Characterization of the Role of Lysine 110 of NADH-Cytochrome $b_5$
Reductase in the Binding and Oxidation of NADH by Site-directed Mutagenesis*

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An expression vector for bovine NADH-cytochrome $b_5$ reductase was used for site-directed mutagenesis of lysine 110, the residue previously implicated in NADH interactions with this flavoprotein. Replacement of this basic residue with an uncharged glutamine resulted in an increase of 3 orders of magnitude in the $K_m$ for NADH and a decrease in $k_{cat}$ of an order of magnitude, strongly implicating lysine 110 in both binding of NADH to the reductase and the orientation of the reduced nicotinamide group for rapid hydride ion transfer to the flavin. Substitution of lysine 110 by histidine, to provide a pH-sensitive positive charge at this position in the neutral pH range, exhibited only a moderate 25-fold increase in $K_m$ and a normal $k_{cat}$ at pH 6.0, whereas at pH 8.5 the $K_m$ for NADH rose to 238 μM with a decrease of 45% over unmodified enzyme in the $k_{cat}$. A similar pH sensitivity in the inhibition constant for adenosine diphosphate ribose, lacking only the nicotinamide moiety of NADH, emphasizes the crucial role of the positive charge at this locus and is consistent with charge-pairing of lysine 110 with the pyrophosphate group of NADH or adenosine diphosphate ribose.

Cytochrome $b_5$ reductase plays a central role in utilizing cytoplasmically generated NADH (1, 2) to reduce cytochrome $b_5$ and initiate a spectrum of reductive reactions on the surface of liver endoplasmic reticulum in which the hemeprotein serves as electron donor (3-7). Two recent studies (8, 9) focused on the details of the interactions between the reductase and cytochrome $b_5$ required for rapid electron transfer from reduced flavoprotein to the heme. In the first (8), the fact that the flavoprotein and heme protein are characterized by a common structural feature of a membrane-binding hydrophobic domain that serves to orient each catalytic domain at the membrane-aqueous surface to permit rapid electron transfer (2, 10), was used to carry out specific cross-linking of active-site lysyl residues of the reductase with complementary carboxylate residues of the cytochrome. Sequence analysis of peptides from such cross-linked preparations revealed that reductase Lys41 and Lys110 were cross-linked to Asp or Glu residues surrounding the exposed ε-amino edge in cytochrome $b_5$. These results, and similar identification of cross-linking of reductase Lys41 and the carboxylate of a heme propionyl group identified complementary charge pairing of these residues as the probable interactions in normal rapid electron transfer from the flavin to the heme of the two proteins. This possibility was subsequently confirmed (9) using an expression vector for cytochrome $b_5$ reductase to carry out site-directed mutagenesis of the 3 lysine residues. The observed marked decreases in catalytic efficiencies by changing the positive charges of each lysine residue to a negative or uncharged residue are consistent with their participation in charge pairing with carboxyl groups at the active site of cytochrome $b_5$.

Early kinetic and chemical modification studies also focused on the involvement of a specific lysine residue in the interaction of NADH with cytochrome $b_5$ reductase (11, 12). The reduction of the reductase by hydride ion transfer from NADH to form a stable reduced flavoprotein-NAD complex (11) was shown to protect the ε-amino group of a single lysine residue from reaction with either ethyl acetimidate or acetic anhydride (12). Moreover, whereas amidination of the reductase in the absence of NADH resulted in only a modest increase in the $K_m$ for NADH and decrease in $k_{cat}$, removal of the positive charge of lysine residue by acylation in the absence of NADH increased the $K_m$ for NADH approximately 3 orders of magnitude and decreased the $k_{cat}$ almost an order of magnitude. Since the binding of adenosine diphosphate ribose, which lacks only the nicotinamide ring of NADH, to the reductase (12) was similarly inhibited by these modifications of a single lysine residue, charge pairing of the ε-amino group with the pyrophosphate group of NADH was implied. Subsequent acylation of this lysine residue and sequence analysis of proteolytic peptide fragments identified this residue as Lys110 (13).

In the experiments described below, we have utilized the bacterial expression vector for cytochrome $b_5$ reductase (9) to replace Lys110 with either Glu or His, and to isolate the mutant flavoproteins from transformed Escherichia coli cultures. All reductase preparations, as well as cytochrome $b_5$ preparations employed here, were subjected to mild tryptic digestion to obtain the catalytic domains of each protein, designated flavopeptide and hemepeptide, respectively, to compare the kinetic parameters of native and mutant reductases. These data show a marked alteration in both the $K_m$ for NADH and $k_{cat}$ for the Glu substitution and a lesser, but pH dependent, effect of histidine substitution in the pH range from 6 to 8.5.

EXPERIMENTAL PROCEDURES

Materials and Methods—Beef cytochrome $b_5$ was purified from Black Angus steer liver as described previously (14), and mild tryptic digestion at 4°C was used to prepare hemepeptide (15). NADH-cytochrome $b_5$ and NADH-ferricyanide reductase activities were used

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M81759.

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to monitor reductase concentrations during purification as described (16). Cytochrome b$_5$ and hemepeptide concentrations were determined by the absorbance at 413 nm (10) and reductase and flavopeptide concentrations, by the absorbance at 460 nm (17). The Cyclone DNA synthesizer of Biosearch Inc. was used for oligonucleotide synthesis. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (18). Sephadex G-25-150, NAD-agarose, NADH, and adenosine diphosphate ribose were from Sigma; Centri- con-30 micro-concentrators were from Amicon; TPCK-trypsin was from Worthington; and DEAE-cellulose (DE52) was from Whatman.

Restriction enzymes were purchased from U. S. Biochemical Corp. or New England Biolabs. The Klenow fragment of DNA polymerase I of E. coli was obtained from New England Biolabs; calf alkaline phosphatase and T4 DNA ligase were from Pharmacia; and the Klenow fragment of DNA polymerase I from New England Biolabs was eluted with Tris acetate, pH 8.1. The eluate was then subjected to a second adsorption on a 15 ml column of NADH-agarose in a 2.5-cm column equilibrated with 0.1 M Tris acetate, pH 8.1, or other pH values as indicated. The values for $K_a$ and $k_{cat}$ are expressed in terms of a 1-electron reduction of either electron acceptor. Hemepeptide reduction was followed at 420 nm, and ferricyanide reduction, at either 430 or 420 nm at high NADH concentrations. The inhibition constant for adenosine diphosphate ribose ($K_i^{(DP)}$) was determined by Lineweaver-Burk plots of data obtained in the presence and absence of this nucleotide.

RESULTS AND DISCUSSION

The spectra of the two mutant flavopeptide preparations pR1Δ110Q and pR1Δ110H (Fig. 2), in which Lys$^{110}$ is replaced by either Gln or His, are indistinguishable from the unmodified enzyme isolated from pR1 (9). However, the kinetic parameters for the two enzymes (Table II), with only a single amino acid variation at residue 110, show marked increases in $K_a$ values for NADH and decreases in $k_{cat}$ values with either the hemepeptide of cytochrome b$_5$ or potassium ferricyanide as electron acceptor. Significantly, the $K_a$ values for the electron acceptors are only slightly altered, emphasizing the specific requirement of Lys$^{110}$ for interaction with NADH. This contrasts with the observations that modification of Lys$^{110}$ as the residue that is protected from group specific reagent modification by NADH. This result is consistent with the earlier studies (12, 13) that identified Lys$^{110}$ as the residue that is protected from group specific reagent modification by NADH. This result is consistent with the earlier studies (12, 13) that identified Lys$^{110}$ as the residue that is protected from group specific reagent modification by NADH.

The effect of pH on the kinetics of NADH oxidation catalyzed by flavopeptide isolated from pR1Δ110H is consistent with this assumption (Fig. 3). In the pH range from 6 to 8.5, which has previously been shown to yield approximately 80 to 100% of $V_{max}$ with the unmodified beef liver enzyme (20), the $K_a$ for NADH in the mutant reductase assays increased over 7-fold from pH 6 to 8.5, with a concomitant decrease in $k_{cat}$ of 55%. This results in a decrease in catalytic efficiency of more than an order of magnitude. The most acute change corresponds to the titration of the NADH-agarose column during the wash, prior to elution, that removes significant contaminating proteins.

**TABLE I**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Synthetic oligonucleotides used to isolate mutant flavopeptides</th>
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<tbody>
<tr>
<td>pR1Δ110Q</td>
<td>Lys$^{110}$ → Gln$^{110}$</td>
</tr>
<tr>
<td>pR1Δ110H</td>
<td>Lys$^{110}$ → His$^{110}$</td>
</tr>
<tr>
<td></td>
<td>AAG → TAG</td>
</tr>
<tr>
<td></td>
<td>AAG → CAT</td>
</tr>
<tr>
<td></td>
<td>TGAATGAAACTGGATGACCAGGTC</td>
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<td></td>
<td>TGAATGAAACTGGATGACCAGGTC</td>
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$^a$ The underline indicates the base changes. The abbreviation used is: TPCK-trypsin, Na-$p$-tosyl-$p$-phenylalanine chloromethyl ketone-treated trypsin.

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assayed at pH 8.1. The limited decrease in catalytic efficiency, as the proton of the imidazole group is titrated at higher pH values, suggests that binding of NADH may result in sufficient shielding of the imidazole group from water molecules to affect a transitory increase in its pK during each catalytic cycle of oxidation.

The pH sensitivity of the inhibition of NADH oxidation catalyzed by the His110 flavopeptide by adenosine diphosphate ribose (Table III) provides additional evidence that charge complementation occurs between the pyrophosphate group of FAD, in a comparable position in the catalytic mechanism as proposed previously by Strittmatter (11, 24), but rather, that both cysteine residues homologous to cytochrome bs reductase resulted in a 13-28-fold increase in the $K_m$ for NADH, a 2-fold decrease in $k_{cat}$, and a corresponding decrease in catalytic efficiency of approximately 50-fold. These effects were attributed to a structural role for Ser27 in maintaining the tertiary structure necessary for the NADH-binding site. The second study by Shirabe et al. (23) focused on the possible roles of Cys20, the residue included among the conserved residues in flavoproteins homologous to cytochrome bs reductase, as well as Cys385. Their detailed experiments showed that Ser replacement of either or both Cys residues had little effect on NADH oxidation and Ala substitution at Cys23 resulted in only a 4-5-fold decrease in $k_{cat}$. They concluded that Cys20 was not directly involved in the catalytic mechanism as proposed previously by Strittmatter (11, 21, 24), but rather, that both cysteine residues are near the NADH-binding site.

The recent report on the crystal structure of ferredoxin-NADP reductase by Karplus et al. (25), in which they include a discussion of implied structural similarities in a growing family of flavoprotein reductases based upon sequence similarities (26-28), offers additional opportunity for defining the role of Lys110 of cytochrome bs reductase in hydride ion transfer to FAD. The model for spinach ferridoxin reductase at 3.7-Å resolution identified separate FAD and NADP-bind-
phospho-AMP binding to ferridoxin reductase involves ionic or  the reduced nicotinamide binding data do suggest that Lys can serve to directly complex with cytochrome reductase was not obtained for technical reasons, the analog AMP. At the same time, the peptide bond NH and CO groups completion of the initial x-ray structure including the NADH interactions of Lys with the 5'phosphate of 2'-phospho-AMP (25) with corresponding that includes 3 identical and 4 similar amino acids. Of particular interest in the ferridoxin reductase structure is that 2'phospho-AMP binding to ferridoxin reductase involves ionic interactions of Lys with the 5'phosphate of 2'-phospho-AMP. At the same time, the peptide bond NH and CO groups of Lys are also involved in hydrogen bonding to FAD. Although the structure of the NADP complex with ferridoxin reductase was not obtained for technical reasons, the analog binding data do suggest that Lys may serve to directly orient the reduced nicotinamide C4 atom opposite the N5 atom of FAD for rapid hydride ion transfer as was shown in the case of glutathione reductase (30, 31). Our experiments have identified Lys of NADH-cytochrome b reductase as the residue that may fulfill this function. Fig. 4 shows that the amino acid sequence is again similar to that of Lys of ferridoxin reductase. Of 9 residues, 3 are identical and 4 represent conserved substitutions. A rigorous test of this suggested role for Lys of cytochrome b reductase awaits detailed binding studies of NADP to ferridoxin reductase and completion of the initial x-ray structure including the NADH complex with cytochrome b reductase (32).

REFERENCES
29. Deleted in proof

FIG. 4. Comparison of the sequences of ferridoxin-NADP reductase involved in FAD binding and interactions with the 5’-phosphate of 2’-phospho-AMP (25) with corresponding sequences of NADH-cytochrome b reductase (9). Boxed residues are identical and underlined residues show conservative differences.