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

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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.

Haematologica 2006; 91:1542-1545

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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.[1,2] 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.[3] 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.[4]

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)[5] 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.[6] Lymphoid cell lines were established and cultured as previously described.[7]

Methemoglobin analysis

Methemoglobin and sulfhemoglobin levels were determined.[8] The absence of Hb-M was verified by isoelectric focusing and high performance liquid chromatography.[9]

Preparation of detergent-treated lymphoid cell lines

Cultured lymphoid cell line extracts were made by homogeneizing approximately 5×10^7 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 (Bio-Tek, 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 ferricyanide ($K_3Fe(CN)_6$), 10mM Tris-Cl pH 8.0, 0.5 mM EDTA and 0.2mM NADH.

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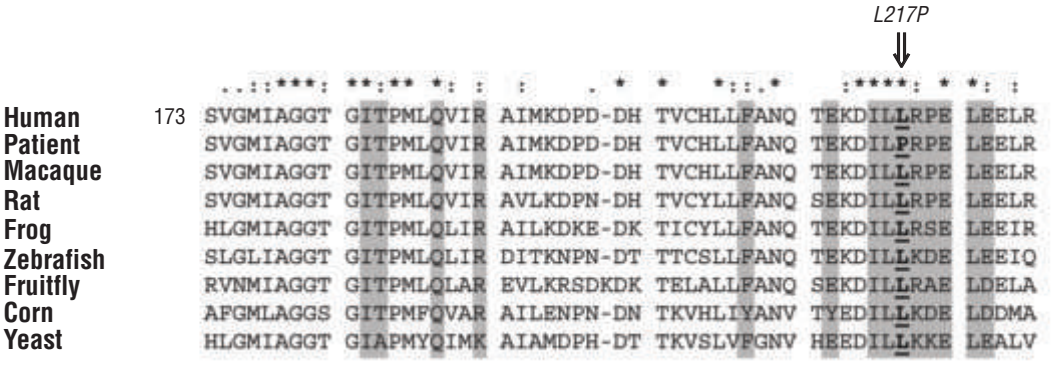

Figure 1. Multiple sequence alignment of cb5r. An acquired mutation in the 8th exon of cb5r that leads to methemoglobinemia in an Indian patient has been identified. A single T-to-C transition at nucleotide of 25985 of the *DIA1* gene was found and leads to a *L217P* substitution. Multiple sequence alignment of the patient DIA1 gene and different species was performed as described in materials and methods, numbering refers to amino acid positions in the human soluble (erythrocyte specific) form. The line of symbols at the top of each alignment indicate 100% identity (*), high physical chemical conservation (:) and, less physical chemical conservation (.). The arrow points to the novel *L217P* mutation described here, corresponding amino acids are in bold character and underlined. The shaded background highlights those residues that are part of the hydrophobic pocket as determined by analysis of the human cb5r structure (1UMK) and are described in the text.

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.[10] 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[11] and manually edited to improve the alignment with the BioEdit v.7.0.5.2 program.[12] The GI numbers are: human GI:56554196; macaque GI:62510917; rat GI:17943396; frog GI:28278121; zebrafish 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

$\Delta G = -RT\ lnKA$ (2)

Rearranging 1 and 2:

$lnK_A = -\Delta H/RT + \Delta S/R$ (3)

An Arrhenius plot of lnK_A vs. $1/T$ yields a straight line with slope=$-\Delta HR$. At Tm, K_A=1 and ΔG=0, therefore $\Delta S=\Delta H/Tm$.

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.

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Table 1. Enzyme characterization.

[a]Sample (genotype)	NADH $^{b}K_m$	NADH:FR $K3Fe(CN)_6$ $^{b}K_m$	Slope (lnK_A vs. 1/T)	Thermodynamic coefficients ΔH (kJ/mol)	ΔS (kJ/mol . K^{-1})	ΔG_{STAB} (37°C) (kJ/mol)	Tm (°C)	$^{c}R^2$
1 (T/T)	19	42	37340	-310.4	-979.5	-6.96	44.1	0.966
2 (T/T)	24	46	37980	-315.8	-996.3	-7.01	44.0	0.987
I.1 (T/C)	28	38	24500	-203.7	-647.4	-3.12	41.8	0.996
II.1 (C/C)	42	46	17340	-144.2	-463.4	-0.64	38.4	0.989

[a]*Samples 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;* [b]K_m *values are given in µmoles/L substrate;* [c]R^2 *is the fitting coefficient from the linear regression of the plot* lnK_A *vs. 1/T.*

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 *Km* 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 *Km* 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 (ΔG_{stab}), as a function of temperature, was calculated and plotted for the two controls, the patient and his heterozygous father (Figue 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

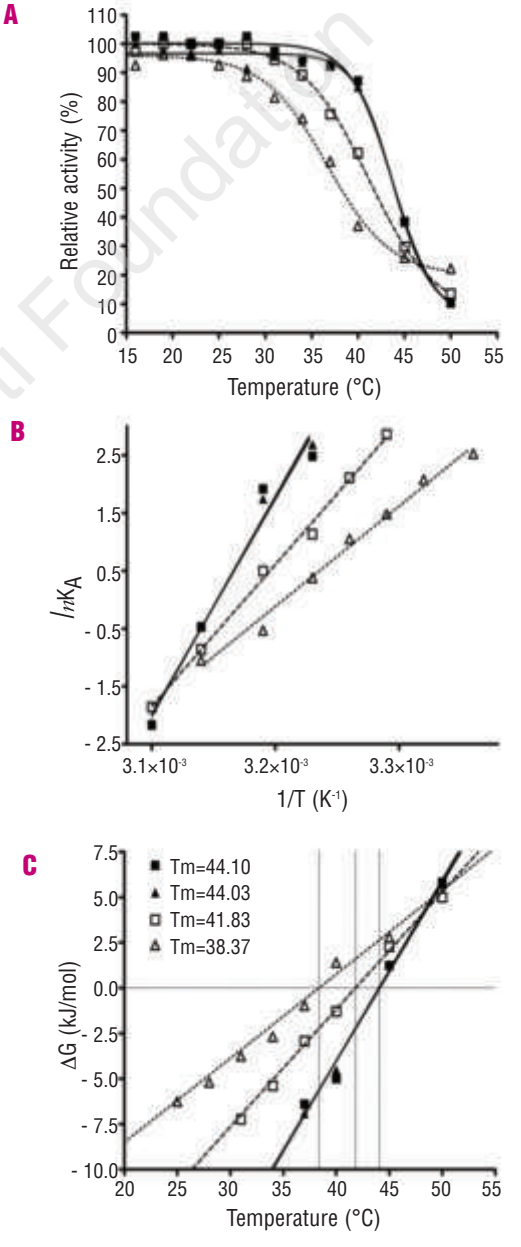

Figure 2 (right). Thermostability and thermodynamics of wild-type and mutant cb5r. Temperature stability and thermodynamic characteristics were investigated in two different controls (■ and ▲), the heterozygous father (□) and propositus (△). **A.** Relative activity of NADH:FR after 30 minute pre-incubation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

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plotting lnK_A vs. $1/T$ (Figure 2B, Table 1). ΔH and ΔS 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 (ΔG_{stab}) was calculated at three different temperatures. The ten-fold higher ΔG 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 ΔH indicates weaker internal interactions, while the change observed in ΔS points to a larger contribution of conformational entropy versus hydrophobic entropy. Estimated values for ΔG were calculated and plotted as ΔG 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 *I215T*, located in the same region as *L217P* were identified in type I RCM with cb5r deficiency.[13,14] 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.[15] 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.

RHN: co-designed the experiments. Performed most of the research, wrote the initial draft of the manuscript, critically participated in all manuscript revisions; HBL: provided clinical care of the patient, cloned and identified the mutation, helped design experiments, participated in all manuscript revisions; AG: performed and designed experiments, reviewed manuscript; QZ: assisted in the computer modeling and interpretation of the mutant protein structure. reviewed the manuscript; NJ: assisted in the computer modeling and interpretation of the mutant protein structure, reviewed the manuscript; JP: designed all the experiments, intellectual concepts, critically analyzed all the data, supported the research ideas and reviewed/edited the manuscript.
Acknowledgments: We are grateful to Yongli Guan and Enli Liu for technical help throughout this work.

Funding: This work was supported by a NIH Grant (HL-050077, JTP). RHN, HBL and AG were supported by a T-32 training grant from the NIH (DK-60445).

Manuscript received April 22, 2006. Accepted August 29, 2006.

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References

1. Bull PC, Shephard EA, Povey S, Santisteban I, Phillips IR. Cloning and chromosomal mapping of human cytochrome b5 reductase (DIA1). Ann Hum Genet 1988;52:263-8.
2. Tomatsu S, Kobayashi Y, Fukumaki Y, Yubisui T, Orii T, Sakaki Y. The organization and the complete nucleotide sequence of the human NADH-cytochrome b5 reductase gene. Gene 1989;80:353-61.
3. Jaffe E, Hultquist D. The Metabolic and Molecular Basis of Inherited Diseases. 8th ed. New York: McGraw-Hill. 2001.
4. Leroux A, Junien C, Kaplan J, Bamberger J. Generalised deficiency of cytochrome b5 reductase in congenital methaemoglobinaemia with mental retardation. Nature 1975; 258:619-20.
5. Bando S, Takano T, Yubisui T, Shirabe K, Takeshita M, Nakagawa A. Structure of human erythrocyte NADH-cytochrome b5 reductase. Acta Crystallogr D Biol Crystallogr 2004; 60:1929-34.
6. Liu E, Jelinek J, Pastore YD, Guan Y, Prchal JF, Prchal JT. Discrimination of polycythemias and thrombocytoses by novel, simple, accurate clonality assays and comparison with PRV-1 expression and BFU-E response to erythropoietin. Blood 2003;101:3294-301.
7. Prchal JT, Borgese N, Moore MR, Moreno H, Hegesh E, Hall MK. Congenital methemoglobinemia due to methemoglobin reductase deficiency in two unrelated American black families. Am J Med 1990;89:516-22.
8. Evelyn KA, Malloy HT. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J Biol Chem 1938; 126:655-62.
9. Ou CN, Rognerud CL. Diagnosis of hemoglobinopathies: electrophoresis vs. HPLC. Clin Chim Acta 2001; 313: 187-94.
10. Maran J, Guan Y, Ou CN, Prchal JT. Heterogeneity of the molecular biology of methemoglobinemia: a study of eight consecutive patients. Haematologica 2005;90:687-9.
11. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497-500.
12. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999; 41:95-8.
13. Jenkins MM, Prchal JT. A novel mutation found in the 3' domain of NADH-cytochrome B5 reductase in an African-American family with type I congenital methemoglobinemia. Blood 1996; 87: 2993-9.
14. Grabowska D, Plochocka D, Jablonska-Skwiecinska E, Chelstowska A, Lewandowska I, Staniszewska K, et al. Compound heterozygosity of two missense mutations in the NADH-cytochrome b5 reductase gene of a Polish patient with type I recessive congenital methaemoglobinaemia. Eur J Haematol 2003;70:404-9.
15. Shih P, Holland DR, Kirsch JF. Thermal stability determinants of chicken egg-white lysozyme core mutants: hydrophobicity, packing volume, and conserved buried water molecules. Protein Sci 1995;4:2050-62.