Hemoglobin D Iran $\alpha_4^4\beta_4^4$Glu in Association With Thalassemia

By Ronald A. Rohe, Vijay Sharma, and Helen M. Ranney

A 25-year-old Indian (Asiatic) woman investigated for a life-long anemia was found to have a hitherto undescribed structural hemoglobin variant $\alpha_2^4\beta_2^4$Glu $\rightarrow$ Gln, which was found independently and designated Hb D Iran by Rahbar in members of a family from Iran. In the present case, Hb D Iran was found in association with high $A_2$ thalassemia. The replacement of glutamic acid by glutamine at $\beta 22$ (helical residue B4) was demonstrated by thin-layer chromatography after automated Edman sequencing. This is the fourth substitution to be described at $\beta 22$; the previous substitutions were (1) G Coushatta (ala); (2) E Saskatoon (lys); (3) G Taipei (gly). Helical residue B4 is an external residue, does not participate in $\alpha$-$\beta$ or protein-heme contacts and hemoglobin D Iran resembles Hb A in visible spectra, $O_2$ equilibria in dilute solutions and heat stability. Since the propositus and her mother who was also heterozygous for $\beta$ thalassemia had similar degrees of anemia, no interaction between this variant and $\beta$ thalassemia was evident. The presence of this Hb variant in Iranians and in Western Indians may reflect the migrations of populations in these areas centuries ago.

The first example of the group of hemoglobins classified as hemoglobin D (Hb D) was described by Itano$^1$ in 1951 in a white family. Hemoglobin D closely resembles Hb S in its electrophoretic mobility at alkaline pH, but it lacks the solubility properties of unliganded Hb S, and its presence is not associated with sickling. The D hemoglobins are further distinguished from Hb S by their mobility in citrate-agar at pH 6.2 in which they migrate with Hb A rather than with Hb S. Hemoglobin variants based upon one of several different amino acid substitutions in either the $\alpha$ or the $\beta$ polypeptide chains may be designated as hemoglobin D; some of these variants, e.g., Hb D$_{P}$J in which glutamate replaces the normal $\beta$ 121 glutamic acid$^2$ or G$_{St. Louis}$ in which lysine is substituted for asparagine in the 68th residue of the $\alpha$ polypeptide chain,$^3$ are encountered rather frequently, while others appear to be uncommon. The present report is an account of the structural identification and some of the properties associated with an uncommon Hb D, designated Hb D Iran, which was found in association with $\beta$ thalassemia in a young woman from India.

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MATERIALS AND METHODS

Hemolysates were prepared from washed red cells by lysis with distilled water and toluene, followed by high-speed centripugation. Vertical starch gel electrophoresis was carried out at 4°C in EDTA-Tris-Borate buffer at pH 8.6, and citrate-agar electrophoresis was carried out at pH 6.4. For isoelectric focusing, ampholyte at 4°C, pH 6.00–8.00 was used. Hemoglobin components were quantified by elution from starch block or from cellulose acetate. Fetal hemoglobin determinations were performed by 1-min alkaline resistance according to Singer. Heat stability was measured at 50° and 55° in 0.1 M phosphate buffer at pH 7.4. Separation into α and β chains by paramercuribenzoate (PMB) was carried out according to Geraci and Parkhurst with minor modifications.

For studies of primary structure, globin was prepared by acid-acetone precipitation at –20°C. The α and β chains were separated by carboxymethyl-cellulose chromatography in 8 M urea at pH 7.0, digested with trypsin, and fingerprinted according to Clegg, Naughton, and Weatherall with the following modifications: (1) ascending chromatography was used; (2) following acid hydrolysis (at 110°C for 18 hr in 6 N HCl containing 9 mg per 100 ml phenol), the peptides were dried in vacuo prior to amino acid analysis. A Beckman Spinco Amino Acid Analyzer Model 120C was used for amino acid analysis.

A Beckman Sequence Model 890 was employed with protein program using Quadrol buffer and Beckman Sequence grade reagents. The β chains were converted to the carboxymethylated form as described by Crestfield, Moore, and Stein. Five milligrams of the dried sample was introduced into the sequence reaction cup, and the first "degradation cycle" was carried out without phenylisothiocyanate. This procedure gives more uniform coating and was found to be helpful in reducing overlap of residues in later degradation cycles. The thiazolinone derivatives were converted to phenylthiodyantoin (PTH) derivatives by heating at 80° for 10 min in 1 M HCl and extracting the products into ethyl acetate. The PTH derivatives were identified with thin layer chromatography and a Beckman GC-45 gas chromatograph. Thin-layer chromatography was carried out on Eastman chromatogram sheets type 6060 with fluorescent indicator. The solvent systems used have been described by Sjoquist.

Studies of O2 equilibria in dilute solution at 20° were carried out by a modification of the methods of Allen, Guthe, and Wyman. Visible spectra of the oxy and deoxy forms were ascertained using a Cary 14R with tonometer and spacers. 16

RESULTS

Propositus

The patient was a 25-yr-old woman who had been born in Kohat, Western India (now Pakistan), and had completed her training as a psychologist. She had had a life-long anemia which was unresponsive to parenteral iron during her childhood. The patient was asymptomatic and there was no history of jaundice or pigmenturia. There had been no major medical or surgical illnesses. Physical examination was unremarkable: evident growth retardation, jaundice, or hepatosplenomegaly were not observed. The results of hematologic studies are shown in Table 1 and the peripheral blood smear is shown in Fig. 1.

Family Studies (Table 1)

Studies were available only on the parents although apparently reliable historical data was obtained on some other members of the family who lived in India. Two siblings had been anemic, and a maternal niece had died in childhood of anemia historically compatible with thalassemia major. The red cell indices and morphology of the mother were compatible with β thalassemia minor, and her hemoglobin had increased proportions of Hb A2 and F. The father had no anemia or abnormalities on blood smear, but on electrophoresis
HEMOGLOBIN D IRAN $^a_{2}^2$-Glu $\rightarrow$ Gin

Table 1. Hematologic Data on Propositus and Parents

<table>
<thead>
<tr>
<th></th>
<th>Father ($D_{in}$ Heterozygote)</th>
<th>Mother ($\beta$ Thalassemia Trait)</th>
<th>Propositus ($D_{in}$/$\beta$ Thalassemia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (gm/100 ml)</td>
<td>14.9</td>
<td>10.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Mean cell volume ( $\mu^3$)</td>
<td>88</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>-</td>
<td>-</td>
<td>3-5.0</td>
</tr>
<tr>
<td>Erythrocyte morphology</td>
<td>Normal</td>
<td>Microcytic hypochromia</td>
<td>Microcytic hypochromia</td>
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<tr>
<td></td>
<td></td>
<td>Targets</td>
<td>Targets</td>
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<tr>
<td></td>
<td></td>
<td>Polychromasia</td>
<td>Polychromasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basophilic stippling</td>
<td>Basophilic stippling</td>
</tr>
<tr>
<td>Hemoglobin composition (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>55.0</td>
<td>91.8</td>
<td>0.0</td>
</tr>
<tr>
<td>$A_2$</td>
<td>0.8</td>
<td>5.2</td>
<td>4.2</td>
</tr>
<tr>
<td>$F$</td>
<td>0.7</td>
<td>3.0</td>
<td>4.3</td>
</tr>
<tr>
<td>$D_{IRAN}$</td>
<td>43.5</td>
<td>0.0</td>
<td>94.5</td>
</tr>
<tr>
<td>Heat Stability 50˚ and 55˚</td>
<td>-</td>
<td>-</td>
<td>Same as Hb A</td>
</tr>
</tbody>
</table>

of his hemoglobin about half of the pigment moved in the position of hemoglobin D (or S).

Electrophoretic Studies: On starch gel (Fig. 2) the propositus had a homogeneous band moving in the position of Hb D (or Hb S) with a high Hb $A_2$. The mother had Hb $A$ and high Hb $A_2$ and the father was apparently heterozygous for hemoglobin D. The $\beta$ chain of the PMB-treated hemoglobin of the propositus moved slower than the $\beta$ chain of normal hemoglobin; this finding, together with the absence of a double minor component, indicated that the abnormality was in the $\beta$ chain. On citrate-agar electrophoresis, the hemoglobins of the propositus, of the mother, and of the father had the same mobility as Hb $A$ (Fig. 3). On isoelectric focusing the hemoglobin variant was indistinguishable from Hb S with an isoelectric point 7.25.

Fig. 1. Blood smear of propositus showed hypochromicity, target cell formation, and basophilic stippling, the latter not apparent in this field (Wright Stain).
Fig. 2. Vertical starch gel electrophoresis. Tris-EDTA-Borate buffer pH 8.6. Amido black stain. Hb S and Hb D Iran (propositus) have identical mobilities. PMB treated Hb D Iran shows normal mobility of \(\alpha\) chains and cathodal of \(\beta\) chains. Sample from father was aged hemolysate—isolectric focusing of fresh hemolysate revealed approximately equal quantities of Hb A and Hb D Iran.

Chromatography of globin in 8 M urea at pH range 7.0–7.4 revealed only two peaks: one in the position of \(\beta\) chains and the other in the position of \(\alpha\) chains (Fig. 4). In approximately 20 runs, small “shadow peaks” were occasionally demonstrated but no fraction in the expected position of \(\beta^A\) (e.g., preceding or in close proximity to the \(\beta^D\) polypeptide chain) was demonstrated on any of the separations. Peptide maps of the tryptic digest of the isolated \(\alpha\) chains were identical with normal \(\alpha^A\). Peptide maps of the \(\beta^D\) chains showed absence of the normal \(\beta TP3\) and a “new” cathodal peptide (Fig. 5).

On amino acid analysis of the acid hydrolysates, the amino acid composition of \(\beta TP3\) of the variant was identical with normal \(\beta TP3\). The normal amino acid composition of \(\beta TP3\) together with its fast cathodal migration could result from an amide substitution at B21, B22, or B26. For final identification, isolated \(\beta\) chains of the variant were carboxymethylated and subjected to automatic sequencing on a Beckman 890B sequencer. The PTH-AA derivatives were identi-
fied by gas-liquid and/or thin-layer (TLC) chromatography. No abnormalities were revealed through residue B21. Examination of residue B22 revealed replacement of glutamic acid by glutamine, as identified by thin-layer chromatography. Thus the sequences in the normal and the variant β peptide 3 were as follows:

Residue Number:  18  19  20  21  22  23  24  25  26  27  28
Normal: VAL ASN VAL ASP GLU VAL GLY GLY GLU ALA LEU
D Iran: VAL ASN VAL ASP GLN VAL GLY GLY GLU ALA LEU

The oxygen equilibria of dilute solutions of Hb D did not show significant variation from normal Hb (Fig. 6). In heme–heme interaction the variant hemoglobin closely resembles HbA (N values of 2.7–3.2). The alkaline Bohr effect was normal over pH range of 7.80 to 6.78. (P50 values were 3.6 and 12.0 mm Hg
at 20°, respectively.) The visible spectra of both oxy and deoxy forms were identical with those of Hb A.

**DISCUSSION**

Heterozygotes for this hemoglobin variant were found independently in members of an Iranian family by Rahbar who designated the variant Hb D Iran. Its occurrence in association with β thalassemia in the present family afforded an unusual opportunity to examine the properties of the hemoglobin variant, since it comprised about 95% of the hemoglobin from lysed red cells.

The oxygen equilibria did not differ significantly from Hb A in any of the three properties, oxygen affinity, cooperative interactions, or Bohr effect. The normal properties which were observed are consistent with the replacement of an external residue (B4), which, according to Perutz, is not in contact with the heme, nor at an area of subunit contact.

The absence of effect on oxygenation function is reflected in the normal hematocrit of the father, a heterozygote for Hb D Iran as well as in the heterozygous subjects observed by Rahbar. The anemia observed in the propositus is comparable to that of her mother, and may be presumed to reflect the β thalassemia present in each. The availability of a sequencer considerably simplified the detection of the substitution at β22, although the nature of the substitution could have been established with other techniques. (The readiest use of the automatic sequencing in the study of hemoglobin variants at present is in the initial 35 or 40 residues of either polypeptide chain).

The thalassemia encountered in this family apparently led to complete suppression of β chain synthesis, since we failed to find any βA polypeptide chain when 200 mg of globin were separated into α and β chains by chromatography in urea. This type of thalassemia has been described in Ferrara, Southeast
HEMOGLOBIN D IRAN \( \alpha_2^H \beta_2^H \text{Glu} \rightarrow \text{Gln} \)

Asia\(^{18}\) including Thailand,\(^{19}\) and other areas, but its occurrence in India has not to our knowledge been described.

The presence of Hb D Iran, like most other hemoglobin variants, did not alter remarkably the manifestations of \( \beta \) thalassemia. The notable exception is Hb E, \( \alpha_2 \beta_2^E \text{ lys} \rightarrow \text{glu} \). The simultaneous presence of hemoglobin E and \( \beta \) thalassemia in Indonesians is clinically manifest as a moderately severe form of thalassemia.\(^{18,20}\) Even though the thalassemia described in this family led to complete suppression of \( \beta^A \) chain synthesis, no such interaction with Hb D Iran was observed.

The finding of the same hemoglobin variant in subjects from western India and from Iran may reflect common ancestry, for as early as 1500 B.C. the Aryans “Eastern Stream” subdivided, one branch settling in Persia, the other in India, or it may reflect migration patterns between 500 and 400 B.C. when Darius I of Persia invaded northern India.\(^{21}\)

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