Hemoglobin Rush [β101 (G3) Glutamine]: A New Unstable Hemoglobin Causing Mild Hemolytic Anemia

By J. G. Adams, III, W. P. Winter, K. Tausk, and P. Heller

A mild hemolytic anemia in a 43-yr-old black woman was attributed to the presence of an abnormal hemoglobin (Hb Rush) which migrated cathodically to Hb A at pH 8.0. Its structural abnormality was found to be in the β-chain, β101 (G3) glu → glu. Another electrophoretic band at pH 8.0 proved to be a hybrid tetramer (α2β2 Rush). Hb Rush is heat unstable. A likely explanation of the instability is the presence of an uncovered positive charge in the central cavity where normally glutamic acid in position 101 neutralizes arginine in position 104 contributing to the net neutrality in this region. This neutrality is disturbed by the substitution of glutamic acid by glutamine in Hb Rush.

The determination of the primary structure of abnormal hemoglobins with abnormal function has been most helpful for our understanding of the normal structural conditions that maintain the hemoglobin molecule stable, soluble, and capable of oxygenation and deoxygenation. Depending upon the position in the primary sequence and on the physicochemical properties of the replaced and replacing amino acid, the mutational substitution may interfere with the hydrophobic character of the interior of the molecule or may change the hydrophilic properties of the molecular surface; it may alter the strength of bonds along the interchain contacts essential for the conformational interaction between chains during oxygen exchange or may actually influence heme function itself. In most unstable hemoglobin variants, one neutral residue in the interior of the molecule has been found to be replaced by another neutral one, but of different dimensions, and the electrophoretic mobility is usually the same as that of normal hemoglobin.

The following is a report on a new unstable hemoglobin which causes mild hemolytic anemia. The reason for the instability of this hemoglobin is most likely the substitution of a neutral amino acid residue for a negatively charged one in the region of the central cavity, thus affecting the net neutrality in this part of the molecule.

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MATERIALS AND METHODS

Hemoglobin Electrophoresis

Blood was drawn into Vacutainer tubes containing Na$_2$EDTA as anticoagulant. Hemolysates were prepared by standard techniques and the stroma extracted with toluene. Cellulose acetate electrophoresis was carried out in the Beckman Microzone apparatus using a Tris-EDTA-borate buffer, pH 8.6. For electrophoresis at pH 8.0, the proportions of tris and boric acid were changed without altering the ionic strength. Preparative cellulose acetate electrophoresis was carried out in the Gelman apparatus using the pH 8.0 buffer. The hemoglobin components were eluted from the cellulose acetate strips according to the method of Glynn et al. The method of Robinson et al. for agar gel electrophoresis was also employed using Bacto-Agar (Difco Laboratories) and a 0.04 M citrate buffer, pH 6.2. The relative proportions of the hemoglobin components were estimated by densitometry of the dry, unstained agar plate in a Densicord densitometer.

Separation of the α- and β-chains

Separation of the α- and β-chains of the whole hemolysate was accomplished on CM-cellulose columns by a modification of the preparative procedure of Clegg et al. Approximately 300 mg of globin was applied to a 2.5 x 10-cm column. The gradient consisted of 500 ml of starting buffer (0.005 M Na$_2$HPO$_4$, 0.05 M 2-mercaptoethanol, 8 M urea, pH 6.7) and 500 ml of end buffer (0.045 M Na$_2$HPO$_4$, 0.075 M 2-mercaptoethanol, 8 M urea, pH 6.7). The fractions containing the abnormal polypeptide chain and its normal counterpart were aminoethylated, desalted on a 90 x 2.5 cm column of Biogel P-2 equilibrated with 0.5% formic acid, and freeze dried.

Peptide Analysis

Approximately 30 mg of normal and abnormal β-chain were suspended in 0.2 M NH$_4$HCO$_3$ and digested with trypsin at 37°C for 2 hr. Trypsin was added at the beginning of digestion and 1 hr later. The trypsin to protein ratio was 1 : 100 (w/w) at the first addition and 1 : 200 (w/w) at the second. Peptide maps were prepared according to the method of Clegg et al. except that 46 x 57-cm papers were used. The papers were stained with 0.2% ninhydrin in acetone. Also specific stains for histidine and arginine were used. Peptides for amino acid analysis were eluted after identification by staining with 0.2% trinitrobenzene sulfonic acid in acetone. The abnormal peptide and its normal counterpart (βT-11) were eluted with distilled H$_2$O and hydrolyzed in 5.7 N HCl in vacuo for 22 hrs at 110°C. The hydrolysate was then analyzed in a Beckman 120 C amino acid analyzer. Larger quantities of normal and abnormal βT-11 were isolated by peptide chromatography of the tryptic digest on a 0.9 x 13-cm column of Beckman PA-35 resin at 50°C with automatic alkaline hydrolysis and ninhydrin development. The purified normal and abnormal peptides were further split at the aspartic acid-proline bond by hydrolysis in dilute acid as described by Schroeder et al. with the following modification: The peptides were refluxed with 0.25 M acetic acid for 6 hr and the resulting hydrolysate containing the two peptide fragments was dried in a rotary evaporator, redissolved in 0.1 M acetic acid and spotted on Whatman 3 MM paper which was prewashed with distilled H$_2$O. Peptide maps were then prepared as described above. The paper was stained for arginine and histidine as well as with ninhydrin. A duplicate paper was stained only with trinitrobenzene sulfonic acid, and the peptide fragments of normal and abnormal βT-11 eluted and subjected to automatic amino acid analysis.

Heat Denaturation

Hemolysates from individuals with Hb A/A and Hb A/Rush were diluted to approximately 2.0 mg/ml with 0.1 M phosphate buffer, pH 7.4, and heated at 50°C, 55°C, and 60°C for 15, 30, and 60 min. Samples were centrifuged, and the precipitate was washed in the phosphate buffer at the same temperatures. Protein determinations were done on the whole hemolysates and the heat precipitates by the method of Lowry.

Oxygen Affinity

Oxygen affinity was only performed on whole hemolysates, because the separation of normal and abnormal hemoglobin by column chromatography was only possible after conversion to cyan-
methemoglobin. Hemolysis was accomplished with distilled H2O and the stroma removed by centrifugation, and the supernate was diluted to 1 mg/ml in 0.1 M phosphate buffer, pH 7.2. Oxygen equilibrium curves were determined according to the method of Rossi-Ilanelli and Antonini, but at 22°C.

CASE REPORT

The 43-yr-old black proband came to our attention because she was found to have a mild hemolytic anemia following cholecystectomy. The operation was performed because of biliary colic caused by multiple stones visualized by cholecystography. The recovery was uneventful, except for the decline of the hemoglobin level from 12 g/100 ml on admission to 10.3 g/100 ml on the fourth postoperative day. During the same period the hematocrit dropped from 37% to 29.8%, and the reticulocyte count increased from 2% to 5%. The peripheral blood smear showed moderate anisocytosis and poikilocytosis. There were many target cells and marked basophilic stippling. Plasma haptoglobin was markedly decreased. Serum iron was 161 mg/100 ml, and the total iron binding capacity was 472 mg/100 ml. Bone marrow aspiration revealed a reactive marrow with erythroid hyperplasia and a normal amount of stainable iron. A few siderocytes and sideroblasts were present. Hemoglobin electrophoresis, as performed by the clinical laboratory, was reported to be normal. Erythrocyte enzyme studies showed increased glucose-6-phosphate dehydrogenase, pyruvate kinase, and glutathione peroxidase consistent with decreased mean red cell age. 51Cr red cell half-life was slightly reduced to 19.5 days (normal: 25-30 days).

The pedigree of the proband's family is shown in Fig. 1. Only the three daughters and one granddaughter were available for examination. One daughter and her child had the abnormal heat-unstable hemoglobin. They also had hematologic findings similar to that of the proband (Table 1). Heinz bodies could not be demonstrated in the peripheral blood of any of these individuals.

RESULTS

Hemoglobin electrophoresis at pH 8.6 on cellulose acetate did not reveal an abnormal hemoglobin component. However, electrophoresis at pH 8.0 in the same medium re-

<table>
<thead>
<tr>
<th>Table 1. Hematologic Data</th>
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<td><strong>Individual</strong></td>
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</tr>
<tr>
<td>II-2</td>
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<td>III-2</td>
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<td>III-4</td>
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<td>IV-1</td>
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revealed two abnormal bands migrating cathodically to Hb A (Fig. 2). Each of the abnormal bands was eluted from preparatory cellulose acetate strips and again analyzed by electrophoresis at pH 8.0. The more cathodic of the two bands gave rise to only one band. Reelectrophoresis of the more anodic of the two bands, however, revealed a pattern identical to that of the whole hemolysate, suggesting that this band consisted of a hybrid tetramer. In hemolysates stored for 3 wk or longer, and containing 25%–30% methemoglobin, the hybrid tetramer could not be demonstrated. The same was true if a fresh hemolysate was converted to cyanmethemoglobin. Hb A₂ was present as a single band, even when large amounts of hemolysate were applied.

Acid agar electrophoresis at pH 6.2 showed a single abnormal band migrating anodically to Hb A and comprising about 35% of the total hemoglobin. The presence of more than a single abnormal band, even with fresh hemolysate, could not be demonstrated under these conditions.

The whole hemolysate of the proband was separated into its component chains by CM-cellulose column chromatography revealing an abnormal β-chain more basic than β₄.

The peptide map of an aminoethylated tryptic digest of β Rush (Fig. 3) revealed βT-11 to be displaced toward the cathode. Amino acid analysis of the abnormal peptide was identical with that of normal βT-11 (Table 2). Since the new peptide was more basic than its normal counterpart, it was concluded that the substitution was either 99 asp — asn or 101 glu — gln. Splitting of the aspartic acid-proline bond between residues 99 and 100 by dilute acid hydrolysis of βT-11 permitted the distinction between these two possibilities (Table 2). Figure 4 shows that the peptide fragment containing arginine (βT-11b) was displaced toward the cathode. Since this fragment includes residues 100–104 (Table 2), it can be concluded that in the abnormal hemo-
globin glutamic acid is replaced by glutamine at β101 (G3). It also should be stated that dilute acid hydrolysis would not have affected an asparagine-proline bond in case the substitution would have been β99 asp → asn. Dilute acid hydrolysis is specific for aspartic acid bonds. This new hemoglobin has been designated Hb Rush because the proband was hospitalized at the Rush-Presbyterian-St. Luke's Medical Center in Chicago.

Heat stability studies (Fig. 5) indicated that whole hemolysate from a Hb Rush heterozygote was more unstable than the normal control for all temperatures tested.

The oxygen dissociation curve of the hemolysate of whole blood of the proband was identical to that of the normal hemolysate (Fig. 6).

### Table 2. Amino Acid Analysis

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<thead>
<tr>
<th>Amino Acid</th>
<th>Hb A</th>
<th>Hb Rush</th>
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<tbody>
<tr>
<td></td>
<td>βT-11</td>
<td>βT-11α</td>
</tr>
<tr>
<td>His</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>0.0</td>
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<tr>
<td>Asp</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Glu</td>
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<td>0.1</td>
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<tr>
<td>Pro</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
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<td>1.0</td>
</tr>
<tr>
<td>Leu</td>
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<td>1.1</td>
</tr>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
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βT-11 (Hb A)

- leu-his-asp
- pro-glu-asn-phe-arg

<table>
<thead>
<tr>
<th>βT-11α</th>
<th>βT-11b</th>
</tr>
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<tr>
<td>96 97 98 99</td>
<td>100 101 102 103 104</td>
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</table>
DISCUSSION

Hemoglobin Rush migrates as Hb A on cellulose acetate electrophoresis at pH 8.6 and, thus, would be missed by routine electrophoretic screening. However, on closer scrutiny the A band seemed broader than normal Hb A suggesting that at a pH other than pH 8.6 separation might occur. Indeed, when the pH was changed to 8.0 in the same medium three distinct bands appeared. The reason for this electrophoretic peculiarity is obscure. The more cathodic of these bands consisted of the abnormal hemoglobin (α\textsuperscript{A} β\textsubscript{Rush}) and the more anodic one of the hybrid tetramer (α\textsuperscript{A} β\textsuperscript{A} β\textsuperscript{Rush}). It is likely that the hybrid tetramer forms under these circumstances because glutamine at position G3 that belongs to the α\textsubscript{1} β\textsubscript{2} contact forms a stronger interchain hydro-
phobic bond with αG3 valine, shifting the dimer-tetramer equilibrium in favor of the tetramer. Hybrid tetramers have also been observed in two other hemoglobins involving mutations of α1 β2 contact residues, Hb Richmond, β102 (G4) lysine,14 and Hb Ypsilanti β99 (G1) tyrosine.2,13

Stored hemolysates did not contain the hybrid tetramer. It is likely that this was due to methemoglobin formation (25%-30%) resulting in a shift of the equilibrium toward dimers. This probability is strengthened by the finding that the hybrid tetramer band is not demonstrable in fresh samples if the hemolysate is converted to cyanmethemoglobin. The absence of the hybrid tetramer at pH 6.2 is attributable to increased dissociation of the tetramer into dimers at this pH.

The amino acid substitution in Hb Rush results in a disturbance of the charge equilibrium in the region of the central cavity. The central cavity extends through the molecule along the twofold axis, from the area of α-α contact to the area of β-β contact. Normally glutamic acid in position 101 (G3) interacts with arginine in position 104 (G6) contributing to the neutrality in this part of the molecule. Disruption of this interaction by the replacement of the glutamic acid by glutamine would be expected

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**Fig. 5.** Heat denaturation of Hb A/A and Hb A/Rush.

**Fig. 6.** Oxygen dissociation curve of the whole hemolysate of the proband and of the whole hemolysate of a normal individual. These studies were carried out at 22°C.
to result in the presence of an uncovered positive charge in the central cavity which
normally has a balance of positively and negatively charged residues. Thus, the in-
stability is most likely explained by the loss of neutrality of the interior. It should be
noted, however, that the disruption of stability and the resulting hemolytic anemia are
surprisingly mild.

It might be expected that the characteristics of hemoglobin Rush would resemble
those of Hb Kempsey, $\beta^{99}$ (G1) asp $\rightarrow$ asn, which has increased oxygen affinity, and
its carriers have erythrocytosis. In both Hb Kempsey and Hb Rush acidic residues in
the G helix involving $\alpha_1\beta_2$ contact residues are replaced by neutral ones. Moreover,
both $\beta$G1 and $\beta$G3 are involved in Van der Waals interactions with $\alpha$G6 (G3) valine in
the $\alpha_1\beta_2$ contact. However, none of the heterozygotes for Hb Rush have erythro-
cytosis (Table 1), and oxygen affinity studies performed on the whole hemolysate of
the proband demonstrated a normal oxygen dissociation curve (Fig. 6). The reason for
this difference between Hbs Kempsey and Rush is not clear but it can be surmised
from the hemoglobin model of Perutz that the substitution in Hb Rush has its main af-
cect in unbalancing the charges in the central cavity and does not affect the interchain
configurational changes during oxygenation. Furthermore it can be assumed that $\beta$G3
does not normally play as important a role in the contact with $\beta$G3 as does $\beta$G1, since
$\beta$G1 normally contributes 3 atoms to the Van der Waals interaction with $\alpha$G3, whereas
$\beta$G3 contributes only 2.

Hemoglobin Rush thus provides an interesting example of the way in which spatial
orientation of the charge change, as well as its position in the amino acid sequence,
determines the functional properties of a variant hemoglobin.

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