Bull Yamaguchi Med Sch 42(3-4) : 49-53, 1995

Genetic and Biochemical Studies of Hereditary Methemoglobinemia (NADH-Cytochrome b_5 Reductase Deficiency)

Komei Shirabe, Toshitsugu Yubisui** and Masazumi Takeshita***

*Department of Biochemistry, Oita Medical University, Hasama-machi, Oita 879-55 Japan
**Department of Biology, Faculty of Science, Kochi University, Kochi 780, Japan
(Received September 6, 1995, Revised October 4, 1995)

Abstract Hereditary methemoglobinemia is an autosomal recessive disorder caused by a deficiency of NADH-cytochrome b_5 reductase. In most cases enzyme deficiency occurs in red cells and symptom is only cyanosis due to elevated methemoglobin (type I). In about 10% of the cases deficiency is demonstrated in all the examined tissues and is accompanied by methemoglobinemia and severe neurological disorders (type II). Gene analyses of five independent families with type I disease showed that point mutations leading to amino acid substitutions occur in all cases. Mutations found in three patients of type II disease were a point mutation, a deletion of 3 bases that leads to one amino acid deletion, and a mutation of splicing acceptor site of exon 9. Characterization of recombinant type I and type II mutant enzymes showed that type I enzymes retained high enzyme activity but were unstable, whereas type II enzymes had low catalytic ability. Mutations in type I disease on three dimensional structure of the enzyme reside in the marginal portion of the enzyme that seem to participate in maintaining the enzyme structure, while type II mutations were found close to the catalytic center of the enzyme that explain low catalytic efficiency of the mutants.

Key words: hereditary methemoglobinemia, NADH-cytochrome b_5 reductase, genetic diagnosis, x-ray structure, catalytic mechanism.

Introduction

Hereditary methemoglobinemia is an autosomal recessive disorder caused by a deficiency of NADH-cytochrome b_5 reductase (b5R, EC 1.6.2.2.). The enzyme transfers two electrons from NADH to two molecules of cytochrome b_5 (cyt. b5) through enzyme-bound FAD. Reduced cyt. b5 transfer electrons to various electron acceptors, thereby participating in a variety of biological processes. There are two forms in b5R, soluble and membrane bound that are generated by alternative usage of different promoters of the single gene[1]. The soluble form exists in red cells and participates in methemoglobin (non-functional hemoglobin with Fe^{3+}) reduction[2]. In other tissues the membrane bound form localizes in microsomes and mitochodrial outer membranes where this electron transfer system functions in fatty acid desaturation[3] and elongation[4], cholesterol biosynthesis[5], cytochrome P450-mediated drug metabolism[6], and cytidine monophospho-N-acetylneuraminic acid hydroxylation[7].

Table I Summary of NADH-cytochrome b_5 reductase activities of type I and type II patients and recombinant mutant enzymes

Subjects	Mutations	Enzyme activity (% of normal control) Erythrocytes	Lymphocytes	Kcat/Km*	Function of the affected residue
Normal		100	100	100	
Type I					
Kagoshima, Japan	Arg-57→ Gln	7.7	40.1	24.6	Structural (mutant is unstable)
Italy	Val-105→ Met	7.5	NT**	14.5	Structural (mutant is unstable)
Kurobe, Japan	Leu-148→ Pro	1.1	36.1	46.7	Structural (mutant is unstable)
Type II					
Hiroshima, Japan	Ser-127→ Pro	2.1	10.6	3.4	Interact with phosphate of FAD
Yokohama, Japan	Phe-298 deletion	ND***	8.4	0.4	Hydrophobic FAD-binding pocket
Italy	exon 9 splicing acceptor site****				

*Values of recombinant enzymes.
**Not tested.
***Not detected.
****No functional enzyme might be produced.

For the deficiency of b5R that causes hereditary methemoglobinemia, three types had been reported. Type I (erythrocyte type), whose enzyme deficiency is restricted to red cells, causes only cyanosis due to elevated methemoglobin. In type II (generalized type), enzyme deficiency is observed not only in red cells but in fibroblasts, muscles, liver, and brain as well. The patients have severe symptoms including mental retardation, microcephaly, retarded growth, bilateral athetoid movements, and generalized hypertonia[8]. On type III only one detailed report is available[9]. The type III patient has the same symptom as type I but the enzyme activity was reduced in all blood cells such as lymphocytes, neutrophils, and platelets besides red cells. To clarify the molecular mechanism leading to the diversity of hereditary methemoglobinemia, molecular genetic approach was undertaken.

Re-evaluation of classification of hereditary methemoglobinemia

The family in Kurobe, Japan was reported as a sole example having a puzzling phenotype in which b5R activity is defective in all blood cells but is not associated with generalized symptoms and a new classification "type III" was proposed[9]. Nagai et al.[10], however, carefully re-examined the case and demonstrated that the enzyme activities of the leukocytes and platelets of the patient were, indeed, in the range of type I disease. The mutant enzyme, Leu-148 → Pro, expressed in the patient showed marked instability that might be the cause of the disease. From these results we concluded that it is not necessary to have "type III" category in the classification of the disease.

Restriction of enzyme deficiency to red cells in type I

In hereditary methemoglobinemia type I, enzyme deficiency has been reported to be restricted to red cells, as reported for glucose-6-phosphate dehydrogenase[11], glutathione synthetase[12] as well. To understand molecular mechanism for this phenomenon, the nucleotide sequences of the b5R gene of the patients of 8 families of hereditary methemoglobinemia were determined (Table I)[13)-16]. The patients in Kagoshima, Okinawa, and Toyoake, Japan were shown to have the same mutation, Arg-57 → Gln. Patients in Kurobe and Italy had Leu-148 → Pro and Val-105 → Met mutations, respectively. The enzyme activities of Arg-57 → Gln, Leu-148 → Pro, and Val-105 → Met were 62%, 47%, and 77% of the normal, respectively, whereas the activities in erythrocytes of the patients were <10% of the normal. These data suggest that the amount of active enzymes might be reduced in red cells as measured for the Italian patient[17]. The heat instability and proteinase susceptibility were

demonstrated for the recombinant type I mutants. Thus the restriction of the deficiency to erythrocyte might be caused by the instability of the mutant type I enzymes where there can be no compensation by protein synthesis, but not due to an impairment of the erythroid specific promoter, or a failure of release of the membrane-bound ertzyme into soluble fraction in the red cells as formerly postulated.

Why are there two types, type I and type II?

Mutations of type II disease in Hiroshima and Yokohama were Ser-127 → Pro and 3 base-pairs in-frame deletion of codon 298 which results in deletion of Phe-298. kcat/Km values, which reflect the efficiency of the enzyme reaction in vivo, of the recombinant mutant enzymes of type I disease (Table I) were closer to normal comparing with type II. The enzyme activities in type II patients might be reduced to the level that is not sufficient for the cell metabolism due to mutations in critical residues and result in general symptoms. Thus, the extent of the impairment of the enzyme activity might diverge the disease into two types.

Localization of affected residues in hereditary methemoglobinemia in b5R structure

X-ray structure of b5R was determined by authors' group[18] and Miki's group[19] recently. The affected residues in type I are located in a marginal portion in the structure which might be involved in a maintenance of overall enzyme conformation. In contrast, the residues altered in type II exist close to the catalytic center. Ser-127, which was replaced by Pro in type II Hiroshima, hydrogen-bonds to pyrophosphate moiety of cofactor FAD. Phe-298, which was deleted in type II Yokohama, is a part of hydrophobic FAD binding pocket. Thus, the affected residues in type II disease were shown to be important in the enzyme function.

New insights into ascorbate regeneration from hereditary methemoglobinemia

Neurological disorder might be explained by an impaired lipid metabolism which causes altered fatty acid composition in brain[20]. The modified lipid metabolism, however, does not explain some of the symptoms in hereditary methemoglobinemia, such as delayed synostosis of cranial bones, hypertrophy of gums, and absent or underdeveloped teeth[8]. We recently demonstrated that severe type II patient in Italy had a mutation at 3' splicing acceptor site of exon 9 and expressed no immunologically detectable b5R[21]. The previous report on the participation of b5R in semiascorbate (SDA) reduction[22] prompted us to measure SDA reductase activity of fibroblast of this patient to show that the activity was markedly reduced[21]. These results suggest an important role of b5R in ascorbic acid regeneration. Impairment of SDA reductase activity may lower intracellular ascorbate level, thereby hampering collagen biosynthesis.

References

1) Pietrini, G., Aggujaro, D. Carrera, P. Malyszko, J. Vitale, A. and Borgese N.: A Single mRNA, Transribed from an Alternative, Erythroid-specific, Promoter, Codes for Two Non-myristylated Forms of NADH-Cytochrome b_5 Reductase. *J. Cell Biol.*, **117** : 975-986, 1992.

2) Jaffe, E. R.: Methemoglobin pathophysiology. In D.F.H. Wallach,(ed.) *The Function of Red Blood Cells,* Alan R. Liss, New York, 1981. p.133-155.

3) Oshino, N., Imai, Y. and Sato, R.: A function of cytochrome b5 in fatty acid desaturation by rat liver microsomes. *J. Biochem.*, **69** : 155-167, 1971.

4) Keyes, S. R. and Cinti D. L.: Biochemical Properties of Cytochrome b5-dependent Microsomal Fatty Acid Elongation and Identification of Products. *J. Biol. Chem.*, **255** : 11357-11364, 1980.

5) Reddy, V. V. R., Kupfer, D. and Capsi E.: Mechanism of C-5 Double Bond Introduction in the Biosynthesis of Cholesterol by Rat Liver Microsomes *Evidence for the participation of microsomal cytochrome b5 J. Biol. Chem.*, **252** : 2797-2801, 1977.

6) Aoyama, T., Nagata, K. Yamazoe, Y. Kato, R. Matsunaga, E. Gelboin, H. V.

and Gonzale, F. J.: Cytochrome b_5 potentiation of cytochrome P-450 catalytic activity demonstrated by a vaccinia virus-mediated in situ reconstitution system. *Proc. Natl. Acad. Sci. USA.* **87** : 5425-5429, 1990.

7) Kawano, T., Kozutsumi, Y. Kawasaki, T. and Suzuki, A.: Biosynthesis of N-glycolylneuraminic acid-containing glycoconjugates. Purification and characterization of the key enzyme of the cytidine monophospho-N-acetylneuraminic acid hydroxylation system. *J. Biol. Chem.,* **269** : 9024-9029, 1994.

8) Jaffe, E. R., Neumann, G. Rothberg, H. Wilson, F. T. Webster, R. M.and Wolff J. A.: Hereditary methemoglobinemia with and without mental retardation. A study of three families. *Am. J. Medicine,* **41** : 42-55, 1966.

9) Tanishima, K., Tanimoto, K. Tomoda, A. Mawatari, K. Matsukawa, S. Yoneyama, Y. Ohkuwa, H. and Takazakura, H.: Hereditary methemoglobinemia due to cytochrome b5 reductase deficiency in blood cells without associated neurologic and mental disorders. *Blood,* **66** : 1288-1291, 1985.

10) Nagai, T., Shirabe, K. Yubisui, T. and Takeshita, M.: Analysis of mutant NADH-cytochrome b5 reductase: Apparent "type III" methemoglobinemia can be expained as type I with an unstable reductase. *Blood,* **81** : 808-814, 1993.

11) Beutler, E., Mathai, C. K. and Smith, J. E.: Biochemical Variants of Glucose-6-Phosphate Dehydrogenase Giving Rise to Congenital Nonspherocytic Hemolytic Disease. *Blood,* **31** : 131-150, 1968.

12) Mohler, D. N., Majerus, P. W. and Minnich, V.: Glutathione synthetase deficiency as a cause of hereditary hemolytic disease. *N. Engl. J. Med.,* **283** : 1253-1257, 1970.

13) Kobayashi, Y., Fukumaki, Y. Yubisui, T. Inoue, J. and Sakaki, Y.: Serine-Proline Replacement at Residue 127 of NADH-Cytochrome b5 Reductase Causes Hereditary Methemoglobinemia, Generalized Type. *Blood,* **75** : 1408-1413, 1990.

14) Shirabe, K., Yubisui, T. Borgese, N. Tang, C. Hultquist, D. E. and Takeshita, M.: Enzymatic Instability of NADH-Cytochrome b_5 Reductase As a Cause of Hereditary Methemoglobinemia Type I (Red Cell Type). *J. Biol. Chem.,* **267** : 20416-20421, 1992.

15) Katsube, T., Sakamoto, N. Kobayashi, Y. Seki, R. Hirano, M. Tanishima, K. Tomoda, A. Takazakura, E. Yubisui, T. Takeshita, M. Sakaki, Y. and Fukumaki, Y.: Exonic Point Mutation in NADH-Cytochrome b5 Reductase Genes of Homozygotes for Hereditary Methemoglobinemia, Type I and III: Putative Mechanisms of Tissue-dependent Enzyme Deficiency. *Am. J. Hum. Genet.,* **48** : 799-808, 1991.

16) Shirabe, K., Fujimoto, Y. Yubisui, T. and Takeshita, M.: An in-frame deletion of codon 298 of the NADH-cytochrome b5 reductase gene results in hereditary methemoglobinemia type II (generalized type). *J. Biol. Chem.,* **269** : 5952-5957, 1994.

17) Borgese, N., Pietrini, G. and Gaetani, S.: Concentration of NADH-Cytochrome b5 Reductase in Erythrocytes of Normal and Methemoglobinemic Individuals Measured with a Quantitative Radioimmunoblotting Assay. *J. Clin. Invest.,* **80** : 1296-1302, 1987.

18) Takano, T., Bando, S. Horii, C. Higashiyama, M. Ogawa, K. Sato, M. Katsuya, Y. Danno, M. Yubisui, T. Shirabe, K. and Takeshita, M.: The structure of human NADH-cytochrome b5 reductase at 2.5 Å resolution. *In Flavins and Flavoproteins,* Walter de Gruyter, Berlin, 1994, pp.409-412.

19) Nishida, H., Inaka, K. Yamanaka, M. Kaida, S. Kobayashi, K. and Miki, K.: Crystal structure of NADH-cytochrome b5 reductase from pig liver at 2.4 Å resolution. *Biochemistry,* **32** : 2764-2767, 1995.

20) Hirono, H.: Lipids of myelin, white matter and gray matter in a case of generalized deficiency of cytochrome b5 reductase in congenital methemoglobinemia with mental retardation. *Lipids,* **15** : 272-275, 1980.

21) Shirabe, K., Landi, M. T., Takeshita, M.,

Uziel, G., Fedrizzi, E., and Borgese, N.: A novel point mutation in a 3' splice site of the NADH-cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NADH -dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. *Am. J. Hum. Genet.* **57** : 302-310, 1995.

22) Ito, A., Hayashi, S., and Yoshida, T.: Participation of a cytochrome b5-like hemoprotein of outer mitochondrial membrane (OM cytochrome b) in NADH -semidehydroascorbic acid reductase activity of rat liver. Biochem. *Biophys. Res. Comm.* **101** : 591-598, 1981.