Two Novel Mutations in the Reduced Nicotinamide Adenine Dinucleotide (NADH)-Cytochrome b5 Reductase Gene of a Patient With Generalized Type, Hereditary Methemoglobinemia

By Jun-ichi Manabe, Roopen Arya, Hideki Sumimoto, Toshitsugu Yubisui, Alastair J. Bellingham, D. Mark Layton, and Yasuyuki Fukumaki

Hereditary methemoglobinemia due to reduced nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R; EC.1.6.2.2.) deficiency is classified into two types, an erythrocyte (type I) and a generalized (type II). We investigated the b5R gene of a patient with type II from a white United Kingdom (UK) family and found that the patient was a compound heterozygote for two novel mutations. The first mutation was a C-to-A transversion changing codon 42 (TAC: Tyr) to a stop codon in the one allele. From this mutant allele, the product without the catalytic portion of the enzyme is generated. The second one was a missense mutation at codon 95 (CCC→CAC) in the other allele with the result that Pro changed to His within the flavin adenine dinucleotide (FAD)-binding domain of the enzyme. To characterize effects of this missense mutation on the enzyme function, we compared glutathione S-transferase (GST)-fused b5R with the GST-fused mutant enzyme with the codon 95 missense mutation (P95H) expressed in Escherichia coli. The mutant enzyme showed less catalytic activity, less thermostability, and a greater susceptibility to trypsin than did the normal counterpart. The absorption spectrum of the mutant enzyme in the visual region differed from that of the wild-type. These results suggest that this amino acid substitution influences both secondary structure and catalytic activity of the enzyme. The compound heterozygosity for the nonsense and the missense mutations apparently caused hereditary methemoglobinemia type II in this patient.

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Heredity methemoglobinemia is an autosomal recessive disorder characterized by a deficiency in reduced nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R; EC.1.6.2.2.). This enzyme is a flavoprotein that catalyzes the reduction of cytochrome b5, using flavine adenine dinucleotide (FAD) as a prosthetic group. Two forms of this enzyme are known, a membrane-bound form in somatic cells and a soluble form in erythrocytes. The former exists mainly on the endoplasmic reticulum of somatic cells and participates in ionization and desaturation of fatty acid, cholesterol biosynthesis, and drug metabolism. The soluble form is located in a soluble fraction of circulating erythrocytes and plays a role in methemoglobin reduction. The membrane-bound form of b5R with 300 amino acid residues consists of membrane-bound and catalytic portions, whereas the soluble form has only the catalytic portion with 275 amino acids. Both forms of b5R are encoded by a single gene on chromosome 22. The soluble form is generated from either proteolytic processing of the membrane-bound form or by a direct translation from an erythroid-specific transcript encoding the NH2-terminal portion of the soluble form, as demonstrated in rats. He-
years old with motor and cognitive skills at about the level of a 3 to 3.5 year old. Growth is between the third and tenth centiles, and she has a convergent squint, mild tremor, and some degree of hypertonia, which is worse in the lower limbs.

PCR and sequencing. High molecular weight DNA was extracted from peripheral blood leukocytes from the patient and her parents. Genomic DNA (100 ng) was amplified by PCR. Use of two primer sets allowed for amplification of 0.3- to 1.2-kb fragments. The primers and the regions of the amplification were as described previously.\(^1\)\(^2\)\(^3\) The standard cycle for PCR was 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute (63°C for amplification of the fragment containing exon 1 region), and extension at 72°C for 1 minute. The reaction mixture for PCR contained 1 U Taq polymerase (Promega, Madison, WI), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 0.2 mmol/L dextroxyribonucleoside 5'-triphosphate (dNTP), 0.2 mmol/L each primer, 5% dimethyl sulfoxide, and 0.1 μg of genomic DNA. After the first denaturing at 94°C for 5 minutes, Taq polymerase and dNTP were added to the tube. For direct sequencing, a 0.2-μL aliquot of the amplified product was used for the second PCR, which contained only one of the two primers. The amplified products were extracted with phenol-chloroform and then precipitated by ethanol and ammonium acetate. These DNA fragments were subjected to direct sequencing using T7 Sequencing kits (Pharmacia, Uppsala, Sweden).

On the other hand, each first PCR fragment was cloned into the T-vector\(^8\) and the nucleotide sequences of the fragments were determined by the method of Sanger et al.\(^8\) Concentrations of the enzymes were determined on the basis of either molecular extinction at 462 nm (10.4 mmol/L\(^{-1}\) cm\(^{-1}\)) or by the method of Bradford.\(^9\)

Characterization of enzyme properties. Assay of enzyme activity was performed with potassium ferricyanide or recombinant human erythrocyte cytochrome b5 expressed in E. coli, as described before. Enzymatic activity was determined by the method of Lowry et al.,\(^10\) using the NADH-ferricyanide reductase assay. The standard cycle for the assay was 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute (63°C for amplification of the fragment containing exon 1 region), and extension at 72°C for 1 minute. The reaction mixture for the assay contained 5 μmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L CaCl\(_2\) containing 5 mmol/L NADH reduced glutathione. To cleave GST, alternative enzyme from glutathione-S-transferase 4B (Pharmacia) equilibrated with buffer A. After washing in buffer A, the elution was performed with buffer A containing 5 mmol/L reduced glutathione. To cleave GST, alternative enzyme from glutathione-S-transferase 4B was performed using buffer B (50 mmol/L Tris-HCl [pH, 8.0] 100 mmol/L NaCl, 1 mmol/L CaCl\(_2\)) containing 5 mmol/L reduced glutathione. The eluted protein was digested with a 3:100 (w/v) ratio of thrombin for 6 hours at 25°C and purified with 5% AMP-Sepharose 4B (Pharmacia). Enzymes eluted with buffer B containing 5 mmol/L NADH were applied to glutathione-Sepharose 4B to remove undigested GST-fused b5R. NADH contained in the sample was removed by dialysis. Purity of the GST-fused b5R was evaluated by electrophoresis on a polyacrylamide gel (12.5%) in the presence of sodium dodecyl sulfate (SDS). Concentrations of the enzymes were determined on the basis of either molecular extinction at 462 nm (10.4 mmol/L\(^{-1}\) cm\(^{-1}\)) or by the method of Bradford.\(^9\)

**Table 1. NADH-Ferricyanide Reductase Activity**

<table>
<thead>
<tr>
<th>MetHb (total Hb (%))</th>
<th>Patient</th>
<th>Father</th>
<th>Mother</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell b5R activity (EU/gHb)</td>
<td>0.9</td>
<td>9.4</td>
<td>10.5</td>
<td>12.2-21.8</td>
</tr>
<tr>
<td>Platelet b5R activity (μmol/min/mg)</td>
<td>0.11</td>
<td>0.51</td>
<td>0.64</td>
<td>0.71-1.09</td>
</tr>
</tbody>
</table>

**Fig 1.** b5R expression system in E. coli. To construct the expression plasmid of the mutant enzyme, we made use of placZ'-APR-b5R\(^\text{wild-type}\) and pGEX-2T, as described in Materials and Methods. The box with slants indicates lacZ'-APR-b5R cDNA, the filled box indicates position of the missense mutation, and the box with dots indicates the GST-coding region.
by Nagai et al. as electron acceptors. NADH-ferricyanide activity was measured at 25°C in 10 mmol/L potassium phosphate (pH 7.5) and NADH-cytochrome b5 activity was assayed at 25°C in 5 mmol/L Tris-HCl (pH 7.5), as described by Yubisui and Takeshita. Km and kcat values of enzymes were determined by 1/v axis intercept replot of the 1/v versus 1/[NADH] date obtained at varied [ferricyanide] or [cytochrome b5], and vice versa. The kcat values were calculated based on a molecular weight of 58 kDa. Spectrophotometric determinations of the enzymes were made using a Hitachi spectrophotometer, model 557 (Tokyo, Japan). Absorption spectra of the GST-fused wild-type and P95H b5Rs were recorded, using a Hitachi 340 spectrophotometer.

RESULTS

Identification of mutations. Base sequences were determined in both directions for all exons and splicing junctions of the gene for NADH-cytochrome b5 reductase (b5R), as described in Materials and Methods. Two novel mutations were found when we compared sequences of the normal b5R gene, one was a transversion, C to A at the third position in exon 42 within exon 2. This mutation leads to substitution of a TAC codon (Tyr) by an ochre termination codon [Nonsense mutation is considered to have no enzymatic activity].

Preparation of mutant b5R. b5R from the allele with the nonsense mutation is considered to have no enzymatic activity because the product consists of only the 42 amino acids without the catalytic portion. To examine the effect of the nonsense mutation on the biochemical properties of b5R, the substitution was introduced into the normal b5R cDNA by PCR primer-directed mutagenesis and was ligated into the GST fusion gene expression vector (Fig 1). GST-fused wild-type b5R and GST-fused b5R with the substitution. GST-fused P95H b5R, expressed in E. coli were purified, as described in Materials and Methods. Yield of both enzymes during purification was about 50%, based on the ferricyanide reductase activity. Purity of both b5Rs was analyzed by electrophoresis on a polyacrylamide gel in the presence of SDS. A single band with a molecular mass of 58 kDa was obtained (Fig 4).

Kinetic properties. The Km values of GST-fused wild-type b5R for cytochrome b5 and NADH were 19 μmol/L and 0.50 μmol/L, respectively. After removing GST from the recombinant enzyme, wild-type b5R showed Km values for cytochrome b5 and NADH 9.5 μmol/L and 0.5 μmol/L, respectively. Using ferricyanide as the electron acceptor, the kinetic properties of GST-fused wild-type b5R were determined and are shown in Table 2. The kinetic properties of the GST-fused wild-type b5R are essentially the same as those of native b5R32 recombinant b5R33 and b5R, prepared by removing GST from GST-fused b5R. Thus, GST seems to have little effect on the kinetic properties of b5R. The cleavage process of GST from recombinant enzymes led to deterioration of the functional properties of the mutant because of reduced intrinsic stability of the enzyme. Thus, the experiments below were performed using b5Rs with GST.

Heat stability and protease susceptibility of the mutant. When the diluted GST-fused b5Rs (10 μmol/L) were incubated for various times at 37°C and 42°C, distinct differences in heat stability of the wild-type and mutant b5Rs were observed (Fig 5). Using ferricyanide as an electron acceptor, residual activities of the mutant after 10 minutes at 37°C and 42°C were about 60% and 20% of the initial activity, respectively. In contrast, the wild-type retained more than 80% of the initial activity, under the same conditions. After 60 minutes incubation at 37°C and 42°C, activities of the mutant were unchanged, whereas those of the mutant were about 40% and <5% of the initial activity, respectively. Trypsin was used to probe the protease sensitivity of the mutant. The wild-type was resistant to this treatment, whereas the mutant indicated only 30% of the initial activity after incubation for 30 minutes at 25°C with 3 μmol/L trypsin (3 μmol/L enzyme in 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 0.1 mmol/L DTT).

Spectral properties. The absorbance spectrum of GST-fused wild-type b5R derived from the enzyme-bound FAD showed the same findings as those for the native b5R32 and recombinant b5R,24 (Fig 6). The absorbance of recombinant
NOVEL MUTATIONS IN HEREDITARY METHEMOGLOBINEMIA

Fig 2. Identification of the nonsense mutation in exon 2 of the patient and her father. (A) PCR fragments containing exon 2 of the patient were cloned into the T-vector, as described in Materials and Methods. The blank letter indicates position of the substitution. (B) PCR-amplified exon-2 DNAs from the patient, father, mother, and a normal subject were digested with Rsa I and fractionated on agarose (4%) electrophoresis. Marker indicates 4x174 DNA digested with Hae III. The Rsa I map within the 290-bp fragment is shown below (N, the allele without the nonsense mutation; M, the mutant allele).

P95H b5R at the 390 nm peak was almost the same as that at the 460 nm peak, whereas the absorbance of the wild-type was significantly higher at 390 nm than at 460 nm (Fig 6). The absorbance ratio of 390 to 460 nm of the mutant enzyme, 1.04, was lower than that of the wild-type, 1.17. These differences in absorption spectra suggested that conformational changes of the FAD binding domain occurred in this mutant b5R.

DISCUSSION
To date, 10 mutations in the b5R gene have been detected in unrelated patients with hereditary methemoglobinemia; three missense mutations (Arg-57 → Gln, Val-105 → Met, and Leu-148 → Pro) have been found in type I patients, and two missense mutations (Ser-127 → Pro and Cys-203 → Arg), one nonsense mutation (Arg-218 → stop), two deletion mutations (Phe-298, del 3-bp and Met-272, del 3-bp), and two splicing mutations (5’ splice site of exon 5 and 3’ splice site of exon 9) have been found in type II patients.18,24,32,33 The mutations in type I were located in the nonfunctional domain of the enzyme, which seems relevant only to the maintenance of the structure of the enzyme, then type II mutations were identified near the catalytic center of the enzyme. Functional
studies of mutant b5Rs show that instability and increased proteolytic susceptibility of enzymes with relatively high catalytic activities may cause hereditary methemoglobinemia type I, and the loss of catalytic activities of enzymes may lead to a type I1 phenotype. In the present study, we detected two novel mutations in the b5R genes of a white girl in the UK. The first mutation was a replacement for a TAC codon (Tyr) with a TAA codon (Stop codon) at codon 42 in the one allele. As this nonsense mutation results in a truncated b5R, which contains neither FAD- nor NADH-binding domains, this mutation belongs to a group of type II mutations. The second one was a missense mutation in the other allele that causes amino acid change from Pro to His at codon 95. b5R is now considered to be a member of the flavoprotein family of dinucleotide-dependent flavo enzymes, on the basis of homology of the primary structures of the related enzymes. The amino acids corresponding to Pro-95 of b5R are hydrophobic in all
Fig 4. Gel electrophoresis of GST-fused b5Rs expressed in E. coli. A total of 2 μg of GST-fused wild-type b5R and the mutant counterpart purified with glutathione-Sepharose 4B were subjected to electrophoresis on SDS-polyacrylamide gel (12.5%). Molecular weight markers used were rabbit muscle phospholipase B (97 kD), bovine serum albumin (66 kD), egg white ovalbumin (45 kD), and bovine carbonic anhydrase (31 kD).

Table 2. Kinetic Properties of the P95H Mutant

<table>
<thead>
<tr>
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<th>GST-Wild Type b5R</th>
<th>GST-P95H b5R</th>
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<tbody>
<tr>
<td>Ferricyanide as electron acceptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km (μmol/L) for NADH</td>
<td>6.7</td>
<td>6.9</td>
</tr>
<tr>
<td>kcat (S⁻¹)</td>
<td>1,337</td>
<td>745</td>
</tr>
<tr>
<td>kcat/Km (NADH)</td>
<td>1.9 x 10⁶</td>
<td>1.1 x 10⁷</td>
</tr>
<tr>
<td>Cytochrome b5 as electron acceptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km (μmol/L) for NADH</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Km (μmol/L) for cyto. b5</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>kcat (S⁻¹)</td>
<td>497</td>
<td>295</td>
</tr>
<tr>
<td>kcat/Km (NADH) (S⁻¹M⁻¹)</td>
<td>99.6 x 10⁷</td>
<td>56.8 x 10⁷</td>
</tr>
<tr>
<td>kcat/Km (cyto. b5) (S⁻¹M⁻¹)</td>
<td>2.61 x 10⁷</td>
<td>1.48 x 10⁷</td>
</tr>
</tbody>
</table>

of Arg-91 and Try-93 of human b5R in the fourth strand may be hydrogen bonded to maintain the structure of ribitol and phosphate group of flavin.34–36 These results indicate that there are functionally critical residues involved in FAD binding around Pro-95 of human b5R.

To characterize the effect of the amino acid substitution at residue 95, the GST-fused mutant enzyme, P95H b5R, was prepared, using a bacterial expression system. The Km values of P95H b5R for cytochrome b5 and NADH were 20 μmol/L and 0.52 μmol/L, respectively, values essentially the same as those of the wild type, whereas the kcat value of the mutant b5R was approximately half the value of the wild-type. Remarkable differences between the wild-type and mutant b5Rs were observed concerning stability. The mutant was less stable than the wild-type in studies on thermostability and susceptibility to trypsin. In addition, there was a difference in ratios of the absorption spectrum at 390 to 460 nm derived from the FAD chromophore. Thus, replacement members of the flavoprotein family.34,36–39 Pro-95 is conserved among b5R from sheep, rats, mice, and humans.40 The crystal structure of b5R from pig liver microsomes indicated that Pro-67, which corresponds to Pro-95 of human b5R, is located between the fourth and fifth strands in the FAD-binding domain. Several residues in the fifth β-strand and in the subsequent loop region are hydrogen bonded to the flavin molecule, mostly by main chain atoms. Residues

Fig 5. Heat stability of the wild-type and mutant b5Rs. b5Rs were diluted to 10 μmol/L with 50 mmol/L Tris-HCl (pH, 8.0) containing 1 mmol/L EDTA and 0.1 mmol/L DTT. Samples (100 μL) were incubated for various times at 37°C (○, the wild-type; □, the mutant) or 42°C (●, the wild-type; ●, the mutant) and then diluted 10-fold with ice-cold buffer and assayed for activity using ferricyanide as an electron acceptor at 25°C.
the kinetic properties of the mutant enzyme, this substitution probably belongs to type I mutations. However, characteristics of the type II mutations also need attention because the spectrum of the mutant enzyme in the visual region differed from that of the normal counterpart. Therefore, this missense mutation could not be classified into either type I or type II mutations. Compound heterozygosity for this unique mutation and the typical type II mutation apparently led to a type II phenotype in our patient.

ACKNOWLEDGMENT
We are grateful to H. Furuumi for the synthesis of oligonucleotides and to M. Ohara for comments on the manuscript.

REFERENCES

Fig 6. Absorption spectra of the wild-type and mutant b5Rs. Absorption spectra were measured in 50 mmol/L Tris-HCl (pH, 8.0) containing 1 mmol/L EDTA and 0.1 mmol/L DTT.
Two novel mutations in the reduced nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase gene of a patient with generalized type, hereditary methemoglobinemia

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