A Novel Silent Posttranslational Mechanism Converts Methionine to Aspartate in Hemoglobin Bristol (β67(E11) Val-Met → Asp)

By D.C. Rees, J. Rochette, C. Schofield, Brian Green, M. Morris, N.E. Parker, H. Sasaki, A. Tanaka, Y. Ohba, and J.B. Clegg

The first reported case of congenital Heinz body hemolytic anaemia was subsequently shown to be caused by an unstable hemoglobin, Hb Bristol [β67(E11) Val-Asp]. This has become one of the classic models of an unstable hemoglobin, the hydrophilic aspartate disrupting the hydrophobic heme pocket. We have restudied this original case, who remains clinically well after nearly 50 years of severe hemolysis with a hemoglobin level of about 7 g/dL and two unrelated Japanese cases. Surprisingly, all three cases show the same DNA changes, predicting a valine to methionine change at β67, rather than the expected aspartate. Further analysis with electrospray ionization mass spectrometry and globin chain biosynthesis strongly suggests that this anomaly is because of a novel posttranslational mechanism, with slow conversion of the translated methionine into an aspartate residue. The proximity of the heme and oxygen may be important in facilitating the reaction. These findings show the importance of complete characterization of variant hemoglobins using protein, DNA, and biosynthetic analyses.

© 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Hematological analysis. Blood was collected into EDTA. Blood counts were performed on an automated cell counter. Blood films, reticulocyte counts, Heinz body preparations, hemoglobin iso-electric focusing, isopropanol, and heat stability tests were performed using standard methods.

DNA analysis. DNA was extracted from whole blood. The β-globin gene was amplified using the polymerase chain reaction (PCR), with previously published conditions and primers. One of the PCR primers in the reaction was 5'-biotinylated, and a single-stranded template isolated using magnetic beads (Dynabeads M-280 Streptavidin; Dynal, UK; Wirral, UK) and a magnetic particle

From the MRC Molecular Haematology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, the Oxford Centre for Molecular Studies, Oxford; VG Organic, Altrincham, Cheshire; the Department of Haematology, Whittington Hospital, London, UK; the Yokohama City University School of Medicine, Kanazawaku, Yokohama 236; the Niigata University School of Medicine, Niigata-machi; and the Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, Ube 755, Japan. Submitted November 22, 1995; accepted February 21, 1996.

D.C.R. is an MRC Training Fellow.

Address reprint requests to D.C. Rees, MRCP, MRC Molecular Haematology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8801-0004$3.00
by acid-acetone precipitation, the globin dissolved in 8 M urea was precipitated with tritiated leucine was followed over one hour, and the separated pre-β-globin peak (X, see Fig 3). Globin was prepared and the chains separated using cation exchange chromatography. Specific activities were calculated following dialysis or anemia.

Globin chain biosynthesis. A time-course incubation of heparinized reticulocytes with tritiated leucine was followed over one hour, and globin prepared and the chains separated using cation exchange chromatography. Specific activities were calculated following dialysis of the peak fractions against 0.5% formic acid.\(^8\)

Mass spectrometry. Whole blood was diluted 1:500 in 50% aqueous acetonitrile containing 0.2% formic acid, before analysis by electrospray ionization mass spectrometry (ESI-MS). Ten microliters was injected into the electrospray ion source of the Quattro mass spectrometer (VG Organic, Altrincham, UK). Three and a half minutes of data were collected from each sample.

RESULTS

Case reports. In 1952, Cathie reported the case of a 5-year-old boy, with a Heinz body hemolytic anaemia, present from birth, and with no obvious precipitating toxic agents.\(^9\) The child was first seen aged 16 months, when he was jaundiced, with a hemoglobin of 7 g/dL, punctate basophilia and 37% reticulocytes. A diagnosis of congenital acholuric jaundice was made and the spleen removed. He received blood transfusions regularly until he was 15, when they were stopped with no adverse effects.

In the late 1960s, he was re-investigated in the light of the discovery of unstable hemoglobin variants.\(^9\) Hemoglobin instability was demonstrated using the heat stability test and the precipitated hemoglobin analysed using fingerprinting of the tryptic digest. An abnormal βT9 peptide was shown, and amino acid sequencing of this suggested that the N-terminal residue 67 of the P-globin chain; this was called hemoglobin Bristol. The introduction of a charged residue into the hydrophobic heme pocket was said to account for the instability.

The patient is now 47 years old and is in good health. His steady state hemoglobin is 7.5 g/dL. He has suffered one hemolytic crisis following food poisoning in 1991 but did not need a transfusion. He had two subarachnoid hemorrhages in his twenties, with no residual deficit. He has valvular heart disease following rheumatic fever age 16. None of his relatives, including parents and five siblings, suffer from hemolysis or anemia.

Table 1. Sequences of PCR Primers Used in Allele-Specific Amplification of Valine (HbA) and Methionine (Hb Bristol) at Codon 67

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>β 14</td>
<td>GAGTCAAGGCTAGAGATGC</td>
<td>Amplifies control region</td>
</tr>
<tr>
<td>β 15</td>
<td>CAATGATATCATGACTTTTG</td>
<td>Amplifies control region</td>
</tr>
<tr>
<td>Codon 67 valine</td>
<td>ATCACTAAAGGCAACCGAGAC</td>
<td>Amplifies normal codon 67 sequence</td>
</tr>
<tr>
<td>Codon 67 methionine</td>
<td>ATCACTAAAGGCAACGAGGAT</td>
<td>Amplifies Hb Bristol codon 67 sequence</td>
</tr>
</tbody>
</table>

Standard beta globin PCR conditions were used\(^9\) with an annealing temperature of 60°C.

Table 2. Clinical Details of Published Cases of Hb Bristol

<table>
<thead>
<tr>
<th>UK</th>
<th>Japanese 1</th>
<th>Japanese 2</th>
<th>Russian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/ys</td>
<td>47</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Hb/gdL</td>
<td>7-9</td>
<td>7-9</td>
<td>5-6</td>
</tr>
<tr>
<td>% Unstable Hb</td>
<td>36</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>MCV/μL</td>
<td>140</td>
<td>111</td>
<td>107.5</td>
</tr>
<tr>
<td>Retic/μl</td>
<td>1.1</td>
<td>1.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Transfusions</td>
<td>Until age 15</td>
<td>Until age 8</td>
<td>4 units/mo</td>
</tr>
<tr>
<td>Spleen</td>
<td>Splenectomy age 2</td>
<td>Splenectomy age 8</td>
<td>Just palpable</td>
</tr>
<tr>
<td>Ferritin/ng/mL</td>
<td>88</td>
<td>Hemosiderosis</td>
<td>5,000, desferrioxamine</td>
</tr>
<tr>
<td>Bilirubin/mg/dL</td>
<td>Jaundice</td>
<td>desferrioxamine</td>
<td>Jaundice</td>
</tr>
<tr>
<td>Height</td>
<td>Normal</td>
<td>Normal</td>
<td>Growth retarded</td>
</tr>
<tr>
<td>Other</td>
<td>Subarachnoid hemorrhage</td>
<td>Gall stones</td>
<td>Developmentally delayed</td>
</tr>
<tr>
<td></td>
<td>rheumatic fever</td>
<td>Gall stones; hepatitis C</td>
<td>thalassemic facies</td>
</tr>
</tbody>
</table>
In 1985, two further cases of hemoglobin Bristol were reported from Japan. Protein analysis was performed in both of these unrelated cases by precipitation of the unstable chain, tryptic digestion, and amino acid analysis. Both showed abnormalities of the βT9 peptide, and the valine to aspartic acid substitution at the N-terminal end, previously found in hemoglobin Bristol. Again routine hemoglobin electrophoresis at pH 8.6 showed no abnormal band.

A fourth case of hemoglobin Bristol has been reported in a 14-year-old Russian boy, again confirmed by protein analysis. The clinical and hematological features seem similar. The hematological data from these cases are summarized in Table 2.

**Hematological analysis.** Blood films from the UK case showed bizarre anisopoikilocytosis with very marked basophilic stippling. Eighty percent of the cells showed Heinz bodies. Isoelectric focusing showed no abnormal bands. Iso-propanol and heat stability tests were positive after less than a minute.

**DNA analysis.** Both DNA strands of the β-globin gene of the UK case of Hb Bristol were sequenced, and showed the heterozygous mutation GTG to ATG at codon 67 (Fig 1). This predicts an amino acid change of valine to methionine, not the expected aspartic acid. This mutation was confirmed by allele-specific amplification using the primers shown in Table 1, which selectively amplified the ATG codon in position 67 of the Hb Bristol case (Fig 2). No other mutations were found in the rest of the β-globin gene. The coding strands of both Japanese cases of Hb Bristol were also sequenced and the same mutation found as in the UK case.

**Globin chain biosynthesis.** After 60 minutes the total counts biosynthesis ratio was α/β = 1.2, suggesting no significant imbalance in the rate of synthesis of the globin chains. It is interesting that although the o.d. 280 nm trace shows a significant peak in the pre-β region, where one would expect a variant β-chain with a valine-aspartate substitution to run, there is no corresponding peak of radioactivity at this point (Fig 3). The time course incubation of the UK case is shown in Fig 4. The rate of increase of specific activity of both α and β chains is nonlinear, asymptotically approaching maximum values. This is the expected pattern with unstable hemoglobin variants.

**Mass spectrometry.** Analysis by ESI-MS of whole globin showed the anticipated peaks corresponding to the calculated masses for normal α chain (15,126 daltons) and normal β chain (15,867 daltons). In addition, two significant peaks in the β region, one 16 daltons heavier and one 32 daltons...
heavier, in the ratio 4:3, respectively, were apparent (Fig 5). These mass increments are identical to those calculated for the aspartate-67 and methionine-67 variants of normal β-globin. Analysis of the pre-β peak (X, Fig 3), which one would expect to consist mostly of aspartate-67 on the basis of its altered charge, showed a main peak of 15,883 daltons, 16 daltons heavier than normal β-globin, as predicted (Fig 6). Additional peaks corresponding to the 8 mol/L urea-mediated carbamoylation of the β-globin are also apparent. The apparent presence of normal β-globin in the pre-P ESI-MS analysis probably results from incomplete separation of the globins.

DISCUSSION

The UK subject with hemoglobin Bristol has survived in good health for nearly 50 years, suffering no obvious problems related to his hemoglobinopathy, despite his persistent severe hemolysis. He has not accumulated excessive iron. The first Japanese case appears to be following a similar course. The second Japanese and Russian cases are more severely affected, with a lower hemoglobin and growth retardation. The reason for this variable phenotype is not clear; splenectomy resulted in marginal increases in hemoglobin in the UK, Japanese 1 and Russian cases, although clinical improvement was said to follow.

The DNA sequencing appears to disagree with the protein analysis; the latter has been performed on three separate occasions and in two different laboratories, making a technical error unlikely. A possible explanation involves the oxidation of the variant methionine residue to methionine sulfone or methionine sulfone.\(^\text{18}\) Both these reactions are thought to occur commonly in proteins and could explain some of the results: the oxidized derivatives could cause the peak seen on cation-exchange chromatography and travel close to aspartate in many amino acid analyzers, thus “impersonating” aspartate. However, depending on the conditions, the prolonged acid hydrolysis used in the amino acid sequencing might be expected to reduce these derivatives back to methionine, making the oxidation undetectable. The mass spectrometry and globin chain synthesis shed further light. ESI-MS shows there are proteins present with masses corresponding to α-chains, normal β-chains, aspartate-67 and methionine-67 β-chains (Fig 5). It should be noted that more than one modification may result in the same or very similar mass changes: for example a modification producing a homoserinyl y-aldehyde, a possible product resulting from methionine oxidation, would result in a β-globin of mass 15,867 daltons, identical to that of normal β-globin. Further relevant examples of modifications and their associated mass changes are given in Table 3. Separation of the globin chains by cation exchange chromatography shows three main protein peaks, corresponding to α chains, normal β chains (and variants with no charge alteration eg, valine to methionine) and β chains with a valine to aspartate (or glutamate) substitu-

![Fig 3. Globin-chain biosynthesis of Hb Bristol. Incubation at 37°C for one hour. The globin chains are separated by cation-exchange chromatography at pH 6.4. The two traces correspond to the amount of protein present (---) and the rate of radioactive incorporation (-----). The peak X precedes the β-globin peak, in a position consistent with a valine to aspartate change.](image)

![Fig 4. Globin-chain biosynthesis time-course incubation. The lines represent the rate of increase of specific activity of α- (*) and β-globin (•) normal and unstable chains over 1 hour. The nonlinear increase implies hemoglobin instability.](image)
tion. ESI-MS shows that the pre-β peak is 16 daltons heavier than the normal (Fig 6), consistent with a valine to aspartate substitution. After 1 hour, there is little or no radioactive incorporation into the 'aspartate' peak, suggesting that this forms via a slow posttranslational modification of the β-chain (Fig 3). Protein analysis has localized the valine to aspartate change to β67 while the DNA analysis finds the valine to methionine substitution in the same position. Hence, protein, DNA, biosynthetic and mass spectrometric analyses suggest a β67 valine to methionine substitution with subsequent slow posttranslational modification of that methionine to aspartate.

A number of examples of posttranslational modification of hemoglobin have been described. Globin translation is initiated with an unblocked methionine residue that is released when the nascent chain reaches about 30 amino acids in length; the N-terminal can then be cotranslationally acetylated, depending on the amino acid. In globin chains, the N-terminal is valine which strongly inhibits the acetylation. However, mutations involving the N-terminal amino acids
can disrupt this process such that the initiator methionine is retained or significant acetylation occurs, or both; there are examples of this for α- and β-chains. 20,21 Another well-documented example involves Hb Coventry (β141[HI9] Leu deleted), first described in 1978 associated with Hb Sydney (β67[E11] Val-Ala). 22 DNA studies on Hb Atlanta-Coventry (β75[E19] Leu-Pro, 141[HI9] Leu deleted) suggested that this was not the case and that the codon for β141 Leu was present; mass-spectrometry suggested that the β141 Leu was posttranslationally modified, possibly to hydroxyelucine, and that this is the probable molecular basis for ‘Hb Coventry’. 23-25  

Reinvestigation of the original case Yon(p81 [EF5] Leu-His), 28 Hb Providence(p82[E6] Lys-leted) showed that this was not the case and that the codon for p141Leu was present; mass-spectrometry suggested that the β141 Leu was posttranslationally modified, possibly to hydroxyelucine, and that this is the probable molecular basis for ‘Hb Coventry’. 23-25  

Interestingly, the α-globin counterpart of Hb Bristol, Hb Evans (α62[E11] Val-Met) has been identified both by DNA and protein analysis at residue 67, the conversion of methionine-67 to aspartate-67 is not a rapid process, and thus the autocalytic role of a ‘normal’ oxy-heme species in the oxidation is questionable.

Although this type of posttranslational modification has not been described before, it is possible that it occurs in other hemoglobin variants in which a methionine is substituted into one of the heme, or near heme, contact points. In variants characterized only by protein analysis, this may have been described as an amino acid being replaced by an aspartate residue at one of the heme contact points, as for hemoglobin Bristol: this group includes Hb J Altgeld Gardens, 30 Hb Yoshizuka, 37 and Hb J-Rovigo. 38 Similarly, identification of variants involving methionine by DNA sequencing alone might miss a posttranslational modification to aspartate. Interestingly, the α-globin counterpart of Hb Bristol, Hb Evans (α62[E11] Val-Met) has been identified both by DNA and protein characterisation with no evidence of posttranslational modification. 39

Five hemoglobin variants have been described at the β67 position, summarized in Table 4. It was noted in the original characterization of Hb Bristol that it is not possible to derive each of the amino acids found in Hb Sydney, Hb M-Milwaukee, and Hb Bristol from a single base change in any single amino acid. One of the published oxides have shown that the oxidation of internal methionines in hydrophobic regions is very slow or nonexistent. 34 The β67 residue is in a hydrophobic region and is one of the heme contact points. Hence the variant methionine is in close proximity to both the iron and the oxygen of oxyhemoglobin. Thus, it is reasonable to suppose that an oxy-heme complex is involved in the conversion of the methionine to an aspartate residue. Minimally, this requires a four electron oxidation of the γ-carbon of methionine. The mechanism by which this oxidation occurs is open to speculation and may be mediated by free radical (dioxygen) or anionic (superoxide or peroxide) species. In theory the oxidation process may result from reaction of 67-methionine (or its sulfoxide(s) or sulfone) with a dioxygen molecule to produce a thioester and a water molecule (Fig 7). Subsequent hydrolysis of the thioester will produce aspartate. Such a process has a precedent in the enzyme catalyzed oxidation of the substrate analogue L-δ-(α-aminoadipoyl)-L-difluorohomocysteinyl-D-valine to L-δ-(α-aminoadipoyl)-L-difluoroaspartyl-D-valine by the non-heme ferrous-dependent oxygenase isopenicillin N synthase. 39 A number of other mechanisms are possible. It is also noteworthy that because it is possible to isolate β-globin with a methionine at residue 67, the conversion of methionine-67 to aspartate-67 is not a rapid process, and thus the autocatalytic role of a ‘normal’ oxy-heme species in the oxidation is questionable.

![Fig 7. Outline scheme for conversion of methionine-67 to aspartate-67. The sulfur may be at sulfide (n = 0), sulfoxide (n = 1) or sulfone (n = 2) oxidation states.](image-url)
variants, Hb Alesha,
identified in a Russian boy with a phenotype similar to Hb Bristol, revealed the change GTG-ATG in codon 67, predicting a valine-methionine substitution: this is the same DNA change present in the three cases of Hb Bristol analyzed by us. Protein sequencing was not performed for Hb Alesha, although iso-electric focusing showed no abnormal bands (as in the UK case when restudied by us). The results of hemoglobin electrophoresis have been variable in all the three published cases, with abnormal bands sometimes being seen. This may be caused by the extent of the post-translational modification of methionine to aspartate, which will depend on the age of the sample, storage conditions etc. Presumably Hb Alesha and Hb Bristol are the same entity, presenting a problem of nomenclature. We propose that β67(E11)Val-Met → Asp be referred to as Hb Bristol-Alesha.

ACKNOWLEDGMENT

The authors would like to thank Prof Sir David Weatherall for advice, Prof Max Perutz for helpful comments, and Dr Irina Udalova for translation of Russian.

REFERENCES


23. Brenner SO, Williamson D, Symmans WA, Carrell RW: Two unstable haemoglobins in one individual: Hb Atlanta (beta 75 LeuPro) and Hb Coventry (beta 141 deleted). Hemoglobin 7:303, 1983

Table 4. Details of Hemoglobin Variants Involving Codon 67 of the β Chain

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA Analysis</th>
<th>Protein Analysis</th>
<th>Posttranslational Modifications</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>GTG</td>
<td>Valine</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>Hb M-Milwaukee</td>
<td>GAG*</td>
<td>Glutamate</td>
<td>None</td>
<td>Methemoglobininaemia</td>
</tr>
<tr>
<td>Hb Sydney</td>
<td>GGC*</td>
<td>Alanine</td>
<td>β 141 leucine hydroxylation</td>
<td>Mild hemolysis</td>
</tr>
<tr>
<td>Hb Manukau</td>
<td>GGG</td>
<td>Glycine*</td>
<td>β 141 leucine hydroxylation</td>
<td>Severe hemolysis</td>
</tr>
<tr>
<td>Hb Bristol</td>
<td>ATG</td>
<td>Aspartate</td>
<td>β 67 methionine to aspartate</td>
<td>Severe hemolysis</td>
</tr>
<tr>
<td>Hb Alesha</td>
<td>ATG</td>
<td>Methionine*</td>
<td>None described</td>
<td>Severe hemolysis</td>
</tr>
</tbody>
</table>

* Information inferred rather than directly measured.


DC Rees, J Rochette, C Schofield, B Green, M Morris, NE Parker, H Sasaki, A Tanaka, Y Ohba and JB Clegg