A novel mutation of the cytochrome-b5 reductase gene in an Indian patient: the molecular basis of type I methemoglobinemia

We report here a novel mutation in the cytochrome b5 reductase gene resulting in type I methemoglobinemia. A single T→C transition in exon 8 at position 25985 was identified, changing codon 217 from Leu to Pro (L217P). The mutation is located in the NADH binding domain at the base of α-helix Nα3, a region of sequence highly conserved from yeast to man. A quantitative assessment of the thermodynamic cost of this mutation at 37°C revealed a ten-fold drop in the free energy of stability. Alterations in hydrogen bonding and solvent accessibility surrounding residue 217 were predicted based on computer modeling.

Key words: congenital methemoglobinemia, cytochrome b5 reductase deficiency, thermostability, protein structure modeling, thermodynamics.

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Congenital methemoglobinemia due to a deficiency of NADH-cytochrome b5 reductase (cb5r; EC 1.6.2.2) is an autosomal recessive disorder (OMIM 250800). The gene for cb5r (DIA1) is localized to chromosome 22q13-qter covering 31kb in length with nine exons and eight introns. Two major clinical forms of recessive congenital methemoglobinemia (RCM) due to cb5r deficiency are recognized. In the type I form, the enzyme is deficient only in erythrocytes, leading to clinical cyanosis from birth and a normal life expectancy. A type II form is due to cb5r deficiency in all tissues and is characterized by cyanosis and early death due to progressive neurological and cognitive impairment, microcephaly and failure to thrive.

We studied the molecular consequences of a novel mutation in the cb5r gene, DIA1, in an Indian patient with type I RCM. We established the causative mutation of this patient’s type I RCM, as well as thermodynamic parameters using native enzyme. Using the recently released human cb5r crystal structure (1UMK) we studied the structural consequences of the mutation by computer modeling. From these investigations we propose a model that explains type I RCM as being due to an unstable enzyme.

Design and Methods

Blood samples

Informed consent was obtained from all subjects. Peripheral blood was collected from the patient and first degree relatives and different cell fractions separated using standard methods. Lymphoid cell lines were established and cultured as previously described.

Methemoglobin analysis

Methemoglobin and sulfhemoglobin levels were determined. The absence of Hb-M was verified by isoelectric focusing and high performance liquid chromatography.

Preparation of detergent-treated lymphoid cell lines

Cultured lymphoid cell line extracts were made by homogenizing approximately 5×10^6 cells in 0.5mL (10mM Tris-Cl pH8.0, 1mM EDTA, 1% Triton X-100, 0.1mM PMSF), using a sonicator (Misonix Inc., USA). Cells were maintained on ice for 30 min and subsequently centrifuged at 16,000 g for 20 min at 4°C. The supernatant was decanted for use in all assays. The protein concentration was determined by the BCA method (Pierce Biochemicals, USA) and adjusted to 2 mg/mL immediately prior to enzyme assays.

Enzyme assays

NADH-ferricyanide reductase (NADH:FR) activity was measured by following oxidation of NADH at 340nm on a Synergy-HT microplate reader for 10 min at 30°C (BioTek, USA). Reaction rates were determined by linear regression of the linear portion of the change in optical density, sampled at 1 min intervals. Typical reactions (100 µL) consisted of cell extract, 200 µM potassium ferriyanide (K-Fe(CN)₆), 10mM Tris-Cl pH 8.0, 0.5 mM EDTA and 0.2mM NADH.
Polymerase chain reaction (PCR) and DNA sequencing

Genomic DNA from each family member was extracted from whole blood using the Puregene DNA purification kit (Gentra, USA), and PCR amplified. PCR products were purified and sequenced. Both parents and the propositus’ sister were genotyped as well.

Sequence analysis and alignments

Multiple sequence alignment was performed using the Clustal X v1.81 program and manually edited to improve the alignment with the BioEdit v.7.0.5.2 program. The GI numbers are: human GI:56554196; macaque GI:62510917; rat GI:17943936; frog GI:28279135; fruitfly GI:24662934; corn GI:4336205; yeast GI:6322146.

Thermal stability measurements and thermodynamic calculations

Thermal stability profiles for normal and mutant proteins were determined by measuring the loss of NADH:FR activity at different temperatures. Lymphoid cell line extracts, at 2 mg/mL, from two controls (1 and 2), the propositus and his heterozygous father were pre-incubated at increasing temperatures from 16°C to 50°C for either 10 or 30 min in a PTC-200 thermal cycler (MJ Research, USA) and placed on ice. Residual NADH:FR activity was determined as described above. Thermodynamic parameters were calculated by rearranging the equations for the calculation of Gibbs free energy and the van’t Hoff equation. Briefly:

\[ \Delta G = \Delta H - T \Delta S \]  

(1)

and

\[ \Delta G = -RT \ln K_A \]  

(2)

Rearranging 1 and 2:

\[ \ln K_A = - \frac{\Delta H}{RT} + \frac{\Delta S}{R} \]  

(3)

An Arrhenius plot of \( \ln K_A \) vs. \( 1/T \) yields a straight line with slope=\(-\Delta H/R\). At \( T_m \), \( K_A=1 \) and \( \Delta G=0 \), therefore \( \Delta S=\Delta H/T_m \).

Model of cb5r mutant structure

A model of the structure of the mutant protein was built by replacing Leu 217 with Pro in human cb5r (PDB: 1UMK) and subjected to energy minimization.

Results and Discussion

Laboratory parameters of methemoglobinemia

The propositus, a 22-year old, is the son of a consanguineous marriage in the state of Andhra Pradesh, India. This family immigrated to the United States in 1995. The propositus had a methemoglobin level of 16% (normal values between 0-1%) while sulfhemoglobin levels were undetectable (often increased in toxic acquired methemoglobinemia). Erythrocyte cb5r activity was low and estimated at 3.94 IU/g Hb. The propositus’ mother and sister displayed intermediate levels (14.29 and 22.18 IU/g Hb) while the father’s erythrocyte cb5r activity was found to be in the normal range at 35.27 IU/g Hb.

Mutation: Identification and characterization

Sequencing of the DIA1 gene revealed a novel T→C transition at position 25985 in exon 8 (Figure 1 A-C). This mutation results in the replacement of an evolutionarily conserved leucine at position 217 to proline (Figure 1D). The propositus was found to be homozygous for this mutation, while both his parents and sister were heterozygous.
Kinetic properties of wild type and mutant enzymes

Initial rate kinetic analysis of NADH:FR activity in lymphoid cell lines extract from the patient and his heterozygote father was performed. The $K_m$ for each substrate was estimated and results did not show a significant difference between mutant and wild type enzymes, there being, at most, a two-fold increase in $K_m$ for the mutant enzyme (Table 1).

Thermal stability

The mutant enzyme was more sensitive than the wild type enzyme to thermal denaturation (Figure 2A). After 30 min of pre-incubation at 40°C, the patient's sample had a residual enzymatic activity of 37%; in contrast control samples retained >85% enzymatic activity (Figure 2A). Interestingly, the patient’s heterozygous father displayed an intermediate level of thermal instability in spite of normal levels of enzyme activity and methemoglobin in his blood (Figure 2A). We cannot explain this discrepancy between enzyme instability, normal enzyme activity and normal methemoglobin levels in this individual. The values of $T_m$ (50% decrease in NADH:FR activity) for both controls, the heterozygous father and the propositus are shown in Table 1.

Thermodynamic comparison of wild type and mutant enzymes

Changes in Gibbs stability free energy ($\Delta G_{\text{stab}}$), as a function of temperature, was calculated and plotted for the two controls, the patient and his heterozygous father (Figure 2C and Table 1). The slopes of the linear regression were obtained by fitting the data from the 30 min thermostability curve (Figure 2A) to equation 3 and

Table 1. Enzyme characterization.

<table>
<thead>
<tr>
<th>Sample (genotype)</th>
<th>NADH $K_m$ (µM)</th>
<th>NADH:FR $K_m$ (µM)</th>
<th>Slope (lnK vs. 1/T)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (kJ/mol)</th>
<th>$\Delta G_{\text{stab}}$ (37°C) (kJ/mol)</th>
<th>$T_m$ (°C)</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>1 (T/T)</td>
<td>19</td>
<td>42</td>
<td>37340</td>
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<td>-979.5</td>
<td>-6.96</td>
<td>44.1</td>
<td>0.966</td>
</tr>
<tr>
<td>2 (T/T)</td>
<td>24</td>
<td>46</td>
<td>37980</td>
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<td>-996.3</td>
<td>-7.01</td>
<td>44.0</td>
<td>0.987</td>
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<tr>
<td>I.1 (T/C)</td>
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<td>38</td>
<td>24500</td>
<td>-203.7</td>
<td>-647.4</td>
<td>-3.12</td>
<td>41.8</td>
<td>0.996</td>
</tr>
<tr>
<td>II.1 (C/C)</td>
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<td>46</td>
<td>17340</td>
<td>-144.2</td>
<td>-463.4</td>
<td>-0.64</td>
<td>38.4</td>
<td>0.989</td>
</tr>
</tbody>
</table>

*Sample 1 and 2 represent two different controls, I.1 represents the heterozygous father and II.1 represents the propositus. The respective genotypes for each sample are shown between parentheses; $K_m$ values are given in µmoles/L substrate; $R^2$ is the fitting coefficient from the linear regression of the plot lnK vs. $1/T$. 

Figure 2 (right). Thermostability and thermodynamics of wild-type and mutant cb5r. Temperature stability and thermodynamic characteristics were investigated in two different controls (■ and ▲), the heterozygous father (●) and propositus (△). A. Relative activity of NADH:FR after 30 minute pre-incubation at the temperatures indicated. B. Arrhenius plot used to calculate the values for $\Delta H$ and $\Delta S$ as described in methods. C. Plot of estimated and experimentally determined $\Delta G$ values as a function of temperature. The inset shows the $T_m$ values for different samples estimated by the intercept with the x-axis when $\Delta G=0$. 

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plotting $lnK_A$ vs. $1/T$ (Figure 2B, Table 1). $\Delta H$ and $\Delta S$ for the patient were two-fold greater than those found for the controls, whereas the father displayed intermediate values reflecting his heterozygous status (Table 1). The overall free energy of stability ($\Delta G_{\text{total}}$) was calculated at three different temperatures. The ten-fold higher $\Delta G$ at 37°C indicates the high degree of instability of this enzyme (Table 1). Instability of the patient’s enzyme can be explained by increases in both the enthalpic and entropic contributions. An increase in $\Delta H$ indicates weaker internal interactions, while the change observed in $\Delta S$ points to a larger contribution of conformational entropy versus hydrophobic entropy. Estimated values for $\Delta G$ were calculated and plotted as $\Delta G$ vs $T$ (Figure 2C) further demonstrating the father’s heterozygosity despite his normal level of blood enzyme.

**Structural studies**

A model of the $L217P$ mutation was generated by replacing the wild type residue with the mutant one in the human cb5r structure (1UMK). Leucine 217 is located within the NADH binding lobe, at the beginning of helix N-3, and is enclosed in a hydrophobic pocket. Substitution of $L217P$ in the human cb5r structure (1UMK) is characterized by steric hindrance and contacts between the proline side chain and Cα-backbone. In our mutant model, after energy minimization, steric hindrance of the proline residue was no longer observed and main chain deviations were minimal. Leucine 217 is highly conserved throughout evolution, from yeast to man, suggesting that it must play an important structural role (Figure 1D). Indeed, two other mutations, $E212K$ and $L215T$, located in the same region as $L217P$, were identified in type I RCM with cb5r deficiency.11,12 Our studies predict the presence of two pairs of stabilizing hydrogen bond partners (R191 – E220 and E212 – R218) for both the wild type and mutant structures. A decrease in solvent accessibility for residue E220 was observed in our model, indicating that this residue is buried deeper in the hydrophobic pocket. According to the model, a slightly wider hydrophobic cavity is predicted to form in the mutant enzyme, leading to increased entry of water molecules and disruption of conserved hydrogen bonds.13 The results described here further enhance our understanding of type I RCM and restriction of cb5r deficiency to red blood cells. This is the first report of a quantitative evaluation of the destabilizing consequences of mutations in cb5r using thermodynamic parameters.

**References**