The Characterization of Hemoglobin Shimonoseki

By M. Hanada and D. L. Rucknagel

In 1960 Yamoaka et al. described one of the first abnormal hemoglobins to be detected in Japan, found in a survey of approximately 3000 successive clinic patients. This hemoglobin, subsequently designated Hb Shimonoseki (Hb Sh) according to the town of residence of the propositus, was demonstrated in two generations of a large kindred and thus shown to be inherited. The affected individuals were normal hematologically. The abnormal component migrated slightly faster than hemoglobin S (Hb S) on paper electrophoresis at pH 8.6 and on starch gel electrophoresis at pH 8.6 slightly slower than Hb S. By paper electrophoresis at pH 6.5 it did not separate from Hb A. Solubility, absorption spectrum, and alkali denaturation rate, were normal. Mobility on the amberlite IRC-50 chromatographic column was identical with that of Hb S.

In the present paper Hb Shimonoseki is shown to be due to the substitution of arginine for glutamine in the 54th residue of the α-polypeptide chain. It can thus be designated \( \alpha^54 \text{Arg} \beta^A \) in accordance with recommended nomenclature.

A preliminary report on these findings has already been published.

Materials and Methods

Hemolysates of approximately 10 Gm. per cent hemoglobin concentration were prepared by Drabkin's method. Vertical starch gel electrophoresis was performed at pH 8.6 using a 0.03 M borate buffer or at pH 7.1 using a 0.04 M phosphate gel buffer (0.025 M \( \text{KH}_2\text{HPO}_4 \) and 0.015 M \( \text{NaH}_2\text{PO}_4 \)). The apparatus contained the same buffers at tenfold concentration. The samples were applied by inserting small rectangles of Whatman 3 MM filter paper saturated with hemolysate, into an incision in the gel. After electrophoresis the gel was sliced horizontally, the bottom half stained with Amido-Schwartz 10B protein stain and the top with ortho dianisidine. The abnormal components were quantitated by starch block electrophoresis. Agar gel electrophoresis at pH 6.5 was performed by a minor modification of the technic of Robinson et al. Prior to all electrophoreses one drop of 2 per cent potassium cyanide, neutralized with 10 per cent acetic acid, was added per 0.3 ml. of hemolysate to convert any methemoglobin present to the cyanmet form. Column chromatography was performed using amberlite CG-50 resin in a 2 x 30 cm. column, and recording the absorbence of the effluent at 415 m\( \lambda \) wave length using a Vanguard monitor. In order to elute the abnormal components in a smaller volume a pH 7.30 developer containing 0.0375 M \( \text{Na}_2\text{HPO}_4 \), 0.025 M \( \text{NaH}_2\text{PO}_4 \) and 0.01 M potassium cyanide was used. Molecular hybridization was performed by incubation of purified Hb Sh with Hb S or Hb I for 2 hours at 0 C. in 0.2 M acetate buffer (total molarity), pH 4.7, and then dialyzing overnight in the 0.03 M borate gel buffer containing 0.01 M potassium cyanide (pH 8.8). Hybridization was demonstrated by starch gel electrophoresis at pH 8.6. Controls were achieved by treating each hemoglobin separately as described but mixing immediately prior to electrophoresis.

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The heat-denatured hemoglobin was digested with trypsin for 90 minutes and the pH maintained at 8.0 by titrating with 0.05 N sodium hydroxide in a pH stat.\textsuperscript{13} or by use of ammonium carbonate volatile buffer.\textsuperscript{14} Descending chromatography was performed using either the n-butanol-acetic acid-water (4:1:5) solvent of Katz et al.,\textsuperscript{14} the n-butanol-acetic acid-pyridine-water (90:18:60:72) system of Swenson et al.,\textsuperscript{15} or the isopropanol-acetic acid-water (35:35:30) solvent of Baglioni.\textsuperscript{16} Electrophoresis at approximately 2500 volts and 125 ma. (50 volts/cm.) was performed in a lucite tank utilizing Varsol \#3 as the coolant.\textsuperscript{14,17} We used the pyridine-acetic acid-water buffers at pH 3.45 (1:10:289)\textsuperscript{14} or at pH 6.5 (10:0.4:100).\textsuperscript{13} The fingerprints obtained by chromatography with the solvent of Swenson et al. followed by high voltage electrophoresis at pH 6.5 offered the best resolution of the critical peptides of this particular hemoglobin. Chymotryptic digestion of the "core" was performed according to the method of Hunt and Ingram\textsuperscript{18} and fingerprinted as above. The dried fingerprints were stained with 0.2 per cent ninhydrin in water-saturated butanol or were stained for arginine, histidine tyrosine, tryptophane, and sulfur-containing amino acids.\textsuperscript{17} The ninhydrin stained tryptic peptides were eluted from the fingerprints with constant boiling hydrochloric acid, hydrolyzed for 18-20 hours at 105-110 C. and the amino acid composition qualitatively determined by two dimensional paper chromatography.\textsuperscript{17} Quantitative analysis of either the whole tryptic peptides or the amino acid components of peptide hydrolysates was performed by staining the fingerprint or amino acid chromatogram with a ninhydrin-copper complex and determining the optical density of the eluate at a wave length of 504 nm.\textsuperscript{19} Calibration curves were prepared by chromatographing measured amounts of each amino acid. The molar ratios of table 1 were derived from the molar concentrations of each amino acid comprising the peptide analyzed. Purified \(\alpha\)- and \(\beta\)-chains were prepared from globin\textsuperscript{20} by differential precipitation with trichloroacetic acid in 8 M urea\textsuperscript{21} and fingerprinted separately.

RESULTS

The mobility on starch gel electrophoresis at pH 8.6 of Hb Sh is compared with that of Hb S and two other abnormal hemoglobins from western Japan (Hb Umi and Hb Kokura) in figure 1. Hb Sh migrated slightly slower than Hb S. This was not due to concentration differences inasmuch as all of the samples shown in figure 1 had been adjusted to the same total concentration and proportion of abnormal components by addition of Hb A. Not visible in this pattern stained with Amido-Schwartz was a minor component migrating cathodally to Hb A\(_2\), suggesting that a similar electrophoretic abnormality of Hb A\(_2\) was present. Upon starch block electrophoresis the major abnormal component comprised 16.3 per cent of the total hemoglobin. The analogue of Hb A\(_2\) was not measured separately but both components constituted 3.5 per cent of the total hemoglobin, within the normal limits for Hb A\(_2\) in this laboratory. At pH 7.1 Hb Sh migrated slightly anodally to Hb S (fig. 2). It did not separate from Hb A on agar gel electrophoresis at pH 6.5 (fig. 3). Hb Sh was eluted from the amberlite CG-50 chromatographic column by 110 ml. of developer compared with 125 ml. for Hb S chromatographed under the same conditions. The difference was verified by chromatographing a mixture of Hb S and Hb Sh.

The results of the hybridization experiment are shown in figure 4. The control experiments, performed by treating equivalent amounts of hemoglobins Sh, S and I separately and then mixing them together, showed small amounts of Hb A and Hb A\(_2\) due to contamination of the stock of abnormal components. When acid incubation was performed with a mixture of Hb S and Hb Sh, at
Fig. 1.—Starch gel electrophoresis at pH 8.6, stained with Amido-Schwartz, of hemoglobin Shimonoseki compared with Hb S and two other abnormal hemoglobins from Japan (Hb Umi and Hb Kokura).

pH 4.7, the amounts of Hb A and the slowly migrating substance were greatly increased, compared with the corresponding control. The hybridization mixture of hemoglobins Sh and I did not differ from the control. These hybridization results suggest that there has been an exchange of subunits between Hb Sh and Hb S with the formation of a hybrid molecule and of Hb A with corresponding net ionic charges as follows:

\[ \alpha^{Sh} \beta^A + \alpha^A \beta^S \rightarrow \alpha^{Sh} \beta^S + \alpha^A \beta^A \]

This plus the lack of hybrid molecule formation with Hb I indicates that the \( \alpha \)-polypeptide chains of Hb Sh are abnormal.

The fingerprint of Hb Sh was remarkably similar to that of Hb A, performed at the same time (fig. 5). However, the specific stain for arginine showed six positive peptides in Hb Sh compared with only five in Hb A (fig. 5b). The peptide in Hb A designated number 10 by Ingram\(^1\) and number 17 by Chernoff and Liu\(^1\) does not contain arginine. Although this same peptide was not displaced in the Hb Sh fingerprint, it stained positively with the Sakaguchi reaction for arginine. Since trypsin cleaves polypeptide chains following arginine, an additional peptide should have been formed by the substitution of this amino acid into the \( \alpha \)-chain.

The amino acid composition of peptide number 10 of Hb A is shown in table 1. This identifies it as the sixth tryptic peptide from the amino end of the
Fig. 2.—Starch gel electrophoresis in 0.04 M phosphate buffer at pH 7.1 of hemoglobin Shimonoseki compared with hemoglobins S and C; stained with Amido-Schwartz. Hemoglobin Shimonoseki migrated very slightly anodally to Hb S. Not visible with this stain is a minor component migrating cathodally to Hb C which is presumably the abnormal analogue of Hb A₂ (α₂ δ₂).

α-chain (αTpVI) according to the sequences (fig. 6) published by Konigsberg et al. and Braunitzer et al. Peptide number 10 of Hb Sh (Sh-10), compared with that of the analogous peptide in Hb A (A-10) lacks glutamine, lysine, and valine but contains arginine (fig. 6). These changes suggest that residue number 54, normally glutamine, is replaced by arginine. An additional peptide, valyllysine, should be formed (Sh-10b). Valyllysine is already present in the fingerprint of Hb A, as the sixth peptide of the β-chain (βTpVI), number 19 in Ingram’s nomenclature and number 6 by the method of Chernoff and Liu. In Hb Sh this peptide is increased in intensity relative to the remaining peptides. This observation was quantitated by eluting this peptide and three others as controls from the same ninhydrin-copper nitrate stained fingerprint and measuring the absorbence at wave length 504 μμ. The ratio of optical density of peptide 19 to that of the respective control peptides is twice as great in Hb Sh as in Hb A. Peptide number 19 of Hb Sh is therefore composed of peptide 10b, as well as number 19 derived from the β-polypeptide chains.

This has been further demonstrated by fingerprinting the separated α- and
Fig. 3.—Agar gel electrophoresis at pH 6.2 of hemoglobin Shimonoseki compared with hemoglobins S, Umi, Kokura, and F, stained with Amido-Schwartz. AS refers to hemoglobin of subject with sickle cell trait.

β-polypeptide chains of Hb Sh (fig. 7). Peptide number 19 is present in the fingerprint of the β-chains but absent from the α-chains of Hb A. In Hb Sh an identical peptide is present in both the α- (number 10b) and β-chains (number 19). We have determined that both peptides in Hb Sh contain valine and lysine.

A minor peptide, designated number 10c (fig. 5), which was not seen on fingerprints prepared by electrophoresis at pH 3.5, was initially believed to be an artifact since it was inconstantly present on repeated fingerprints at pH 6.5. This peptide is composed of glycine, histidine, lysine, and valine. Adjacent peptide number 20α (αTpVII) contains glycine, histidine, and lysine only, whereas 20β (βTpVII) contains glycine, histidine, lysine, and alanine. Peptide 10c, distinct from 20α and 20β, is not a tryptic peptide of normal
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Fig. 4.—Starch gel electrophoresis of hybridization experiments using hemoglobins Shimonoseki, S, and I. The A and C samples were applied as markers. The gel was stained with o-dianisidine.

hemoglobin. It is formed most likely due to failure of tryptic cleavage at residue 56 or Hb Sh, resulting in a fusion of 10b with αTpVII, the next peptide in sequence (fig. 6).

DISCUSSION

The data presented herein clearly demonstrate that the 54th residue of the α-polypeptide chain of hemoglobin, normally glutamine, is substituted in hemoglobin Shimonoseki by arginine, creating a new tryptic cleavage point. Thus, according to current convention it might be designated α254 AVS.A. The fingerprinting results thus corroborate the evidence for abnormal α-chains gathered from the hybridization studies and the presence of a minor component migrating cathodally to Hb A2. In bloods containing abnormal hemoglobinics due to amino acid substitutions in the α-polypeptide chains, parallel electrophoretic abnormalities of the Hb F24,25 and Hb A225,26,27,28 have been shown.

Of the large number of inherited abnormalities of human hemoglobin described (reviewed in Rucknagel and Neel29 and Baglioni30) in approximately 15 others the amino acid substitutions have been delineated. Apparent clustering of these substitutions at the amino terminal end of the polypeptide chains, between residues 1 and 30 and in the proximity of the heme rings, from residues 54 to 68, suggests that they are non-randomly distributed along the chains. Non-random distributions of mutants along the genetic map of microorganisms, referred to as genetic "hot spots," have been observed.31 It is un-
likely that clustering of the hemoglobin substitutions, if, indeed, the observation is correct, represents regions of mutational lability of the hemoglobin structural genes because of the possibility that selective forces may play decisive roles in determining which mutants are detected. Substitutions in
some areas of the polypeptide chains may so disrupt the physiologic function of the hemoglobin molecule as to make the mutation lethal, leaving only the more benign mutations to persist. Alternatively, substitutions in other parts of the molecule may tend to make the abnormality more readily detectable clinically. Specific examples of the latter may be hemoglobins S and C Georgetown, both of which are responsible for sickling and are due to abnormalities in the amino terminal peptide of the β-chains, as well as the various types of hemoglobin M which were detected because of cyanosis in the heterozygotes. Since Hb Shimonoseki confers no known hematologic consequences on the carriers it presumably would fall into the benign category, despite the proximity of its amino acid substitution to the heme ring of the α-chain.

This is also the first hemoglobin anomaly shown to be due to an amino acid substitution in the sixth tryptic peptide of the α-chains.

A number of technical aspects of this investigation are worthy of elaboration. Glutamine was not actually observed on the amino acid chromatograms of peptide Sh-10 because of its conversion to glutamic acid upon acid hydrolysis. The electrophoretic mobility of Hb Sh indicates a net charge difference of + 2 relative to Hb A. This study confirms the observation that the amino acid normally present at residue 54 is neutral glutamine rather than glutamic acid. The ninhydrin stained fingerprint of Hb Sh was grossly normal. The use of the specific stain for arginine greatly facilitated location of the abnormality. Direct measurement of the relative intensity of the peptides of the fingerprints also proved to be a useful procedure for detecting the two overlapping peptides, Sh-10b and A-19. Upon electrophoresis at pH 3.5 peptide Sh-10c was not separated from 20α and 20β, illustrating the value of examining a given protein by more than one fingerprinting condition. Although each of these three adjacent peptides contain glycine, histidine, and lysine, differentiation is possible because peptide Sh-10c distinctly contains valine whereas number

Table 1.—Amino Acid Analysis of αTpVI Peptides of Hb A and Hb Shimonoseki

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>αTpVI (Ingram No. 10)</th>
<th>Shimonoseki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>1.1</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>Asp</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Glu</td>
<td>1.1</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>His</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Gly</td>
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<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Thr</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Ala</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Val</td>
<td>1.2</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>Leu</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Arg</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The molar ratio of the amino acid is given.
Proline is present but not quantitated.
Fig. 6.—Sequences of peptides αTpVI-αTpVIII, and βTpVI of hemoglobin A and of the corresponding peptides 10, 10b, and 10c of hemoglobin Shimonoseki. The ascending arrows designate tryptic cleavage points.

20β contains alanine as a unique constituent. Thus peptide Sh-10c was formed by only partial tryptic cleavage after the lysine molecule of residue 56 of the α-chain (fig. 6). Similar incomplete cleavage of the polypeptide chain between adjacent lysine residues has been shown at positions 60 and 61 of Hb A and Hb Norfolk. This study shows that tryptic digestion is impaired in hemoglobin by basic amino acids up to two units apart. We have not analyzed peptide Sh-10c to determine whether one or two lysine molecules are present at the carboxy-terminus of the peptide.

Peptide Sh-10 had the same mobility as peptide A-10, presumably because the removal of valyllysine (Sh-10b) resulted in a normal net ionic charge on Sh-10. Despite the fact that peptide Sh-10 was two amino acid residues shorter than A-10, its mobility in the chromatographic phase was nearly normal. Thus, a grossly normal fingerprint does not necessarily exclude the possibility of an abnormality in the primary structure of a protein.

SUMMARY

Hemoglobin Shimonoseki, discovered in western Japan in 1960, has been further characterized as a mutant with abnormal α-polypeptide chains on the basis of:

1. The presence of a minor hemoglobin component migrating cathodally at pH 8.6 to Hb A2, presumably αsβ2A2.
2. Hybridization studies.
3. Fingerprinting of isolated α-polypeptide chains.

Hemoglobin Sh is characterized by the substitution of arginine for glutamine at residue 54 and can therefore be designated as α254Argβ2A.

SUMARIO IN INTERLINGUA

Hemoglobina Shimonoseki, discoperite in Japon occidental in 1960, es characterisate additionemente como un mutante con anormal catenas de polypeptida α, a base de:

1. Le presentia de un componente de hemoglobina minor que migra cathodalmente a pH 8.6 verso hemoglobina A2, presumitmente αs8β2A2.
Fig. 7.—(A). Fingerprint of polypeptide chains of Hb A and Hb Shimonoseki isolated by differential precipitation from 8 M urea solution with trichloroacetic acid.²¹ (B). Tracing of the fingerprint of the isolated α- and β-polypeptide chains of Hb A and Hb Sh.
2. Studios de hybridisation.
3. "Dactylographia" de isolate catenas de polypeptida α.

Hemoglobina Shimonoseki es caracterisate per le substitution de arginina pro glutamina a residuo 54 e, per consequente, pote esser designate como Α54АР2 B2.

REFERENCES
19. Fischer, F. G., and Dörfel, H.: Zur Quantitativen Auswertung der Pa-
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