and
electrophoresed for 20 min at a constant voltage of 450 V in a Tris/
EDTA/boric acid buffer, pH 8.4. The strips were stained with Pon-
ceau S in 5% trichloroacetic acid. For citrate agar electrophoresis,
samples containing approximately 1 g/dl of hemoglobin were applied
to an anilinothiazolinone strip, which remain in the aqueous phase after anilinothiazolinone are
converted to phenylthiohydantoins, the starting buffer was a mix-
ure of 40% Buffer A and 60% Buffer B. The same linear mixing gradient program was used over a
10-min period. Enzymes used in this study were purchased from
Worthington Biochemical Corp. Electrophoretic media and cham-
bers were obtained from the Helena Co. All chemicals were of the
highest purity available.

* The use of trade names is for identification only and does not
constitute endorsement by the Public Health Service or by the

Fig. 1. Pedigree of family with Hb Providence Asn and Hb provi-
dence Asp. The Roman numerals correspond to the generation and the arrow indicates the propositus.

FIG. 2. The electrophoretic patterns of Hb Providence Asn and
Asp on cellulose acetate (pH 8.4, upper panel) and citrate agar (pH
6.2, lower panel). Immediately after blood was drawn, a hemolysate
was prepared, and samples were applied at a hemoglobin concentra-
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have mobilities similar to those of βJ 16 Gly → Asp and βN 95 Lys → Glu, abnormal human chains representing net gains of one and two negative charges, respectively (Fig. 3 upper panel).

Chain separation of globin prepared from the propositus’ hemolysate is shown in Fig. 3 (lower panel). The elution of the abnormal β chains is consistent with the mobilities observed in globin chain electrophoresis. The isolated chains eluting in peaks marked β Providence Asp and β Providence Asn were used for structural analysis. The chain eluting in position βA has an amino acid analysis which is indistinguishable from normal β chains.

Integration of the DEAE-Sephadex chromatography elution pattern and fractions obtained by high pressure liquid chromatography on DEAE-Controlled-Pore Glass yielded 46.2% Hb A, 2.3% Hb A2, 19.3% Hb Providence Asn, and 32.2% Hb Providence Asp (Fig. 4). Hb F, by the Singer method (4), was 2%. The heat denaturation and isopropyl alcohol tests (4) used to demonstrate unstable hemoglobins were negative.

Paper fingerprints of tryptic digests of the aminomethylated β chains of hemoglobin Providence Asn and Asp showed that normal β tryptic peptides VIII-IX, IX, and X were absent (Fig. 5). Instead, other peptides were observed in different positions. Elution of these peptides and subsequent amino acid analysis yielded compositions corresponding to the abnormal peptides of Hb Providence Asn (IX-XI) and Hb Providence Asp (VIII-XI). The amino acid compositions of both peptides indicated a loss of 1 lysine residue and a corresponding gain of 1 aspartic acid residue.

Tryptic peptides from the β chains of Hb Providence Asn and Asp were also prepared by ion exchange chromatography. Relative to normal β chain patterns, both Providence patterns were identical except that β tryptic peptide IX (which elutes between β tryptic peptide XIIa and IV) and β tryptic peptide X and XI (which co-elutes as a single peak after XIV and XV) were absent. Abnormal β tryptic peptide IX-XI migrates as a broad peak between β tryptic peptides II and I (Fig. 6). Amino acid analysis of these peptides also confirmed the loss of 1 lysine residue and the gain of 1 aspartic acid residue (Table I). Similar results were obtained when cyanogen bromide peptide II (residues 56 to 146) was analyzed. The substituent amino acid of hemoglobins Providence Asn and Asp was positively identified by sequenator analysis of cyanogen bromide peptide II and tryptic peptide IX-XI of both the forms of Hb Providence. Table II shows the results. These results indicate a substitution of lysine by asparagine at position β82 in Hb Providence Asn and aspartic acid in Hb Providence Asp.

**DISCUSSION**

The propositus and other members of her family who have Hb Providence Asn and Asp are heterozygotes, possessing
normal Hb A as well as the two forms of the mutant hemoglobin. That the existence of both Hb Providence Asn and Hb Providence Asp can be explained solely on a genetic basis seems improbable, since this would require two mutations in a single codon to produce Hb Providence Asp. However, a single base change can account for Hb Providence Asn. In addition, the probability of gene duplication followed by a second mutation at β82 is remote.

In view of the unlikelihood of a genetic mechanism to support the lysine to aspartic acid substitution, it appears that a more reasonable assumption would be to consider that Hb Providence Asn and Hb Providence Asp are chemically rather than genetically related. It would appear that a possible in vitro deamidation of a first formed peptidyl amide to the acid may be a likely explanation. Support for such an assumption comes from the work of Robinson and co-workers (14-16) who have reported the spontaneous deamidation of asparagine residues in proteins and in synthetic and natural peptides.

These investigators have reported that a number of factors (the anions, the pH, and the ionic strength of the buffering media) can influence the deamidation reaction. Another important factor is the influence of neighboring residues, since both steric hindrance and charge affect the rate of deamidation. It is also noteworthy that asparagine deamidates more rapidly than glutamine, and the presence of adjacent glycyl or histidyl residues (or both) accelerates this process. In Hb Providence Asn some of these conditions exist, β82 lysine has an adjacent glycyl residue at position β83. In addition, the β82 position is in close proximity to histidine β2 and β143, both of which also contribute to the charge cluster which binds anions. These 2 residues are even closer when the hemoglobin molecule is in the deoxy conformation. However, in Hb Korle Bu and Hb C Harlem, although a glycine-asparagine sequence occurs at position 73 and 74, in vivo deamidation is not ob-

### TABLE 1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Expected</th>
<th>Hb Providence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>2</td>
<td>1.4 1.3</td>
</tr>
<tr>
<td>AE-cysteine</td>
<td>1</td>
<td>0.5 0.6</td>
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<tr>
<td>Histidine</td>
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<td>2.5 2.5</td>
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<td>Arginine</td>
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<td>0.7 0.7</td>
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<tr>
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<td>6.8 6.9</td>
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<td>Threonine</td>
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<td>1.9 2.0</td>
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<td>Serine</td>
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<td>2.1 2.1</td>
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<td>Glutamic acid</td>
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<td>Proline</td>
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<td>3.2 3.1</td>
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</tr>
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<td>1.9 1.8</td>
</tr>
<tr>
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<td>6.7 6.7</td>
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In view of the unlikelyhood of a genetic mechanism to support the lysine to aspartic acid substitution, it appears that a more reasonable assumption would be to consider that Hb Providence Asn and Hb Providence Asp are chemically rather than genetically related. It would appear that a possible in vitro deamidation of a first formed peptidyl amide to the acid may be a likely explanation. Support for such an assumption comes from the work of Robinson and co-workers (14-16) who have reported the spontaneous deamidation of asparagine residues in proteins and in synthetic and natural peptides.

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<tr>
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<tr>
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</table>
Automated Edman degradation of CNBr II of Hb Providence Asn and Asp. These values were 95.1% and 95.0%, respectively, for Hb Providence. Hb Providence Asp-CNBr II was 850 nmol.

Repetitive yields were 93.3% for alanine (cycles 7 and 21) and 92.7% for leucine (cycles 13 and 26) in the analysis of Hb Providence Asn. These values were 95.1% and 95.0%, respectively, for Hb Providence Asp.

<table>
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<th>Cycle no.</th>
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<td>Asn</td>
<td>503.8 385.4</td>
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<td>Pro</td>
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<td>79</td>
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<td>82</td>
<td>27</td>
<td>(Lys) Asn</td>
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</tr>
<tr>
<td>83</td>
<td>28</td>
<td>Gly</td>
<td>54.6 54.6</td>
</tr>
</tbody>
</table>

a Quantified by high pressure liquid chromatography.
b Identified and confirmed by back hydrolysis with HI. Initial sample of Hb Providence Asn-CNBr II was 950 nmol. Initial sample of Hb Providence Asp CNBr II was 850 nmol.
c Determined as the trimethylsilyl derivative by on-column silylation.

The charge cluster which binds anions involves eight cationic groups from the two β chains of the hemoglobin tetramer. In previous studies with other abnormal hemoglobins, investigators have probed all the residues thought to be involved in this binding site (21–25) except the β82 lysines which until now were thought to be invariant. Since 2,3-diphosphoglycerate is asymmetric, only one of the two β82 lysine residues is involved in the binding of this cofactor (1). Hb Providence Asn and Hb Providence Asp thus offer us an opportunity to study the significance of position β82 in the binding of anions. We therefore investigated the functional properties of these two new hemoglobins, in which the net positive charge of the diphosphoglycerate binding site is decreased. The functional properties of hemoglobin Providence Asn and Hb Providence Asp are described in the next paper (26).

REFERENCES
12. Sequence No. 7 (1975) l-20, Beckman Instruments, Inc., Palo Alto, California
Hemoglobin Providence. A human hemoglobin variant occurring in two forms in vivo.

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