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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HEREDITARY 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 in the endoplasmic reticulum of somatic cells and participates in elongation 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.

Molecular mechanisms of hereditary methemoglobinemia caused by b5R deficiency has been classified into two types, an erythrocyte type (type I) and a generalized type (type II). In type I, the deficiency of b5R is restricted to erythrocytes of patients with mild cyanosis. On the other hand, type II is characterized by this enzyme deficiency in various tissues of patients manifesting cyanosis and mental retardation. We previously identified three different missense mutations in genes of patients with type I and one missense and one deletion mutation in the genes of type II patients. Characterization of the respective recombinant mutant enzymes indicated that the enzyme resulting in type I had 60% to 70% of the activity of normal and showed heat labilities, findings that suggested restriction of this enzyme deficiency to erythrocytes is caused by degradation of an unstable mutant enzyme in patients' erythrocytes because of the lack of compensation by new protein synthesis during the long life span of erythrocytes. The recombinant type II enzymes had lower Km values compared with the type I mutant, therefore leading to generalized type of the enzyme deficiency in a patient.

To examine the heterogeneity of the molecular basis of hereditary methemoglobinemia and the relation between properties of mutant enzymes and pathogenesis of types I and II phenotypes, we analyzed the b5R gene of a patient with type II, using polymerase chain reaction (PCR)-related techniques. We found a novel nonsense and missense mutation in the b5R gene of this patient. Molecular mechanisms of hereditary methemoglobinemia type II are discussed in terms of biochemical properties of the recombinant mutant enzyme.

MATERIALS AND METHODS

Patient. A white, United Kingdom (UK) girl had cyanosis and neurodevelopmental delay from early infancy. Her parents are Northern Europeans and nonconsanguineous. The diagnosis was confirmed as hereditary methemoglobinemia type II on the basis of clinical symptoms and b5R activities of fractionated blood cells, as shown in Table 1. b5R activities were extremely low in both red blood cells and platelets of the patient, yet these activities were only slightly decreased in her healthy parents. In 1996, the patient is 6...
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.18,19 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 73°C for 1 minute. The reaction mixture for PCR contained 1 U Taq polymerase (Promega, Madison, WI), 10 mM/L Tris-HCI (pH, 8.3), 50 mM/L KCl, 1.5 mM/L MgCl₂, 0.2 mM/L dNTP, 0.2 µM/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-vector20 and the nucleotide sequences of the fragments were determined as described above to confirm the results.

Restriction enzyme analysis of the amplified DNA. As the nonsense mutation abolishes the Rsu I (5'GT/AC3') site, amplified fragments containing exon 2 were purified and digested with Rsu I. To confirm the nonsense mutation, nested PCR was performed with a set of internal primers using PCR fragments containing exons 3 and 4. The downstream primer, (CCTGTGACTCATGCTGTCAGACATG), was the mismatch primer with replacement of G for C at the fourth nucleotide from the 3' terminal. The sequence of the upstream primer was (AGATGATCCTGCCAGGATGTA). The nested PCR fragments were purified and digested with Nsp I (5'ACATGCT/G3'). The digested fragments were electrophoresed on a gel containing both 2% agarose and 2% NuSieve (FMC, Rockland, ME). The gels were stained with ethidium bromide.

Construction of the expression plasmid. To generate cDNA for the mutant enzyme with the codon 95 nonsense mutation, PSH b5R, the plasmid containing the cDNA for normal b5R, placZ'-APR-b5R26 was mutagenized by the method of PCR site-directed mutagenesis,27 using two sets of primers (sense [CCTATACACACATCTCCAGCATGA]; antisense [TCATCGCTGGAGATCTGTATAGG]). The wild-type and mutant b5R cDNA fragments were ligated into the glutathione S-transferase (GST) gene fusion vector, pGEX-2T (Pharmacia), as shown in Fig 1.

Purification of wild-type and mutant b5Rs. Escherichia coli JM109 harboring expression plasmids was incubated overnight at 37°C in 5 mL L-Broth medium containing 100 µg/mL ampicillin. The culture was inoculated into 250 mL sterile broth (TB) medium and incubated at 37°C. After incubation for 2 hours, isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 mM/L) was added to the medium and incubation was continued for 4 hours. The cells were collected by centrifugation (8,000 g for 15 minutes), resuspended in buffer A (50 mM/L Tris-HCl [pH, 8.0], 1 mM/L EDTA, 0.1 mM/L dithiothreitol [DTT]), and sonicated five times with Branson Sonifier Model 200 (Danbury, CT). After centrifugation (20,000 g for 20 minutes), the supernatant was applied to glutathione-Sepharose 4B (Pharmacia) equilibrated with buffer A. After washing in buffer A, the elution was performed with buffer B containing 5 mM/L reduced glutathione. To cleave GST, alternative elution from glutathione-Sepharose 4B was performed using buffer B (50 mM/L Tris-HCl [pH, 8.0] 100 mM/L NaCl, 1 mM/L CaCl₂) containing 5 mM/L reduced glutathione. The eluted protein was digested with a 3:100 (wt/wt) ratio of thrombin for 6 hours at 25°C and purified with 5'-AMP-Sepharose 4B (Pharmacia). Enzymes eluted with buffer B containing 5 mM/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 b5Rs 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 mM/L cm⁻¹) or by the method of Bradford.29

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
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, as one was a transversion, C to A at the third position in codon 42 within exon 2. This mutation leads to substitution of the TAC codon (Tyr) by an ochre termination codon (UAA). The Km 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 Km 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.

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 b5R, recombinant b5R39 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. To characterize the enzyme with the missense mutation, the kinetic properties of GST-b5Rs were examined. b5R activity of P95H b5R, determined under standard assay conditions, was lower than that of the wild-type enzyme. The kinetic properties of the wild-type and P95H b5Rs are summarized in Table 2. There was a difference between the wild-type and mutant b5Rs in Km values, but not in Kcat values. The Kcat value of the mutant b5R using cytochrome b5R as an electron acceptor was 295.6 S⁻¹M⁻¹, which is about 60% of the values of the wild-type. The values of Kcat/Km (NADH) and Kcat/Km (cytochrome b5) for the mutant b5R were 56.8% and 56.7% of those of the wild-type, respectively. The kinetic properties of the muta b5R were similar to those of the wild-type, except that the Kcat value of the mutant was lower than that of the wild-type, indicating that the Pro to His substitution in residue 95 does not affect the affinity for NADH and cytochrome b5, but does cause a significant reduction in the catalytic activity of b5R.

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 wild-type 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 absorption spectrum of GST-fused wild-type b5R derived from the enzyme-bound FAD showed the same findings as those for the native b5R and recombinant b5R,24 (Fig 6). The absorbance of recombinant b5R showed the same findings as those for the native b5R and recombinant b5R,24 (Fig 6).
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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. 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 II phenotype.\textsuperscript{19,21,23,24}

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,\textsuperscript{23} 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.\textsuperscript{24-26} The amino acids corresponding to Pro-95 of b5R are hydrophobic in all
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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
NADH-Cytochrome b5 Reductase

<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>
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<td>6.9</td>
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<tr>
<td>kcat (S⁻¹)</td>
<td>1,337</td>
<td>745</td>
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<tr>
<td>kcat/Km [NADH] (S⁻¹M⁻¹)</td>
<td>1.9 × 10⁶</td>
<td>1.1 × 10⁶</td>
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<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>
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<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 × 10⁷</td>
<td>56.8 × 10⁷</td>
</tr>
<tr>
<td>kcat/Km [cyto. b5] (S⁻¹M⁻¹)</td>
<td>2.61 × 10⁷</td>
<td>1.48 × 10⁷</td>
</tr>
</tbody>
</table>

members of the flavoprotein family. Pro-95 is conserved among b5R from sheep, rats, mice, and humans. 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 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. 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

![Fig 5. Heat stability of the wild-type and mutant b5Rs.](image-url)
of a hydrophobic amino acid with a basic amino acid in residue 95 seems to lead to disruption of the structure of the FAD-binding domain. Pro-95 apparently participates in formation and/or stabilization of the hydrophobic core of the β-barrel structure in the FAD binding domain. According to the kinetic properties of the mutant enzyme, this substitution probably belongs to type I1 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.

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REFERENCES


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