Purification and Analysis of a Flavoprotein Functional as NADH Oxidase from Amphibacillus xylanus Overexpressed in Escherichia coli*

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The gene encoding the Amphibacillus xylanus flavoprotein has been cloned into pITTQ18 and overexpressed in Escherichia coli. The recombinant enzyme has been purified to homogeneity yielding 15 mg of pure enzyme/liter of cell culture. Recombinant flavoprotein is fully active and has an absorption spectrum identical to that of the enzyme purified from A. xylanus. The N-terminal sequence analysis and analytical gel filtration data confirm the structural identity of recombinant and A. xylanus enzymes. The K₅₀ value for oxygen and the K₅₀ value for NADH are 1.7 mm and 33.3 μM, respectively. In the presence of free additional FAD, however, the K₅₀ value for oxygen decreases dramatically. The NADH oxidase activity is accelerated markedly in the presence of additional FAD. The intracellular free FAD concentration of A. xylanus is calculated to be about 13 μM. This FAD concentration would be enough to accelerate the NADH oxidase activity of flavoprotein in cells of A. xylanus. Two-electron reduction of the enzyme FAD by the strong reductive dithionite occurs during the total uptake of 6 electrons. Such behavior usually indicates the presence of non-flavin redox centers. The high degree of homology between this enzyme and alkyl hydroperoxide reductase F52a protein suggests that these centers are the redox-active disulfide adjacent to the FAD and another disulfide, which is able to slowly interchange with the redox-active disulfide. The presence of two disulfides has been demonstrated.

We recently isolated a new group of facultative anaerobic bacteria from an alkaline compost (11). The bacteria have a unique phenotype and chemotaxonomic characteristics, and are named Amphibacillus xylanus (2). A. xylanus grows at the same rate and with the same cell yield, both under strictly anaerobic conditions and under aerobic conditions with shaking, in spite of lacking a respiratory chain. This characteristic growth of A. xylanus is due to the presence of aerobic and anaerobic pathways producing similar amounts of ATP (2, 8). Under aerobic conditions, NADH oxidase is thought to be responsible for maintenance of the intracellular redox balance (8).

The flavoprotein functional as NADH oxidase is purified from cells of A. xylanus grown aerobically (1, 2). The enzyme is a homotetramer of identical subunits that have a molecular weight of 56,000, with one FAD prosthetic group/subunit. The enzyme catalyzes the reduction of oxygen to hydrogen peroxide with β-NADH as the preferred electron donor (1).

The gene coding for the flavoprotein of A. xylanus has been cloned by using a specific antibody and the nucleotide sequence of the gene has been determined. The amino acid sequence deduced from nucleotide sequence has two ADP-binding sites and a FMN-binding site, indicating binding sites for FAD and NADH. The amino acid sequence of A. xylanus NADH oxidase exhibited sequence identities as high as 51.2% and 72.5%, with the alkyl hydroperoxide reductase F52a protein component from Salmonella typhimurium (27) and the NADH dehydrogenase from an alkylphilic Bacillus sp. YN-1 (28), respectively. Alkyl hydroperoxide reductase F52a protein is related to Escherichia coli thioredoxin reductase, but there is an additional domain at the N-terminus of alkyl hydroperoxide reductase F52a protein. Both enzymes contain a FAD and redox-active disulfide, in each monomer, in homologous positions. The N-terminal extension of alkyl hydroperoxide reductase F52a protein contains an additional disulfide, which appears to be able to interchange with the redox-active disulfide (27, 29).

We had attempted to extend the analysis of the functional properties of the flavoprotein from A. xylanus, but only obtained yields of about 0.1 mg of pure enzyme from a liter of culture broth of A. xylanus. Therefore, the gene encoding the flavoprotein from A. xylanus was cloned into a high level expression vector, purified, and analyzed. In this report, we describe the functional properties of flavoprotein and the differences from known NADH oxidases from several microorganisms, which suggested that the enzyme had unique functional properties.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T₄ DNA ligase, and IPTG1 were purchased from Takara Shuzo Co. (Kyoto, Japan). Butyl-Toyopearl 650,
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Toyopel HW-40, HW-55, and HW-60 were obtained from Toyoh (Tokyo, Japan). Preswollen anion exchange resin was from Whatman, and 5'-AMP-Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. The Inertial ODS column was purchased from GL Science Inc. (Tokyo, Japan). FAD and β-NADH were obtained from Sigma. All other reagents and buffer salts were of the highest quality available.

Bacterial Strains and Plasmids—E. coli JM110 and HB101 were used as hosts. Cultures were grown with shaking at 37 °C in Luria-Bertani broth (LB) broth. Ampicillin (50 μg/ml) was used as required.

Plasmid pBLS, which was originally designed for expression of the flavoprotein gene, was partially digested with HindIII to release a 1.9-kb fragment containing the entire flavoprotein gene. This fragment was used as hosts. Cultures were grown with shaking at 37 °C in Luria-Bertani broth (LB broth). FAD and P-NADH were obtained from Sigma. All other methods, and manipulation was performed according to standard methods (4).

Overexpression of Recombinant Flavoprotein—E. coli JM109 was transformed with pNOH1850 was grown overnight at 37 °C in 3 ml of LB broth containing 50 μg/ml ampicillin. Cells were then subcultured into 100 ml of fresh medium. After reaching the log phase of growth, these cultures were used to inoculate 5-liter flasks containing 1 liter of the same medium. Flasks were shaken vigorously at 37 °C, and expression was induced by the addition of 1 mM IPTG (final concentration) at an A600 of 0.5. Cells were harvested by centrifugation after 3 h of cultivation, washed with 30 mM sodium carbonate-sodium bicarbonate buffer at pH 11.3, and then frozen at -80 °C.

Purification of the Recombinant Flavoprotein—All steps of the purification procedure were performed at 4 °C. Approximately 50 g of cells (wet weight) were suspended in 220 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA, 220 μl of 100 mM PMSF, and 500 μl of 40 mg/ml lysozyme. The suspension was stored on ice for 30 min and disrupted by sonication. The supernatant from the centrifugation at 187,000 × g for 90 min was treated with streptomycin sulfate to remove nucleic acids. After centrifugation at 31,000 × g for 30 min, FAD and PMSF (final concentration 25 μM and 200 μM, respectively) were added to the supernatant, and then the supernatant was applied to a column of DE52 (2.6 × 33 cm) equilibrated in 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM EDTA. The column was washed with this same buffer, and the recombinant flavoprotein was eluted by 200 ml of the equilibration buffer containing 160 mM NaCl. Solid ammonium sulfate was added to the pool of active fractions to give a final concentration of 22.6% of saturation, and the solution was stirred for 15 min, the pH of supernatant was adjusted to 7.0 with 1 M Tris and then the supernatant was applied to a column of Toyopearl HW-60 (1.6 × 70 cm) equilibrated in 50 mM sodium phosphate buffer, pH 6.6, containing 0.5 mM EDTA. After centrifugation at 187,000 × g for 90 min, the supernatant was applied to a column of Toyopearl HW-40 (1.6 × 70 cm) equilibrated in 50 mM sodium phosphate buffer, pH 7.0, and free flavin was eluted with the same buffer. The fluorescence (excitation at 450 nm and emission at 530 nm) of the eluate was monitored and then was subjected to HPLC analysis on an Inertial ODS column (0.46 × 25 cm) with methanol in 5 mM ammonium acetate at pH 6.5, as the mobile phase (17). The fluorescence (excitation at 450 nm and emission at 530 nm) of the eluate was then monitored.

Anaerobiosis—All titrations were performed under anaerobic conditions at 25 °C. The anaerobic enzyme sample, in 50 mM sodium phosphate buffer, pH 6.6, containing 0.5 mM EDTA, was prepared in an all glass apparatus by sequential evacuation and re-equilibration with oxygen-free argon (26). Oxygen-free argon was prepared by passing commercially obtained pure argon through a column of Oxyout (Osaka Sanko Co.). Dithionite solutions were prepared in the same buffer.

Determination of Metal Content—Measurements of metal contents were performed in the Research Centre, Inc. using a Hitachi model 2920 graphite furnace atomic absorption spectrophotometer.

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Thiol and Disulfide Quantitation—The flavoprotein was denatured under nonreducing conditions with a final concentration of 6 mM guanidine HCl at pH 5.0. The number of thiol present in the enzyme was then quantitated by reaction with DTNB and measurement of formation of the nitrothiobenzoate anion at 412 nm (9).

The number of disulfide bonds was determined using NTSB as described by Thannhauser et al. (10). The disulfide bonds were cleaved by excess sodium sulfite at pH 9.6. The flavoprotein then was denatured with a final concentration of 2 × guanidine thiocyanate. The number of disulfide bonds present in the enzyme was quantitated by reaction with NTSB and measurement of the formation of the nitrothiobenzoate anion at 412 nm using an extinction coefficient of 19,600 M⁻¹ cm⁻¹.

NADH:DTNB Oxidoreductase Assay—The catalytic activity of NADH: DTNB oxidoreductase was measured under anaerobic conditions at 25 °C. The enzyme was prepared in 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. After the enzyme solution was added to 100 μM NADH containing 0.02% bovine serum albumin, the reaction was started by adding DTNB (400 μM), and the nitrothiobenzoate anion production was monitored at 412 nm (ε = 13,600 M⁻¹ cm⁻¹). Activities were expressed as turnover numbers (mol of substrate catalyzed/mmol of enzyme-bound FAD).

General Procedures—Proteinase group analysis, N-terminal amino acid sequence determination, and other analytical methods followed protocols described previously (1, 8).

RESULTS

Expression and Purification of Recombinant Flavoprotein—The pBLS was partially digested with HindIII to release a 1.9-kb fragment. This fragment was cloned into the high level expression vector pTTQ18 (Fig. 1). The resultant plasmid pNOH1850 contained the complete flavoprotein gene downstream from the tac promoter. The plasmid was transformed into E. coli JM109. The E. coli JM109 transformed with pNOH1850 gave consistent IPTG-induced expression of the flavoprotein, confirmed by SDS-polycrylamide gel electrophoresis, which gave a 56-kDa band after IPTG induction.

The purification scheme of recombinant flavoprotein was outlined under "Experimental Procedures," and results from a
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Activity showed parallel lines (Fig. 2), suggesting a ping-pong type mechanism, and the replot of the y-intercepts versus [oxygen] was linear (data not shown). The $K_m$ value for NADH and $K_m$ for oxygen were 33.3 μM and 1.7 mM, respectively. These $K_m$ values for oxygen were too high to catalyze the reoxidation of NADH by oxygen in the cell of A. xylanus (8). In the presence of free FAD, however, the $K_m$ value for oxygen was greatly diminished, indeed it was too low to allow accurate determination of value by the usual assay method. The NADH oxidase activity was accelerated markedly in the presence of additional FAD (Fig. 3). FAD was the most effective and riboflavin was less effective, whereas FMN had no capability of affecting the NADH oxidase activity. The decrease of $K_m$ value and the acceleration of NADH oxidase activity in the presence of FAD were observed A. xylanus flavoprotein.

Based on the results of HPLC analysis, the intracellular free FAD concentration of A. xylanus was calculated about 13 μM. This FAD concentration was enough to accelerate the NADH oxidase activity of flavoprotein in the cell of A. xylanus.

Dithionite Titration—Titration of flavoprotein with dithionite shows an uptake of 3 eq or 6 electrons (Fig. 4). Since the FAD is able to take up 2 electrons, the 6-electron total shows that other redox-acceptor are present; from data presented later, these are disulfides. These are two apparent phases in the titration as shown in Fig. 4 (inset). In the first phase, involving 4.0–4.6 electrons, the flavin absorbance of the oxidized enzyme diminishes and a new band appears at 570 nm, having a shoulder at 600 nm, typical of the neutral (blue) flavin semiquinone (30, 31). The extinction coefficient of this band is 3,800 m$^{-1}$ cm$^{-1}$ at 585 nm and 3,500 m$^{-1}$ cm$^{-1}$ at 454 nm, and the extinction coefficient of FADH$_2$ is 1,800 m$^{-1}$ cm$^{-1}$ at 454 nm in thioredoxin reductase from E. coli (32). Scaling these extinction coefficients to account for the higher extinction of the FAD in the A. xylanus flavoprotein (4400, 4100, and 2100, respectively) and applying them to the spectra in Fig. 4 indicates that at the break between the phases, the FAD is partitioned approximately as follows: 0.3 eq FADH (0.6 electrons) and 0.6 eq of FADH$_2$ (1.2 electrons). Thus, in this first phase, most (4.0–4.6 – 1.8 = 2.2–2.8) of the electrons have been taken up by the disulfides.

Following each addition of dithionite, the absorbance at 450 nm fell in the mixing time and then rose slowly, recovering part of the lost absorbance at equilibrium. Such behavior has been noted in mercuric reductase, an enzyme with one disulfide adjacent to the FAD and another further away but able to interconvert with the adjacent disulfide-dithiol (33). The titration behavior observed in the titration of mercuric reductase titration indicates that the redox potential of the distant disulfide-dithiol is higher than that of the disulfide-dithiol adjacent to the FAD since the distant disulfide is more reduced at equilibrium than is the adjacent disulfide (33) (see "Discussion" for further details). Reduction of the three redox centers is completed in the second phase of the titration with the remaining FAD and FADH$_2$ being reduced to FADH$_2$.

NADH Oxidase—The flavoprotein with NADH proceeds in three apparent phases of absorbance loss at 450 nm, as shown in Fig. 5. The changes at long wavelengths are more complex than those observed in the dithionite titration. Semiquinone accumulates in the first phase but only approximately 0.1 eq in contrast to 0.3 eq in the dithionite titration, apparently due to a kinetic barrier. This phase involves the uptake of 1.7–2.0 reducing equivalents from NADH. As would be expected from the less strong reductant, NADH, the absorbance at 450 nm is higher at this point in the titration than at the end of the first phase of the dithionite titration. This indicates that
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**Table I**

<table>
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<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
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<th>Yield</th>
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<td>19.9</td>
<td>56.9</td>
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<tr>
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<td>436.8</td>
<td>21.0</td>
<td>60.0</td>
<td>7.0</td>
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**Fig. 2.** Lineweaver-Burk plots of the steady-state kinetic analyses. Assay conditions were 50 mM sodium phosphate buffer, pH 6.6, containing 0.5 mM EDTA at 25 °C. Filled squares, 121 μM oxygen; open triangles, 187 μM oxygen; filled triangles, 255 μM oxygen; open circles, 607 μM oxygen; filled circles, 1214 μM oxygen.

**Fig. 3.** Effect of flavin on NADH oxidase activity. Assay conditions were 50 mM sodium phosphate buffer, pH 6.6, containing 0.5 mM EDTA at 25 °C. Circles, FAD; triangles, riboflavin; squares, FMN. There is less FADH₂ at this stage of the NADH titration (Fig. 5, spectrum 5). Thus, most of the reducing equivalents have been taken up by the disulfides in the first phase.

Flavin reduction continues in the second apparent phase and as FADH₂ accumulates the familiar FADH₂-NAD⁺ charge transfer complex forms with its broad long wavelength absorbance extending beyond 800 nm (34). This apparent phase ends with spectrum 7 (Fig. 5), with uptake of approximately 3 reducing equivalents from NAD⁺. In the third apparent phase, the reduction proceeds as excess NADH accumulates as shown by increased absorbance at 350 nm. The stability of the FADH₂-NAD⁺ complex in the presence of excess NADH indicates very tight binding of NAD⁺ relative to NADH.

**Determination of Metal Content**—Metal contents were 0.0954 mol/mol of flavin for iron, 0.0060 mol/mol of flavin for zinc, and 0.0002 mol/mol of flavin for molybdenum. None of these metals are present in quantities sufficient to account for redox activity within the enzyme. Manganese, copper, and cobalt were not detected in the enzyme.

**Thiol and Disulfide Quantitation**—The results of dithionite or NADH titration indicate that the flavoprotein has two non-flavin redox centers, accounting for the total uptake of 6 electrons. Although the enzyme contained neither heme nor other metallic compounds, the enzyme has 5 cysteine residues/subunit as deduced from DNA sequence (1). The number of thiols present in the enzyme was quantitated by reaction with DTNB under nonreducing conditions with a final concentration of 6 mM guanidine HCl, and measurement of formation of the nitrothiobenzoate anion at 412 nm (9). Only 1 of 5 cysteine residues of the enzyme reacted with DTNB. Subsequently, two disulfide bonds were determined by reaction with NTSB.
**Table II**

The number of thiols and disulfides of flavoprotein

<table>
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<tr>
<th>Reaction</th>
<th>Reaction with DTNB</th>
<th>Reaction with NTSB</th>
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</thead>
<tbody>
<tr>
<td>Cysteine residues</td>
<td>5.0^a</td>
<td>1.0</td>
</tr>
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</table>

^a Taken from Ref. 1.

(From Table II). It was obvious that the flavoprotein has one free thiol and two disulfides.

**NADH:DTNB Oxidoreductase Activity**—When the number of cysteine residues present in the NADH-reduced recombinant flavoprotein was quantitated by reaction with DTNB (without guanidine HCl at pH 8.0), the amount of nitrothiobenzoate anion obtained was greater than that expected for 5 cysteine residues as deduced from the nucleotide sequence. This result indicated the flavoprotein catalyzed a thiol-disulfide interchange reaction. The turnover number of NADH:DTNB oxidoreductase activity was 61.5 min^-1. NADH:thioredoxin oxidoreductase activity was negligible using thioredoxin from E. coli. The NADH:DTNB oxidoreductase activity was also observed in the enzyme purified from *A. xylanus*.

**DISCUSSION**

The very low yield of the flavoprotein (0.1 mg/liter of cell culture) (1) precluded a thorough study of this enzyme as isolated from *A. xylanus*. The gene encoding the flavoprotein was cloned into pT7Q18, and when this plasmid was transformed into *E. coli* JM109, the yield of pure enzyme was over 15 mg from 1 liter of cell culture. The recombinant flavoprotein was fully active and was identical to the enzyme originally isolated from *A. xylanus* in the broad range of characteristics tested including spectra and stimulatory activity by excess FAD.

*A. xylanus* metabolizing either anaerobic and aerobic pathways produces similar amounts of ATP. We have proposed that NADH oxidase regenerates NAD^+ from NADH using thioredoxin from *E. coli*. The NADH:DTNB oxidoreductase activity appeared to occur in two phases. The first phase involved the uptake of approximately 4 electrons. The maximal amount of flavin semiquinone observed, 0.3 eq, was formed in this phase together with 0.6 eq of FADH^2_. Thus, in the first phase most of the reducing equivalents have been taken up by the disulfide; the redox-active disulfide adjacent to the FAD and a disulfide in the N-terminal extension, which can presumably interconvert slowly with the redox-active disulfide. In mercuric reductase, the redox link between the FAD and the redox-active disulfide is spectrally observable during reduction. This makes it possible to distinguish the two disulfides on the basis of their redox potentials, the redox-active disulfide having the lower potential. Thus, this is not possible in the present study because any interaction between the redox-active disulfide and the FAD is not spectrally observable. The second 2-electron reduction phase completed the reduction of the flavin and the disulfides.

Ahmed and Clairoke (19, 20) proposed that the cysteinyl redox centers of the dimer NADH oxidase were not equivalent; only one of the cysteine-sulfenic acid centers is reduced by dithionite in the absence of pyridine nucleotide.

The 2-electron reduction of FAD in flavoprotein from *A. xylanus* with the strong reductant dithionite in this study, required 6 electrons/FAD (subunit) for full reduction. The reduction appeared to occur in two phases. The first phase involved the uptake of approximately 4 electrons. The maximal amount of flavin semiquinone observed, 0.3 eq, was formed in this phase together with 0.6 eq of FADH^2_. Thus, in the first phase most of the reducing equivalents have been taken up by the disulfide; the redox-active disulfide adjacent to the FAD and a disulfide in the N-terminal extension, which can presumably interconvert slowly with the redox-active disulfide. In mercuric reductase, the redox link between the FAD and the redox-active disulfide is spectrally observable during reduction. This makes it possible to distinguish the two disulfides on the basis of their redox potentials, the redox-active disulfide having the lower potential. Thus, this is not possible in the present study because any interaction between the redox-active disulfide and the FAD is not spectrally observable. The second 2-electron reduction phase completed the reduction of the flavin and the disulfides. Two disulfide bonds were quantitated in the flavoprotein.

The short segment containing Cys-337 and Cys-340 of the flavoprotein showed a high degree of identity with the segment of *E. coli* thioredoxin reductase from *E. coli*. Identical and related amino acid residues are indicated by asterisks and dots, respectively. The redox-active cysteines of thioredoxin reductase, Cys-135 and Cys-138, are indicated by arrows. No active or Tphase indicate *A. xylanus* flavoprotein and *E. coli* thioredoxin reductase, respectively.

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The 2-electron reduction of FAD in flavoprotein from *A. xylanus* with the strong reductant dithionite in this study, required 6 electrons/FAD (subunit) for full reduction. The reduction appeared to occur in two phases. The first phase involved the uptake of approximately 4 electrons. The maximal amount of flavin semiquinone observed, 0.3 eq, was formed in this phase together with 0.6 eq of FADH^2_. Thus, in the first phase most of the reducing equivalents have been taken up by the disulfide; the redox-active disulfide adjacent to the FAD and a disulfide in the N-terminal extension, which can presumably interconvert slowly with the redox-active disulfide. In mercuric reductase, the redox link between the FAD and the redox-active disulfide is spectrally observable during reduction. This makes it possible to distinguish the two disulfides on the basis of their redox potentials, the redox-active disulfide having the lower potential. Thus, this is not possible in the present study because any interaction between the redox-active disulfide and the FAD is not spectrally observable. The second 2-electron reduction phase completed the reduction of the flavin and the disulfides. Two disulfide bonds were quantitated in the flavoprotein.

The short segment containing Cys-337 and Cys-340 of the flavoprotein showed a high degree of identity with the segment of *E. coli* thioredoxin reductase containing Cys-135 and Cys-138 (22) (Fig. 6). Characterizations of two active site mutations of thioredoxin reductase and of their apoproteins reconstituted with analogs of FAD have revealed that Cys-135 is the electron transfer or flavin-interacting thiol, and it was suggested that Cys-135 might be the interchange thiol on the basis that in other members of this enzyme family, one thiol interacts with the FAD and the other with the disulfide substrate (21, 24). The x-ray crystal structure shows that Cys-135 is indeed close to the flavin, 3.0 Å from the C4a position of isosalloazine ring, whereas Cys-135 is 4.4 Å from the C5a position of isosalloazine ring (6, 23). From these results, we hypothesize that Cys-337 and Cys-340 of the flavoprotein may create the disulfide adjacent to the FAD and involved in the flow of electrons. In order to confirm our hypothesis, site-directed mutations of flavoprotein have been engineered at Cys-337 and Cys-340, and their properties are being investigated.
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REFERENCES