Role of Thr<sup>66</sup> in Porcine NADH-cytochrome <i>b</i><sub>5</sub> Reductase in Catalysis and Control of the Rate-limiting Step in Electron Transfer*  

Shigenobu Kimura,† Masanori Kawamura, and Takashi Iyanagi  
From the Department of Life Science, Graduate School of Science, Himeji Institute of Technology, Kouto 3-2-1, Kamigori, Hyogo 678-1297, Japan  

Site-directed mutagenesis of Thr<sup>66</sup> in porcine liver NADH-cytochrome <i>b</i><sub>5</sub> reductase demonstrated that this residue modulates the semiquinone form of FAD and the rate-limiting step in the catalytic sequence of electron transfer. The absorption spectrum of the T66V mutant showed a typical neutral blue semiquinone intermediate during turnover in the electron transfer from NADH to ferricyanide but showed an anionic red semiquinone form during anaerobic photoreduction. The apparent <i>k</i><sub>cat</sub> values of this mutant were 10% of that of the wild type enzyme (WT). These data suggest that the T66V mutation stabilizes the neutral blue semiquinone and that the conversion of the neutral blue to the anionic red semiquinone form is the rate-limiting step. In the WT, the value of the rate constant of FAD reduction (<i>k</i><sub>red</sub>) was consistent with the <i>k</i><sub>cat</sub> values, and the oxidized enzyme-NADH complex was observed during the turnover with ferricyanide. This indicates that the reduction of FAD by NADH in the WT-NADH complex is the rate-limiting step. In the T66A mutant, the <i>k</i><sub>red</sub> value was larger than the <i>k</i><sub>cat</sub> values, but the <i>k</i><sub>red</sub> value in the presence of NAD<sup>+</sup> was consistent with the <i>k</i><sub>cat</sub> values. The spectral shape of this mutant observed during turnover was similar to that during the reduction with NADH in the presence of NAD<sup>+</sup>. These data suggest that the oxidized T66A-NADH-NAD<sup>+</sup> ternary complex is a major intermediate in the turnover and that the release of NAD<sup>+</sup> from this complex is the rate-limiting step. These results substantiate the important role of Thr<sup>66</sup> in the one-electron transfer reaction catalyzed by this enzyme. On the basis of these data, we present a new kinetic scheme to explain the mechanism of electron transfer from NADH to one-electron acceptors including cytochrome <i>b</i><sub>5</sub>.  

NADH-cytochrome <i>b</i><sub>5</sub> reductase (EC 1.6.2.2) is a member of the large family of flavin-dependent oxidoreductases that transfer an electron from two-electron carriers of nicotinamide dinucleotides to one-electron carriers such as heme proteins and ferredoxins. This enzyme catalyzes the electron transfer from NADH to cytochrome <i>b</i><sub>5</sub> (b5) (1–3), and participates in fatty acid synthesis (4, 5), cholesterol synthesis (6), and xeno-biotic oxidation (7) as a member of the electron transport chain on the endoplasmic reticulum. In erythrocytes, this enzyme participates in the reduction of methemoglobin (8).  

The outline of the catalytic cycle of the solubilized catalytic domain of NADH-cytochrome <i>b</i><sub>5</sub> reductase (b5R) is understood as follows (3) (Scheme I). At first, two electrons are transferred from NADH to FAD by hydride (H<sup>−</sup>) transfer. Then the two-electron reduced enzyme-NAD<sup>+</sup> complex (E-FADH<sup>−</sup>-NAD<sup>+</sup>) transfers two electrons to two one-electron acceptors one by one via the anionic red semiquinone form (E-FAD<sup>−</sup>-NAD<sup>−</sup>), and the reduced enzyme returns to the oxidized state. Strittmatter (9–11) suggested that the reduction of FAD by NADH is the rate-limiting step in electron transfer catalyzed by b5R. Iyanagi et al. (3, 12) found that the anionic red semiquinone of FAD in Pb5R is stabilized by binding of NAD<sup>−</sup>. Kobayashi et al. (13) analyzed the conversion of the neutral blue to the red semiquinone in the presence of NAD<sup>−</sup> using a pulse radiolysis technique. Meyer et al. (14) also demonstrated that NAD<sup>−</sup> stabilizes the red semiquinone of the human b5R and modulates the electron transfer to b5. These studies suggest the importance of the anionic red semiquinone form of the b5R-NAD<sup>−</sup> complex in the electron transfer.  

The preliminary tertiary structure of human erythrocyte b5R (15, 16), and the detailed tertiary structures of porcine and rat liver b5Rs at 2.1 Å resolution have been determined by x-ray crystallography (17–21). These structural studies revealed that NADH-cytochrome <i>b</i><sub>5</sub> reductase belongs to the structurally related so-called “ferredoxin reductase family” (22, 23) together with other flavoenzymes such as ferredoxin-NADP<sup>+</sup> oxidoreductase (FNR) (22), pthalate dioxygenase reductase (24), flavodoxin reductase (25), NADPH-cytochrome P-450 reductase (26), and the cytochrome <i>b</i> reductase domain of nitrate reductase (27). Enzymes of this family contain a flavin-binding domain and a pyridine nucleotide-binding domain. The former domain has a highly conserved flavin-binding amino acid sequence motif, RXY(T/S). In the Pb5R, Arg<sup>63</sup>, Tyr<sup>65</sup>, and Thr<sup>68</sup> comprise this sequence motif (19). Using site-directed mutagenesis, we demonstrated that the positive charge of Arg<sup>63</sup> is critical for the affinities of Pb5R for both NADH and NAD<sup>−</sup>, and the specific arrangement between the side chain of Tyr<sup>65</sup> and FAD contributes to protein stability and electron transfer (28). Marohnic and Barber (29) also reported the effects of mutations of the corresponding Arg<sup>91</sup> in rat b5R.  

The Thr<sup>66</sup> residue in Pb5R is positioned near both the N5 atom of the isoalloxazine ring of FAD and the potential binding...
site of the nicotinamide ring of NADH (20, 28) (Fig. 1). This position corresponds to threonine or serine residues in the other members of the ferredoxin reductase family (22, 24–27). Ser96 in spinach leaf FNR is critical to the reductive half-reaction of FAD (30), and Ser93 in the C-terminal Tyr280 mutant of pea leaf FNR forms a hydrogen bond with the amide moiety on the nicotinamide ring of the pyridine nucleotide in both the enzyme-NADP+ and enzyme-NADPH complexes (31). However, b5R and cytochrome b reductase domain of nitrate reductase do not have an aromatic ring corresponding to that of the C-terminal Tyr280 in pea leaf FNR, which contacts with the re-side of the isosalloxazine ring of FAD and moves away accompanied with the binding of nicotinamide (31). In addition, the main physiological role of leaf FNR is the reduction of the oxidized pyridine nucleotide, and the direction of the electron transfer between FAD and pyridine nucleotide is different from that of b5R. Therefore, it is considered that Thr66 in Pb5R contributes to the reduction of FAD and/or the stabilization of the reduced FAD. Shibabe et al. (32) reported that mutations of the corresponding Thr94 in human b5R affect oxidation of FAD, but the effects of the mutations on the properties of reduced FAD in the catalytic cycle have not been clarified.

To analyze the role of Thr66 in catalysis in Pb5R, we replaced Thr66 in Pb5R with serine (T66S), alanine (T66A), and valine (T66V) and analyzed the redox properties of FAD in the catalytic cycle using a stopped flow spectrophotometer. We present the catalytic cycle were modulated by the mutations of Thr66 in the oxidized enzyme-NADPb5R. In addition, we present a new model of the reaction scheme that the conversion of the neutral blue to the red semiquinone codon followed by the nucleotide sequence encoding the N-terminal tetrapetide of natural bovine liver b5 (39). The reverse primer was 5′-CTTCGGTTCATCCTTCTTCTGAGC-3′. This nucleotide sequence was complementary to the nucleotide sequence located 12–35 bases downstream after the stop codon in the cDNA of bovine liver b5 (39). The amplified DNA fragment was blunt-ended and inserted into the HindII site of plasmid pUC18, and plasmid pUSP5 was selected. Plasmid pUSP5 contained the nucleotide sequence encoding 133 amino acid residues from the N-terminal Ala3 to the C-terminal Asn133 of porcine liver b5. The deduced amino acid sequence was identical to that previously reported except for the difference at position 3 (40–42). The deduced amino acid residue at position 3 was glutamic acid rather than glutamic acid. The polypeptide containing 87 amino acid residues from Ala3 to Lys93 was prepared as recombinant Pb5. The cDNA encoding recombinant Pb5 was amplified from pUSP5 with the forward primer 5′-AGGAGGATATGCGGTAGAAGGAGTCC-3′ and the reverse primer 5′-CCGGCA-AGCCCTACTTGGCAATCTTGATC-3′. The fragment was inserted into the plasmid pCWori containing a HindIII site (underlined) and the mutagenic common reverse primer, 5′-HINCCGCCA-3′ (Fig. 1). This nucleotide sequence was complementary to the nucleotide sequence located 12–35 bases downstream after the stop codon in the cDNA of bovine liver b5 (39). The amplified DNA fragment was blunt-ended and inserted into the HindII site of plasmid pUC18, and plasmid pUSP5 was selected. Plasmid pUSP5 contained the nucleotide sequence encoding 133 amino acid residues from the N-terminal Ala3 to the C-terminal Asn133 of porcine liver b5. The deduced amino acid sequence was identical to that previously reported except for the difference at position 3 (40–42). The deduced amino acid residue at position 3 was glutamic acid rather than glutamic acid. The polypeptide containing 87 amino acid residues from Ala3 to Lys93 was prepared as recombinant Pb5. The cDNA encoding recombinant Pb5 was amplified from pUSP5 with the forward primer 5′-AGGAGGATATGCGGTAGAAGGAGTCC-3′ and the reverse primer 5′-CCGGCA-AGCCCTACTTGGCAATCTTGATC-3′, which corresponds to the C-terminal peptide, a stop codon, and a HindIII site (underlined). The resultant fragment was inserted into pCWori using HindIII and HindIII sites to construct pCPb5. E. coli TG1 cells containing pCPb5 were cultured in Luria-Bertani medium containing 50 μg/ml ampicillin at 37 °C. When the absorbance at 600 nm was ~0.3, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM, and the cultivation was continued for 14 h. The cells were lysed by sonication in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 2 mM phenylmethylsulfonil fluoride. The lysate was subjected to centrifugation at 15,000 × g for 20 min, and the supernatant was separated on a Seph-ax G-100 (Pharmacia) column equilibrated with 10 mM potassium phosphate (pH 7.0) (buffer A). Red colored fractions containing recombinant Pb5 were concentrated and desalted with a Sephadex G-25 (fine) (Pharmacia) column equilibrated with 100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 2 mM phenylmethylsulfonil fluoride. The yield of the purified protein from 1 liter of culture fluid was 5.9 mg. The N-terminal amino acid sequence analyzed with a Shimadzu PSQ-1 protein sequencer was Ala1–Val14–Lys17–Tyr19, and most of the additional N-terminal methionine residues were cleaved. The oxidized and reduced absorption spectra at 300–700 nm and the ability to accept electrons from the Pb5R were almost identical to those of the natural Pb5 (43).

**Protein Concentrations**—The molar extinction coefficients of the mutant proteins were evaluated by direct curve measurement using the appropriate molar extinction coefficient at 460 nm (ε460) determined by a method similar to that described by Aliverti and Zanetti (44), as previously described (33). The molar concentration of the WT was determined from the absorbance at 460 nm using the molar extinction coefficient, 1.02 × 105 M−1 cm−1 (33). The protein concentration of recombinant Pb5 was determined using the molar extinction coefficient of natural Pb5, 1.13 × 105 M−1 cm−1 at 415 nm (45).

**Spectral Analyses**—Absorption spectra were measured on a Hitachi U-2010 spectrophotometer. CD spectra were measured on a Jasco J-700 spectropolarimeter. Fluorescent emission spectra were measured in 10 mM potassium phosphate (pH 7.0) at 25 °C on a Hitachi F-3010 Fluoroscope spectrophotometer. The excitation wavelength was 460 nm, and 150 nm was observed. The CD spectra were measured at 22°C.

**Enzymatic Activity**—Steady-state enzymatic activities were measured as previously described (33). The apparent Km values for NADH (KmH for Pb5), ferricyanide (KmC), and recombinant Pb5 (KmPb5) and the catalytic constants (kcat, kcatH, kcatC, kcatPb5) were evaluated by direct curve

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fitting of the data according to the Michaelis-Menten equation. The $k_{\text{cat}, \text{NADH}}$, $k_{\text{cat}, \text{FAD}}$, and $k_{\text{cat}, \text{NO}}$ values were determined from the experiments to evaluate the $K_{\text{M}, \text{NADH}}$, $K_{\text{M}, \text{FAD}}$, and $K_{\text{M}, \text{NO}}$ values, respectively. The kinetic data were measured in 10 mM potassium phosphate (pH 7.0) at 25 °C. For the measurement of the $K_{\text{M}}$ values, 100 μM potassium ferricyanide was used as an electron acceptor. For the measurements of the $K_{\text{M}}$ values, 300 μM NADH was used as an electron donor. The reduction rate of ferricyanide was measured at 420 nm using a molar extinction coefficient of 1.02 × 10^4 M^−1 cm^−1. The reduction rate of recombinant Pb5 was measured at 556 nm using a difference in molar extinction coefficient of natural Pb5 between the oxidized and reduced states, 1.9 × 10^4 M^−1 cm^−1 (43). The concentration of NADH was determined using a molar extinction coefficient, 6.3 × 10^3 M^−1 cm^−1 at 340 nm.

Stopped Flow Measurements—Rapid reaction was analyzed with a Phtal RA-401 stopped flow spectrophotometer (Otsuka Electronics) equipped with a Lauda RMS thermostatically regulated circulating water bath in 10 mM potassium phosphate (pH 7.0) at 25 °C. The spectral changes of enzymes during and after the electron transfer from NADH to ferricyanide were analyzed as follows. Equal volumes of the enzyme solution containing potassium ferricyanide and the NADH solution were rapidly mixed, and the rapid scan spectra at 460–800 nm and the time courses of the absorbance changes at 460, 530, and 620 nm were measured. The initial concentrations of NADH, oxidized enzyme, and potassium ferricyanide in the reaction mixtures were 1 mM, 10 mM potassium phosphate (pH 7.0). The solutions were made anaerobic with 5 mM EDTA, 200 mM NADH, and 10 mM potassium phosphate (pH 7.0). The solutions were made anaerobic by successive flushing with oxygen-free argon gas with gentle agitation for more than 50 min. The absorption spectra were observed before and after illumination at 25 °C with a 300 W halogen lamp at room temperature.

Measurement of Dissociation Constants—The dissociation constant of the oxidized enzyme for NAD⁺ ($K_{\text{D,NAD}^+}$) was determined by measuring the perturbation of the flavin spectrum as previously described (28). Approximately 40 μM of oxidized mutant proteins were titrated with NAD⁺ in 10 mM potassium phosphate (pH 7.0) at 25 °C. After the successive addition of NAD⁺ into both the sample and reference cells, the absorption spectra were measured. The $K_{\text{D}}$ values were determined by direct curve fitting of the difference in the absorbance at the wavelength, where the absorbance change caused by the addition of NAD⁺ was largest in the 400–500-nm region, with the theoretical equation for a 1:1 binding mechanism taking into account the dilution.

RESULTS

Purification of Mutant Proteins—All of the purified mutant Pb5Rs showed a single band on an SDS-PAGE gel that was located at the same position as the WT. The yields of the purified T66S, T66A, and T66V mutants from 1 liter of culture fluid were 15.7, 20.3, and 14.5 mg, respectively. In all mutant proteins, the only bound flavin detected on the TLC plate was FAD. The molar extinction coefficients of the T66S, T66A, and T66V mutants at 460 nm were similar to that of the WT and were 1.05 × 10^4, 1.01 × 10^4, and 1.06 × 10^4 M^−1 cm^−1, respectively.

Spectral Properties of Oxidized Proteins—The absorption spectrum of the T66S mutant was blue-shifted with a 300 W halogen lamp at 25 °C with a 300 W halogen lamp at 25 °C.

Steady-state Kinetic Parameters and Dissociation Constant for NAD⁺—The apparent steady-state kinetic parameters and the $K_{\text{cat}}$ values are shown in Table I. The $K_{\text{m}}$ values of the T66S, T66A, and T66V mutants were similar to that of the WT, indicating that the mutations in these proteins did not affect the apparent affinity for NADH. The $K_{\text{cat}}$ values of the T66S and T66A mutants were almost identical to that of the WT, but slight spectral changes were observed (Fig. 2, panels b and c). The absorption spectrum of the T66V mutant was blue-shifted with a 300 W halogen lamp at 25 °C with a 300 W halogen lamp at 25 °C.

The changes in the $K_{\text{cat}}$ values of the three mutants were similar to that of the WT. The changes in the $K_{\text{cat}}$ values of the three mutants were similar to that of the WT. The changes in the $K_{\text{cat}}$ values of the three mutants were similar to those in the $K_{\text{d,NAD}^+}$ values. Only the mutation of Thr⁶⁶ to valine significantly affected the electron transfer from NADH to ferricyanide.

The apparent $K_{\text{m}}$ and $K_{\text{cat}}$ values were evaluated using recombinant Pb5. The $K_{\text{m}}$ value of the T66S mutant was similar to that of the WT. The $K_{\text{m}}$ values of the T66A and T66V mutants were 6.3- and 5.6-fold of that of the WT, respectively. The $k_{\text{cat}}$ value of the T66S mutant was similar to that of the WT, but the $k_{\text{cat}}$ value of the T66A and T66V mutant were 34 and 4.2% of that of the WT, respectively. The mutations of Thr⁶⁶ to alanine and valine affected not only the rate of the electron transfer but also the apparent affinity for Pb5.
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Fig. 2. Absorption and CD spectra of oxidized Pb5Rs. A, absorption spectra of the T66S mutant (panel a), the T66A mutant (panel b), and the T66V mutant (panel c) (solid line) are shown in comparison with the spectrum of the WT (broken line). The concentrations of proteins were 10 μM. B, CD spectra of the WT (broken line), the T66S mutant (solid line), and the T66A mutant (dotted line). For the spectra at 200–250 nm, the protein concentration was 10 μM, and the optical path length was 2 mm. For the spectra at 250–550 nm, the protein concentration was 20 μM, and the optical path length was 10 mm. Values of a molecular ellipticity were calculated based on the molar concentrations of the protein solutions. The absorption and CD spectra were measured in 10 mM potassium phosphate (pH 7.0) at 25 °C.

Table I

| Enzyme | NAD<sup>H</sup> | Ferricyanide | Pb5<sup>b</sup> | NAD<sup>AD</sup> |
|--------|-----------------|--------------|-----------------|-----------------|
|        | Km<sup>cat</sup> | k<sup>cat</sup> | K<sup>D</sup> | k<sup>D</sup> | K<sup>AD</sup> |
|        | μM              | μM s<sup>-1</sup> | s<sup>-1</sup> | μM          | μM          |
| WT     | 3.1 ± 0.3       | 1100 ± 84     | 2.5 ± 0.3       | 822 ± 29      | 19.8 ± 26    | 911 ± 92    | 77 ± 22*    |
| T66S   | 2.8 ± 0.5       | 1150 ± 160    | 3.1 ± 0.4       | 976 ± 42      | 13.3 ± 3.7   | 705 ± 74    | 128 ± 4     |
| T66A   | 2.9 ± 0.2       | 984 ± 30      | 2.2 ± 0.7       | 684 ± 46      | 125 ± 5.3    | 314 ± 8.0   | 59 ± 3      |
| T66V   | 2.8 ± 0.4       | 100 ± 3.0     | 1.6 ± 0.3       | 77 ± 2.0      | 111 ± 15     | 38 ± 3.0    | 394 ± 61    |

* 100 μM ferricyanide was used as an electron receptor.

b 300 μM NAD<sup>H</sup> was used as an electron acceptor.

* The value previously reported (28).

The K<sup>AD</sup> value of the T66V mutant was 5.1-fold of that of the WT, whereas those of the T66S and T66A mutants were similar to that of the WT. The substitution of Thr<sup>66</sup> by valine caused a decrease in the affinity for NAD<sup>H</sup>.

Rapid Scan Spectra during and after Turnover—The spectra of the WT and mutant enzymes during and after the turnover were directly analyzed by rapid scan analysis (Fig. 3). In this experiment, a turnover phase and a subsequent reduction phase by NADH after the consumption of ferricyanide were observed.

In the case of the WT and the T66S and T66A mutants, the rapid scan spectra in the turnover phase showed significant absorption at 460–540 nm in addition to the broad absorption around 620 nm (Fig. 3A, panels a–c). Absorption at these wavelengths decreased simultaneously (Fig. 3B, panels a–c). The rate constant (k<sub>cat</sub>) values were consistent with the k_cat values (the k<sup>cat</sup>(<sup>AD</sup>) values and k<sub>cat</sub>(<sup>F</sup>) values) (Table II). These data indicate that in the WT and the T66S and T66A mutants, the spectrum observed in the turnover phase is due to the intermediate, which contains oxidized FAD, and that the conversion of the intermediate is the rate-limiting step.

In the turnover phase, the T66V mutant showed peaks at 530 and 620 nm but did not show significant absorption at ~460 nm (Fig. 3A, panel d). This spectral shape was obviously different from those of the WT and the other mutants and was characteristic of a neutral blue semiquinone form. Spectra similar to that of a neutral blue semiquinone, which has peaks at ~500 and 600 nm, were observed in the flavodoxins and their mutants (46–48). Accompanied with the consumption of ferricyanide, both peaks disappeared simultaneously (Fig. 3B, panel d). The k value at 530 nm was almost identical to that at 620 nm (Table II). These data indicate that in the T66V mutant, the spectrum observed in the turnover phase is due to the neutral blue semiquinone and that the conversion of the neutral blue semiquinone is the rate-limiting step.

Photoreduction—The enzymes were anaerobically photoreduced in the presence of NAD<sup>H</sup> to analyze the statically stable semiquinone form (Fig. 4). The enzymes showed biphasic absorbance changes at 375 and ~460 nm by successive photoreduction and resulted in the spectra of fully reduced forms. In the case of the WT, absorbance at 460 nm was decreased, and peaks at 375 nm and near 530 nm were increased after photoreduction for 5 min (Fig. 4a). This indicates the formation of the anionic red semiquinone (3). The peaks near 375 nm, which were characteristic of the anionic red semiquinone, were observed in the spectra of the T66S, T66A, and T66V mutants after photoreduction for 3, 5, and 4 min, respectively (Fig. 4,
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Fig. 3. Rapid scan spectra of Pb5Rs and time courses of the spectral changes. A, rapid scan spectra of the WT (panel a), the T66S mutant (panel b), the T66A mutant (panel c), and the T66V mutant (panel d) in the turnover phase (spectra 2) and after the turnover (spectra 1) and after the turnover (spectra 2) in 10 mM potassium phosphate (pH 7.0) at 25 °C. B, time courses of the absorbance changes at 460, 530, and 620 nm accompanied with the consumption of ferricyanide. The WT (panel a), the T66S mutant (panel b), the T66A mutant (panel c), and the T66V mutant (panel d). The dotted lines are the results of single exponential curve fitting. In a–d, spectra 1 and 2 in A were measured at the time indicated by arrows 1 and 2 in B, respectively. The initial concentrations of NADH, oxidized enzyme, and potassium ferricyanide in the solution just after the mixing were 1 mM, 10 μM, and 150 μM, respectively.

TABLE II
Apparent rate constants evaluated from absorbance changes

| Enzyme | 460 nm | 530 nm | 620 nm | k_{cat}^{NAD^+} | k_{red}^{NAD^+} | k_{cat}^{NADH} | k_{red}^{NADH} |
|--------|--------|--------|--------|--------------|---------------|-------------|--------------|
| WT     | 492 ± 24 | 647 ± 101 | 597 ± 52 | 553 ± 2.8 | 505 ± 13 | 1100 ± 84 | 822 ± 29 |
| T66S   | 548 ± 34 | 597 ± 37 | 554 ± 46 | 492 ± 6.8 | 465 ± 15 | 1150 ± 160 | 976 ± 42 |
| T66A   | 535 ± 21 | 535 ± 39 | ND     | 447 ± 18 | 758 ± 80 | 984 ± 30 | 684 ± 46 |
| T66V   | 38 ± 13  | 21 ± 1.0 | 22 ± 2.0 | 516 ± 25 | 439 ± 22 | 100 ± 3.0 | 77 ± 2.0 |

a Data in Table I.
ND, not determined because of the very small absorbance change.

b–d). In these three mutants, the stable one-electron reduced form was an anionic red semiquinone.

Reduction of Enzymes without Turnover—The WT and mutant enzymes were reduced with NADH in the presence and absence of NAD^+ to identify the intermediates observed in the rapid scan spectra of the WT and the T66S and T66A mutants during the turnover with ferricyanide (Figs. 5 and 6). In the presence of 1 mM NAD^+, more than ~70% of the T66V mutant and 90–95% of the other enzymes are in the oxidized enzyme-NAD^+ complex as judged from the K_{cat}^{NAD^+} values in Table I, and these complexes were reduced with NADH. In these experiments, only the last 10–20% of the reduction phases were observed just after the dead time of the instrument (Figs. 5A and 6A).

In both the presence and the absence of NAD^+, spectra of the WT observed during the reduction with NADH showed a large absorption peak at 460 nm with a shoulder at 490 nm and a broad absorption around 620 nm, all of which decreased simultaneously (Figs. 5B, panel a, and 6B, panel a). These spectral features were identical to that observed in the turnover phase (Fig. 3A, panel a). In the WT, the values of the rate constant of reduction (k_{red}) in the presence of NAD^+ (k_{red}^{NAD^+}) and in the absence of NAD^+ (k_{red}^{NAD}) were in good agreement with the halves of k_{cat} values and were consistent with the k_{red} values (Table II). These data suggest that the spectrum of the WT observed in the turnover phase is due to the oxidized WT-NADH complex. The absorbance changes at 460 nm, which were observed after the flow was stopped, both in the presence and absence of NAD^+, were ~0.1 and 0.06, respectively (Figs. 5A, panel a, and 6A, panel a). These differences in the absorbance changes indicate that the reduction of the oxidized WT-NADH complex was delayed in the presence of 1 mM NAD^+.

In the case of the T66S mutant, the spectrum observed during the reduction with NADH in the absence of NAD^+ showed an obvious shoulder at 490 nm and a broad peak of absorption at ~620 nm (Fig. 6B, panel b). This spectral feature
was identical to that observed in the turnover phase (Fig. 3A, panel b). In the T66S mutant, the $k_{\text{red}}^{\text{NAD}}$ and $k_{\text{red}}^{\text{NAD}}$ values were in good agreement with the halves of $k_{\text{cat}}$ values (Table II). These data indicate that the spectrum observed in the turnover phase is due to the oxidized T66S-NADH complex. The shoulder at 490 nm was not observed in the spectrum during the reduction with NADH in the presence of NAD$^+$ (Fig. 5B, panel b). The absence of the shoulder was probably due to the formation of the oxidized T66S-NADH-NAD$^+$ ternary complex. Such a ternary complex is considered in the T66A mutant.
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Fig. 6. Reduction of Pb5Rs with NADH in the absence of NAD\textsuperscript{+}. A, absorbance changes of the WT (panel a), the T66S mutant (panel b), the T66A mutant (panel c), and the T66V mutant (panel d) at 460 nm. In a–d, mixing of the enzyme solution and the NADH solution was started at time 0, and the flow was stopped at the time indicated by the arrows. Initial concentrations of oxidized enzyme and NADH in the reaction mixture were 50 \mu M and 1 mM, respectively. The broken lines are the results of single exponential curve fitting of the data corresponding to the reduction of FAD. Evaluated reduction constants (k_{red}\textsuperscript{NAD}) are shown in Table II. B, rapid scan spectra; spectra of the WT at 13,15, 17, 19, 21, 25, 35 ms (panel a), the T66S mutant at 13,15, 17, 19, 21, 25, and 35 ms (panel b), the T66A mutant at 13,15, 17, 19, and 27 ms (panel c), and the T66V mutant at 13, 15, 17, 19, 21, 25, 29, and 35 ms (panel d). Insets, magnified views of the spectra at 580–720 nm. The directions of the spectral changes are indicated with arrows. The spectra and the absorbance changes were measured in 10 mM potassium phosphate (pH 7.0) at 25°C. Also (see below). The absorbance changes at 460 nm, which were observed after the flow was stopped, both in the presence and absence of NAD\textsuperscript{+}, were $\sim$0.12 and 0.08, respectively (Figs. 5A, panel b, and 6A, panel b). The spectra of the T66A mutant observed during reduction with NADH in the presence of NAD\textsuperscript{+} showed absorption peaks at 460 nm without an obvious shoulder at 490 nm (Fig. 5B, panel c) as seen in the spectrum in the turnover phase (Fig. 3A, panel c). In the T66A mutant, the k_{red}\textsuperscript{NAD} value was roughly half of the k_{cat} values and was consistent with the k_{cat} values (Table II). However, in the absence of NAD\textsuperscript{+}, an obvious shoulder at 490 nm was observed in the spectra of the T66A mutant (Fig. 6B, panel c). This shoulder was observed neither in the spectrum in the turnover phase (Fig. 3A, panel c) nor in the spectrum of the oxidized T66A-NAD\textsuperscript{+} complex (Fig. 7A, panel c). In the T66A mutant, the k_{red}\textsuperscript{NAD} value was 1.7-fold of the k_{red}\textsuperscript{NAD} value and larger than the halves of k_{cat} values (Table II). The absorbance changes at 460 nm, which were observed after flow was stopped, both in the presence and absence of NAD\textsuperscript{+}, were $\sim$0.13 and 0.045, respectively (Figs. 5A, panel c, and 6A, panel c). These differences in the absorbance changes are consistent with the result that the k_{red}\textsuperscript{NAD} value was larger than the k_{red}\textsuperscript{NAD} value. These results suggest that the T66A mutant in the turnover phase (Fig. 3A, panel c) is neither the spectrum of the T66A-NADH complex nor that of the T66A-NAD\textsuperscript{+} complex. Probably, the spectrum of the T66A mutant observed in the turnover phase is due to the T66A-NADH-NAD\textsuperscript{+} ternary complex, and the release of NAD\textsuperscript{+} from the ternary complex is the rate-limiting step. It is considered that the ternary complex has such a structure that both NADH and NAD\textsuperscript{+} bind simultaneously to the oxidized T66A mutant mainly with the 5\textsuperscript{-}ADP-ribose moiety of NADH and with the oxidized nicotinamide moiety of NAD\textsuperscript{+}, respectively.

In the case of the T66V mutant, the spectra observed during reduction with NADH in the presence and absence of NAD\textsuperscript{+} showed a large absorption peak at 460 nm, with a shoulder at 490 nm and a broad region of absorption around 620 nm, all of which decreased simultaneously (Figs. 5B, panel d, and 6B, panel d). These spectral features are similar to those of the WT and are different from the spectrum observed in the turnover phase (Fig. 3A, panel d). The k_{red}\textsuperscript{NAD} and k_{red}\textsuperscript{NAD} values of the T66V mutant were larger than the k_{cat} values of this mutant and roughly similar to the k_{red}\textsuperscript{NAD} and k_{red}\textsuperscript{NAD} values of the WT and T66S mutant (Table II). These results indicate that the substitution of Thr\textsuperscript{66} by valine had very little effect on the reduction of the enzyme.

Spectral Changes Caused by Binding of NAD\textsuperscript{+}, 5\textsuperscript{-}ADP-ribose, and NADH—To analyze the contribution of the 5\textsuperscript{-}ADP-ribose moiety to the binding of pyridine nucleotide, NAD\textsuperscript{+}, 5\textsuperscript{-}ADP-ribose, and NADH were added to the oxidized enzymes as shown in Fig. 7. The spectral changes of the T66S and T66A mutants caused by the addition of NAD\textsuperscript{+} were large, whereas those of the WT and T66V mutant were small (Fig. 7A). These spectral changes are due to the formation of the oxidized enzyme-NAD\textsuperscript{+} complexes. The features of the spectral changes caused by the addition of NAD\textsuperscript{+} were similar, but the degrees of the spectral changes were different. The difference spectra of the WT and mutant enzymes showed a positive peak around 513 nm and negative peaks around 459 and 490 nm. These spectra of the oxidized enzyme-NAD\textsuperscript{+} complexes were changed...
by the addition of 5'-ADP-ribose, which lacks a nicotinamide moiety. The spectra of the WT and mutant enzymes obtained after the addition of 1 mM NAD\(^+\), 1 mM 5'-ADP-ribose (broken line), and 1 mM NADH (broken line with dot) in this order to the WT (panel a), the T66S mutant (panel b), the T66A mutant (panel c), and the T66V mutant (panel d) (solid line). B, spectra after the addition of 1 mM 5'-ADP-ribose (dotted line), 1 mM NAD\(^+\) (broken line), and 1 mM NADH (broken line with dot) in this order to the WT (panel a), the T66S mutant (panel b), the T66A mutant (panel c), and the T66V mutant (panel d) (solid line). Insets, difference spectra calculated by subtracting the spectra of the oxidized enzymes (solid line).
form is the rate-limiting step. However, the anaerobic photoreduction spectra of the T66V mutant in the presence of NAD$^+$ indicated the presence of the anionic red semiquinone form (Fig. 4). It is considered that the neutral blue semiquinone form converts to the red semiquinone form during turnover, and this step is the rate-limiting step. In the T66V mutant, only the hydroxyl group of the Thr$^{66}$ in the WT was replaced with a methyl group. The blue neutral semiquinone of FAD (FADH$^+$) has a hydrogen atom on the N5 atom of the isoxaloxazine ring, and release of a proton from the N5 position is required for the conversion of the blue neutral semiquinone to the anionic red semiquinone (49).

The substitution of Thr$^{66}$ by valine is considered to be unfavorable for the effective proton release from the N5 position of the neutral blue semiquinone of FAD. Kobayashi et al. (13) observed the conversion of the blue semiquinone to the red semiquinone form of the WT using pulse radiolysis below pH 6.5, and only the stable red semiquinone appeared at 200 μs after pulse above pH 7.0. These data suggest that at pH 7.0 the blue semiquinone form of the WT is unstable, and the conversion of the blue to red semiquinone is very fast.

The spectrum of the T66A mutant, which was observed during the turnover (Fig. 3A, panel c), was assigned to be the oxidized enzyme-NADH-NAD$^+$ ternary complex. Those of the WT and the T66S mutant (Fig. 3A, panels a and b) were assigned as the oxidized enzyme-NADH complexes, but the spectra of the T66S mutant observed during the reduction with NADH in the presence of 1 mM NAD$^+$ were assigned as the oxidized T66S-NADH-NAD$^+$ ternary complex (Fig. 5B, panel b). These data suggest that the oxidized enzyme-NADH complex is produced via the oxidized enzyme-NADH-NAD$^+$ complex, and these complexes could be involved in the catalytic cycle of Pb5R.

Based on these data, we present here a new model of the reaction sequence of b5R containing the neutral blue semiquinone form and the oxidized enzyme-NADH-NAD$^+$ ternary complex as intermediates (Scheme II). This model contains the following processes: (i) formation of the oxidized enzyme-NADH complex (E-FAD-NADH), (ii) conversion of E-FAD-NADH to a form that has the ability to transfer H$^+$ (E-FAD-NADH$^+$), (iii) H$^+$ transfer from NADH to FAD, (iv) the first one-electron transfer from the two-electron reduced enzyme complex (E-FADH$^+$-NAD$^+$), (v) rapid conversion of the neutral blue semiquinone form (E-FADH$^+$-NAD$^+$) to the anionic red semiquinone form (E-FADH$^+$-NAD$^+$), (vi) the second one-electron transfer from E-FADH$^+$-NAD$^+$, (vii) formation of the oxidized enzyme-NADH-NAD$^+$ ternary complex (E-FADH-NADH-NAD$^+$) by binding of NADH, and (viii) release of NAD$^+$. Although there is no direct evidence for the conversion of E-FAD-NADH to E-FADH-NAD$^+$ (process (ii)), the existence of the E-FAD-NADH complex, which has no ability to transfer H$^+$, is a reasonable assumption. This is because H$^+$ transfer itself is generally very fast, and a two-step mechanism for pyridine nucleotide binding has been proposed for the related family enzymes, nitrate reductase (50), pthalate dioxygenase reductase (51, 52), and FNR (31). In the WT and the T66S mutant, the rate-limiting step is process (ii). In the T66A mutant, the rate-limiting step is process (vii), and the rate of process (i) is faster than that of process (viii). In the T66V mutant, the rate-limiting step is process (v).

This model reasonably interprets the data presented here, but more investigations are required before this model is established.

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