Structural Role of Serine 127 in the NADH-binding Site of Human NADH-Cytochrome b₅ Reductase*

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Serine 127 of human NADH-cytochrome b₅ reductase was replaced by proline and alanine by site-directed mutagenesis. The former mutation has been found in the genes of patients with hereditary deficiency of the enzyme. Both the mutant enzymes (Ser 127 → Pro mutant and Ser-127 → Ala mutant) were overproduced in Ercherichia coli and purified to homogeneity. The two purified mutant enzymes showed indistinguishable spectral properties which differed from those of the wild-type enzyme. The mutant enzymes showed higher molecular extinction coefficients at 462 nm than those of the wild-type enzyme. Quenching of the binding site in the enzyme.

Furthermore, circular dichroism spectra of the mutant enzymes were different, in both the visible and ultraviolet regions, from that of the wild-type enzyme. The spectra of the mutant enzymes in the visible region were restored to almost the same spectrum as the wild type upon reduction with NADH. Ser-127 → Pro mutant and Ser-127 → Ala mutant showed very low Kₘ/ν (NADH) values (5 × 10⁻⁷ and 3.5 × 10⁻⁷ M⁻¹, respectively) with cytochrome b₅ as an electron acceptor, while that of the wild-type enzyme (Kₘ/ν (NADH) = 179 × 10⁻⁷ M⁻¹) value for each enzyme was similar. The mutant enzymes were less thermostable than the wild-type enzyme. These results indicate that serine 127 plays an important role in maintaining the structure of the NADH-binding site in the enzyme.

Hereditary deficiency of NADH-cytochrome b₅ reductase (b₅R, EC 1.6.2.2) is known as hereditary methemoglobinemia and is classified into three types: an erythrocyte type (type I), a generalized type (type II), and a blood cell type (type III). Among these, type II is the most severe disease, and it is accompanied by mental retardation and neurological defects. In order to clarify the mechanisms which cause various types of the hereditary b₅R deficiency and also to understand the reaction mechanism of b₅R, we determined first the complete amino acid sequence of the soluble form of human erythrocyte b₅R (5, 6) and then the base sequences of cDNAs for the membrane-bound form of b₅Rs in human liver and placenta. Human genomic DNA of b₅R was also cloned to determine its whole structure (8). These studies have shown that the primary structure of the soluble form of the enzyme is the same as that of the membrane-bound form except that it lacks an NH₂-terminal hydrophobic segment consisting of 25 amino acid residues, and two forms of the enzymes are encoded by a single gene. Recently, we analyzed the gene structure for b₅R of type II patients and found that the TCT codon for serine 127 of the enzyme was changed to CCT for proline (9). Since the expression levels of mRNA and protein for the enzyme in blood cells of the patient were shown to be almost comparable to those of a normal individual (9), the mutant b₅R (S127P) expressed in the patient tissues was expected to have reduced catalytic activity. In the present study, we have prepared and characterized the mutant enzymes in which Ser-127 is replaced by Pro (S127P) or by Ala (S127A) by utilizing the expression system of the soluble form of the enzyme in Escherichia coli (10) and by site-directed mutagenesis. This study was undertaken in an attempt to analyze the molecular details of the mutant enzyme produced in patient tissues, to understand how the genetic defect(s) of the single gene can cause these three different types of hereditary methemoglobinemia, and to better understand the catalytic mechanism of the enzyme. We have prepared a Ser-127 → Ala mutant to establish whether the hydroxyl group of Ser-127 is important or not for the enzyme function. Analyses of the structure and function of the mutant enzymes as described below revealed that Ser-127 in b₅R plays a critical role in maintaining the structure of the NADH-binding site. Characterization of the naturally occurring mutant b₅R described in this study is the first case for this disease.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, the Klenow fragment of DNA polymerase I of E. coli, and T₄ polynucleotide kinase were purchased from Takara Shuzo Co. (Kyoto, Japan). An oligonucleotide-directed in vitro mutagenesis system and [α-³²P]dCTP (400 Ci/mmol) were the products of Amersham Corp. Oligonucleotides for mutagenesis were synthesized with the aid of a DNA synthesizer (model 8600; Biosearch, Inc., New Brunswick Scientific Co., San Rafael, CA).

DEAE-Toyopearl was obtained from Tosoh (Tokyo, Japan), and 5'-AMP-Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. Bovine α-thrombin was a product of Sigma. Other agents were obtained commercially. Human erythrocyte cytochrome

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§ The abbreviations used are: b₅R, NADH-cytochrome b₅ reductase; S127P, the mutant whose serine was replaced by proline; S127A, the mutant whose serine was replaced by alanine.

5 Numbering of Ser-127 is for the membrane-bound form b₅R (300 residues), and Ser-127 corresponds to Ser-102 when the Phe-26 is the NH₂ terminus for the soluble enzyme (276 residues).
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Ser-directed Mutagenesis—The HindIII-PstI fragment (503 base pairs), which includes the NH₂-terminal portion of b₅R cDNA, was excised from expression plasmid placZ'-APR-b₅R (10) and cloned in M13mp19 to give M13mp19-b₅R/A (Fig. 1). Site-directed mutagenesis was performed with a synthetic mixed primer, 5'-TCCCTTCTAC(G or C)GAGCTGATGG-3', and by the strand selection method of Taylor et al. (12), using the Amersham mutagenesis system. M13 phage DNA was prepared from 10 independent plaques, and mutant clones were selected by dideoxy sequencing (13). Nucleotide sequence of the HindIII-PstI fragment was verified. The fragment was excised from mutant M13mp19-b₅R/A and cloned into pUC-b₅R/B to construct the mutant expression plasmid placZ'-APR-b₅R (Fig. 1).

Purification of Wild-type and Mutant Enzymes—Wild-type and mutant b₅Rs were expressed in E. coli RB791 as described previously as an a-thrombin-cleavable fusion protein (10), except that the incubation time for cleavage was decreased to 3 h. Expression of b₅Rs was induced by adding 0.25 mM isopropl-β-D-thiogalactopyranoside to the culture medium. Mutant b₅Rs were also purified by an affinity chromatography on a 5'-AMP-Sepharose column (10). Purity of the b₅Rs was examined by electrophoresis on polyacrylamide gel (12.5%) in the presence of sodium dodecyl sulfate (14).

Assay of Enzyme Activity—Enzyme activity of b₅R during purification was monitored by assaying NADH-ferricyanide reductase activity. NADH-cytochrome b₅ reductase activity was assayed as described previously in 5 mM Tris-HCl buffer (pH 7.5) (15). Kₘ and Vₘₐₓ values were determined by 1/ν-axis intercept replots of the 1/V versus 1/[NADH] data obtained at varied [cytochrome b₅] and vice versa. Kₐ (Vₘₐₓ/mol of enzyme) was calculated by using the molecular weight of 32,000.

Spectrophotometric Determinations—Spectrophotometric determinations were carried out with a Union spectrophotometer (SM 401; Union Giken, Osaka, Japan) and also with a Hitachi spectrophotometer, model 200. Fluorescence analyses were performed with a Shimadzu spectrophotofluorometer, model RF-540. CD spectra of b₅Rs were measured at 25 °C with a Jasco 5-600 automatic recording polarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The magnitude of CD spectra was expressed as a molar ellipticity (θ), deg·cm²/dmol, based on the molecular weight of b₅R (32,000). The NADH-reduced form of b₅Rs was produced in a Thumberg's tube by repeating gas exchange with N₂ gas of high purity as described previously (10).

Amino Acid Sequence—NH₂-terminal amino acid sequences of the purified b₅Rs were determined by Edman degradation with an Applied Biosystems model 477A coupled with an Applied Biosystems phenylthiohydantoin-derivative detector model 120A (17).

RESULTS

Purification of b₅R—b₅R expressed in E. coli was purified as described previously (10). As described above, mutant enzymes S127P and S127A were produced in this study to examine the role of Ser-127 in the functioning of the enzyme. Mutant b₅Rs and wild-type b₅R were not significantly inactivated during α-thrombin cleavage at a high concentration of proteins (6-12 mg/ml). Yield of the mutant enzymes during purification was about 50% for both S127P and S127A based on the ferricyanide reductase activity. From a 1-liter culture of E. coli, about 80, 25, and 30 mg of the purified b₅Rs were obtained for the wild type, S127P, and S127A, respectively.

Purity of b₅R—Purity of b₅Rs was examined by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate as shown in Fig. 2. Final preparations of both the S127P and S127A showed a single band of molecular weight of 32,000. The NH₂-terminal amino acid sequences of mutant b₅Rs (from Phe-1 to Pro-18) were identical to that of human erythrocyte b₅R.

Spectral Properties—Absorption spectra of both the mutant b₅Rs differed from that of the wild-type b₅R (Fig. 3). The molecular extinction coefficient of each mutant b₅R was 10.8 mM⁻¹ cm⁻¹ (Fig. 3 and Table I). For each mutant, b₅R absorbance at the 390 nm peak is almost the same as at the 462 nm peak, while the absorbance of the wild-type b₅R is significantly higher at 390 nm than at 462 nm. Absorbance ratios of 390-462 nm were the same (1.01) for each of the mutant b₅Rs, but that for the wild-type b₅R was 1.13. These differences of absorption spectra suggested that some conformational changes around FAD have occurred in the mutant b₅Rs. To examine this possibility, fluorescence spectra of the mutant b₅Rs and the wild-type b₅R were determined. As shown in Fig. 4, less quenching of FAD fluorescence occurred in the mutant b₅Rs. Intensities of fluorescence of FAD are 8% in the wild type, 13% in S127P, and 18% in S127A, respectively, compared with that of free FAD. Spectral properties of these b₅Rs are summarized in Table I. These results suggest that the conformation of mutant b₅Rs is modified so that bound FAD is more exposed to solvent and thus exhibits stronger

![Fig. 1. Construction of mutant expression plasmid. A mutation expression plasmid, placZ'-APR-b₅R', was constructed from the wild-type expression plasmid, placZ'-APR-b₅R, as described under "Experimental Procedures." Filled box in placZ'-APR-b₅R indicates b₅R cDNA fused with the NH₂-terminal portion of lac Z followed by the α-thrombin recognition sequence, Ala-Pro-Arg (APR). Hatched boxes indicate the fragment A'(HindIII-PstI) mutagenized from S127T (TCT) to E (CCT) or A (GCT).](image-url)

![Fig. 2. Gel electrophoresis of mutant b₅R expressed in E. coli. Lanes: a, crude extract; b, eluates from DEAE-Toyopearl; c, fractions from 5'-AMP-Sepharose; d, α-thrombin-digested fraction; e, purified S127P; f, purified S127A; g, purified wild-type enzyme; h, molecular weight markers (bovine serum albumin, 68,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; chymotrypsinogen, 25,000; horse heart cytochrome c, 12,400).](image-url)
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**FIG. 3. Absorption spectra of wild type and mutant b₅Rs.** Absorption spectra were measured in 50 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA and 0.1 mM dithiothreitol. —, wild type; ---, S127P.

**TABLE I**

| Enzymes | A₄₆₂/A₅₅₀ | E (mm⁻¹ cm⁻¹) | Fluorescence* |
|---------|-----------|--------------|---------------|
| Wild    | 1.13      | 10.4         | 8.3           |
| S127P   | 1.01      | 10.8         | 13.1          |
| S127A   | 1.01      | 10.8         | 17.9          |

* Absorbance ratio.  
** Molecular extinction coefficients.  
** Fluorescence intensity of the enzyme-bound FAD was expressed as the intensity relative to that of free FAD. Relative fluences at 522 nm were calculated from the data shown in Fig. 4 and normalized.

**FIG. 4. Fluorescence spectra of wild-type and mutant b₅Rs.** Fluorescence spectra of FAD bound to the wild type and mutant enzymes were measured in 50 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA and 0.1 mM dithiothreitol. The excitation wavelength was set at 460 nm. Fluorescence was compared with that of free FAD. From bottom to top: wild type (8.2 μM), S127P (7.5 μM), S127A (7.9 μM), and free FAD (7.7 μM).

fluorescence than that of the wild-type b₅R.

These structural changes in the mutant enzymes were also examined by CD spectra measurements. As shown in Fig. 5a, the CD spectrum of the oxidized form of the wild-type enzyme from 300 to 550 nm was closely similar to that which we reported previously with the rabbit erythrocyte enzyme (16),

while those of the mutant enzymes are different from that of the wild-type enzyme. Here, it should be noted that the altered CD spectra of mutant b₅Rs are very similar to each other, in spite of the different mutations from Ser to Pro or Ala. In contrast, when the enzymes were reduced with an equivalent amount of NADH anaerobically, the spectra of the mutant enzymes were almost the same as that of the wild type (Fig. 5b). CD spectra of the b₅Rs in the ultraviolet region (200-250 nm) suggest that secondary structures are modified significantly by the replacement from Ser-127 to Pro or Ala. In spite of such changes in the protein structures around the NADH- and/or FAD-binding sites in the mutant enzymes, the typical semiquinone absorption peak at 375 nm was recognized by anaerobic titration of the dithionite-reduced b₅Rs with potassium-ferricyanide in the presence of NAD⁺ (data not shown) as described by Iyanagi (18). These results apparently indicate that the electron transfer process between NADH and FAD in both the mutant b₅Rs is essentially the same as in the wild-type b₅R.
Enzyme Activities—bR activities of both the mutant enzymes determined under the standard assay conditions were significantly lower than that of the wild-type enzyme. To characterize the low enzyme activities of the mutant enzymes, their kinetic properties were examined. Kinetic values for NADH of 6.3 and 14 µM were determined for mutant bRs S127P and S127A, respectively; these values are more than 10-fold higher than the value of 0.5 µM observed for the wild-type enzyme. Kinetic values for cytochrome b were 6.5 µM with S127P and 7.1 µM with S127A, respectively; these values are almost the same as that of 6.6 µM observed with the wild-type enzyme. Kinetic values were 315 and 496 s⁻¹ with S127P and S127A, respectively. These values correspond to about 35 and 55%, respectively, of the value of 896 s⁻¹ observed with the wild-type bR as summarized in Table II. As shown in Table II, Kcat/Km (NADH) values of the mutant bRs are less than 3% of that of the wild-type bR, while Kcat/Km (cytochrome b) values are similar to that of the wild-type. These results apparently indicate that Ser-127 is involved in NADH binding and that the replacement of Ser-127 by Pro or Ala did not affect the binding of cytochrome b.

Thermostability—During purification of bRs produced as an α-thrombin-cleavable fused protein as described under “Experimental Procedures,” no appreciable decrease of enzyme activity was observed after the incubation of bR at 37 °C for 3 h at high protein concentration (more than 6 mg/ml). When diluted bR (10 µM, 0.32 mg/ml) was incubated at various temperatures, however, distinct differences between the thermostability of mutant bRs and wild-type bR were observed (Fig. 6a). Although the wild-type bR is fairly stable even at 50 °C (more than 90% active after 30 min), mutant bRs are heat-inactivated rapidly to less than 20% of the initial activities under the same conditions (Fig. 6b). These results are likely due to the modified structure of mutant bRs as described above. During the incubation, the mutant bRs precipitated at temperatures higher than 40 °C, while the wild-type bR did not aggregate until the temperature was raised to 55 °C. Addition of NADH up to 100 µM in the incubation mixture only slightly protected mutant S127P against the inactivation (data not shown). Likewise, no significant protection of the mutant enzymes against heat treatment was observed by anaerobic incubation in the presence of excess NADH.

**DISCUSSION**

Previously, we analyzed the DNA structure of the type II patients of this disease and showed that a point mutation of TCT for Ser-127 to CCT for Pro occurred in the 5th exon of the bR gene. Western blotting analysis of the patient’s leukocytes and lymphocytes with anti-bR antibody indicated that normal amounts of bR protein are expressed in tissues of the patients (9). A linkage was found between occurrence of the disease and incidence of the family. A significant amount of inactive bR exists in the blood cells of Ser-127 in bR. Since we have previously shown that the primary structure of the soluble form of the enzyme from red blood cells is the same as the catalytic domain of the membrane-bound form of other tissues, the experimental results obtained in this study of the soluble form of the mutant bR are valid to explain the enzymatic defect which occurred in the tissues of the patient.

bR activities determined with blood cells obtained from patients were about 15% of those of normal subjects (9) under the standard assay conditions. The Km value for NADH of the S127P enzyme was determined in this study to be about 10-fold higher than that of the wild-type enzyme. As the concentrations of NADH in human erythrocytes and in other tissues are, however, about 10-fold higher (40-50 µM) (19) than the Km value of the S127P, the low Km of the S127P (about 35% of that of the wild-type bR, Table II) may be a more important factor in causing the disease than the higher Km value for NADH. Second, the instability of the mutant enzyme protein might partially contribute to the impaired activity, although the Western blotting analysis indicated that a significant amount of inactive bR exists in the blood cells of patients.

To test whether the altered enzymatic properties of the S127P are due to replacement by Pro which could disrupt the secondary structure of the enzyme or due to the loss of hydroxyl side chain, S127A was also prepared and characterized. Both of the purified mutant bRs showed different spectral properties from that of the wild-type bR, but those of the mutant bRs are very similar to each other as revealed by the absorption, fluorescence, and CD spectra. These facts suggest that the backbone conformations of the mutant bRs prepared in this study differ from that of the wild-type bR and that this is due to loss of the hydroxyl group of Ser-127. Very interestingly, however, a difference between the CD spectra of the mutant and wild-type bRs was observed for

![Graph showing thermostability of wild type and mutant bRs.](image)

**Table II**

Kinetic properties of mutant NADH-cytochrome (cyt.) b5 reductases

| Enzymes | Km (NADH) | Km (cyt. b) | Kcat | Km/N | Km/Km (NADH) | Km/Km (cyt. b) |
|---------|-----------|-------------|------|------|--------------|---------------|
|         | µM        | µM          | s⁻¹  | M⁻¹  | s⁻¹ M⁻¹     | s⁻¹ M⁻¹       |
| Wild    | 0.5       | 6.6         | 896  | 179.0 | 7.0 × 10⁹   | 7.0 × 10⁹     |
| S127P   | 6.3       | 6.5         | 315  | 50.0  | 4.9 × 10⁸   | 4.9 × 10⁸     |
| S127A   | 14.0      | 7.1         | 496  | 3.5   | 7.0 × 10⁷   | 7.0 × 10⁷     |
the oxidized forms of the enzyme but not after reduction with NADH as shown in Fig. 5. These facts suggest that Ser-127 is involved in the NADH-binding site, and its hydroxyl group is critically important for the function, since the low $K_{cat}$ values and low thermostability of the mutant b$_5$Rs were not restored upon reduction with NADH. The clearly altered CD spectra in the region from 200 to 240 nm as shown with the mutant b$_5$Rs in this study are not commonly observed for mutant proteins prepared by a single amino acid substitution. For example, CD spectra in the far ultraviolet region (200–240 nm) of all 18 mutant a subunits of tryptophan synthetase substituted at position 49 were indistinguishable from that of the wild-type a subunit (20).

Ser-127 in the wild-type b$_5$R is the second amino acid in the $\alpha$-helix extending from Met-126 to Ile-131 in the predicted nucleotide-binding site (6) and is also very close to Lys-110, which is argued by Hackett et al. (21) to be a residue involved with NADH binding. Since residues in the COOH-terminal half are also expected to be in the NADH-binding site (22, 23), the COOH-terminal portion is considered to be folded to make close contact with the NH$_2$-terminal half in the b$_5$R protein to form a crevice for NADH binding. Thus, NADH bound to b$_5$R is expected to be in direct contact with an isoalloxazine ring of FAD which allows for an effective electron transfer between them. Disruption of the tertiary structure in this region, therefore, can cause the functional damage to b$_5$R as revealed in this study. The hydroxyl group of Ser-127 probably makes hydrogen bonds with the folded poly peptide chains to form a stable tertiary structure. Properties of the mutant b$_5$Rs, such as their low $K_{cat}/K_m$ (NADH) values (Table II) and vulnerability to heat treatment (Fig. 6) are all well explained by the disruption of the structure in the nucleotide-binding region. The electron transfer process between NADH and FAD in both of the mutant b$_5$Rs, however, seems to be essentially the same as in the wild-type b$_5$R as revealed by the appearance of the semiquinone spectra. Therefore, more information other than that revealed in this study is required to know the true reason why the mutant b$_5$Rs show low $K_{cat}$ values.

Analysis of secondary structures of the mutant b$_5$Rs as well as of the wild-type b$_5$R by Fourier-transformed infrared spectroscopy is now in progress to assess the conformation differences in the mutant enzymes. The x-ray crystallographic analysis, now progressing, will lead us to further understanding of the role of Ser-127. Genetic analyses of the type I and III mutations are also under progress, and characterization of those mutant enzymes will promote further the understanding of the disease.

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REFERENCES
1. Scott, E. M., and Griffith, I. V. (1959) Biochim. Biophys. Acta 134, 584–586
2. Hultquist, D. E., and Passon, P. G. (1971) Nature 229, 252–254
3. Leroux, A., Junien, C., and Kaplan, J. C. (1975) Nature 266, 619–620
4. Tanishima, K., Tanimoto, K., Tomoda, A., Mawatari, K., Matsukawa, S., Yoneyama, Y., Okuwa, H., and Takazakura, E. (1985) Blood 66, 1288–1291
5. Yubisui, T., Miyata, T., Iwanaga, S., Tamura, M., Yoshida, S., Takeshita, M., and Nakajima, H. (1984) J. Biochem. (Tokyo) 96, 579–582
6. Yubisui, T., Miyata, T., Iwanaga, S., Tamura, M., and Takeshita, M. (1986) J. Biochem. (Tokyo) 99, 407–422
7. Yubisui, T., Naitoh, Y., Zenno, S., Tamura, M., Takeshita, M., and Sakaki, Y. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3609–3613
8. Tomatsu, S., Kobayashi, Y., Fukumaki, Y., Yubisui, T., Orii, T., and Sakaki, Y. (1989) Gene (Amst.) 80, 353–361
9. Kobayashi, Y., Fukumaki, Y., Yubisui, T., Inoue, J., and Sakaki, Y. (1990) Blood 75, 1408–1413
10. Shirabe, K., Yubisui, T., and Takeshita, M. (1989) Biochim. Biophys. Acta 1008, 189–192
11. Yubisui, T., Murakami, K., Takeshita, M., and Takano, T. (1988) Biochim. Biophys. Acta 936, 447–451
12. Taylor, J. W., Ott, J., and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8785
13. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Yubisui, T., and Takeshita, M. (1980) J. Biol. Chem. 255, 2454–2456
16. Yubisui, T., and Takeshita, M. (1984) Biochem. Int. 8, 319–327
17. Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. (1983) Methods Enzymol. 91, 399–413
18. Iyngar, T. (1977) Biochemistry 16, 2725–2730
19. Zerez, C. R., Lee, S. J., and Tanaka, K. R. (1987) Anal. Biochem. 164, 367–373
20. Yutani, K., Ogashara, K., Tajiita, T., and Sugino, Y. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4441–4444
21. Hackett, C. S., Novoa, W. B., Kensil, C. R., and Strittmatter, P. (1988) J. Biol. Chem. 263, 7539–7543
22. Strittmatter, P. (1989) J. Biol. Chem. 234, 2661–2664
23. Hackett, C. S., Novoa, W. B., Ozols, J., and Strittmatter, P. (1986) J. Biol. Chem. 261, 9864–9867