Reaction Mechanism of Amphibacillus xylanus NADH Oxidase/Alkyl Hydroperoxide Reductase Flavoprotein

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NADH oxidase from Amphibacillus xylanus is a potent alkyl hydroperoxide reductase in the presence of the small disulfide-containing protein (AhpC) of Salmonella typhimurium. In the presence of saturating AhpC, $k_{cat}$ values for reduction of hydroperoxides are approximately 180 s$^{-1}$, and the double mutant flavoprotein enzyme C337S/C340S cannot support hydroperoxide reduction (Niimura, Y., Poole, L. B., and Massey, V. (1995) J. Biol. Chem. 270, 25645–25650). Kinetics of reduction of wild-type and mutant enzymes are reported here with wild-type enzyme; reduction by NADH was triphasic, with consumption of 2.6 equivalents of NADH, consistent with the known composition of one FAD and two disulfides per subunit. Rate constants for the first two phases (each approximately 200 s$^{-1}$) where FAD and one disulfide are reduced are slightly greater than $k_{cat}$ values for AhpC-linked hydroperoxide reduction. The rate constant for the third phase (reduction to the 6-electron level) is too small for catalysis. Only the first phase of the wild-type enzyme occurs with the mutant enzyme. These results and the stoichiometry of NADH consumption indicate Cys$^{337}$ and Cys$^{340}$ as the active site disulfide of the flavoprotein and that electrons from FADH$^{2}$ must pass through this disulfide to reduce the disulfide of AhpC.

Amphibacillus xylanus, isolated from alkaline compost (1), has unique phenotypic and chemotaxonomic characteristics (2) as well as unique bioenergetic properties (3). A. xylanus, lacking a respiratory system and the hemeproteins, catalase and peroxidase, grows well and has the same growth rate and cell yield under strictly anaerobic and aerobic conditions (2). This growth characteristic is due to the presence of anaerobic and aerobic pathways producing similar amounts of ATP (4). NADH oxidase is thought to function in vivo to regenerate NAD from NADH produced in the aerobic pathway. A flavoprotein functional as NADH oxidase was purified from aerobically grown A. xylanus. The enzyme is a homotetramer composed of subunits ($M_r = 56,000$) each containing 1 mol of FAD. The amino acid sequence of A. xylanus NADH oxidase exhibits 51.2% identity with an alkyl hydroperoxide reductase F-52a component (AhpF) from Salmonella typhimurium (5, 6). Together with the 22-kDa protein component (AhpC) of the S. typhimurium alkyl hydroperoxide reductase, AhpF was reported to catalyze the NADH-dependent reduction of alkyl hydroperoxides but not of hydrogen peroxide (7). A. xylanus NADH oxidase showed extremely high scavenging activity for both alkyl hydroperoxides and hydrogen peroxide in the presence of S. typhimurium AhpC (8). Such behavior indicates that the A. xylanus NADH oxidase has unique functional properties that are different from other known NADH oxidases (9–15).

The complete reduction of enzyme by dithionite requires 6 electrons/subunit (16). Such behavior indicates the presence of redox centers in addition to the FAD, and these were postulated to be disulfides (16). To assess the catalytic role of disulfides in the enzyme, two of the cysteines, Cys$^{337}$ and Cys$^{340}$, which show a high degree of homology to thioredoxin reductase, were changed to serines by site-directed mutagenesis of the cloned NADH oxidase gene (individually and in a double mutant) (17). Titration of three mutant enzymes, lacking Cys$^{337}$, Cys$^{340}$, or both cysteines, required only 2 electron equivalents to reach the reduced flavin state (17). When coupled with the 22-kDa AhpC of S. typhimurium, the wild-type NADH oxidase of A. xylanus showed extremely high NADH-alkyl hydroperoxide reductase activity, but very little activity with hydrogen peroxide or cumene hydroperoxide was found with the single mutants (C337S and C340S), and none with the double mutant (C337S/C340S) (8). These results indicate that Cys$^{337}$ and Cys$^{340}$ of the flavoprotein NADH oxidase participate in the scavenging activity for hydrogen peroxide and alkyl hydroperoxide in the presence of AhpC, and demonstrate the involvement of Cys$^{337}$ and Cys$^{340}$ as the redox-active disulfide (8). To provide information about the reaction mechanism of A. xylanus NADH oxidase in its role as the alkyl hydroperoxide reductase flavoprotein component, the reductive and oxidative half-reactions of the wild-type and the mutant (C337S/C340S) NADH oxidase were studied, and the results are reported here.

EXPERIMENTAL PROCEDURES

Enzyme—The A. xylanus NADH oxidase and its mutant C337S/C340S were purified as described previously (5, 17).

Stopped-flow Absorbance Spectrophotometry—The stopped-flow apparatus has been described previously (18, 19). In all experiments except those of Fig. 2, an optical cell of 1 cm path length was used. In the experiment of Fig. 2, the optical path length was 2 cm. Anaerobiosis of the flow system was achieved by equilibration overnight with an anaerobic solution of 3,4-dihydroxybenzoate and protocatechuate-3,4-dioxygenase (a generous gift from Dr. David P. Ballou, University of Michigan). Rate constants were obtained by exponential fits using the software “Program A” (developed by Chung-Jen Chiu, Rong Chung, Joel Dinverno, and Dr. David P. Ballou, University of Michigan), which permits the analysis of experimental data by exponential fits based on the Marquardt algorithm (20). This program permits the fixing of as many as five rate constants and subsequent curve fitting to evaluate the absorbance of any intermediate species. It also permits the simulation.
of model kinetic pathways for comparison with the experimental data.

Anaerobiosis for Reductive Half-reaction—The enzyme solution (6 ml in volume) containing 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM EDTA, and approximately 15 μM enzyme was loaded into a tonometer. After anaerobiosis was established by repeated evacuation and equilibration with oxygen-free argon and equilibration at 25 °C, the enzyme was introduced into the stopped-flow spectrophotometer and reduction of the enzyme flavin was monitored by mixing the enzyme solution with various concentrations of NADH and following the reaction at various wavelengths, but in particular at 350 and 450 nm. The NADH solution, containing 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM EDTA, and 100-1000 μM NADH (concentrations were halved after mixing in the stopped-flow spectrophotometer), was bubbled with oxygen-free argon at 25 °C for 15–20 min before being loaded into the stopped-flow instrument.

Anaerobiosis for Oxidative Half-reaction—To 5 ml of approximately 15 μM enzyme in the main compartment of the tonometer, 2 μM NADP, 600 μM glucose 6-phosphate, and 10 μg of catalase were added. After anaerobiosis, a catalytic amount of glucose-6-phosphate dehydrogenase was added from the side arm, and the reduction was carried out at room temperature and monitored in a spectrophotometer cell attached to the tonometer. This procedure, using the nonphysiological reductant, NADPH, resulted in slow reduction of the enzyme over a period of an hour, permitting its use in oxidative half-reaction experiments, typically complete within seconds, without complications from re-reduction.

The oxidative half-reaction was followed after mixing the enzyme solution with various concentrations of oxygen in the stopped-flow instrument. The oxygen solution, containing 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM EDTA, and 30–1230 μM oxygen, was prepared by bubbling the buffer solution with commercial N2/O2 gas mixtures (Matheson), compressed air, or pure O2.

RESULTS

The Reductive Half-reaction of Wild-type NADH Oxidase—The process of reduction of wild-type enzyme by NADH is illustrated in Fig. 1 at wavelengths of 450 nm, which monitors the reduction of enzyme-bound flavin, and at 350 nm, at which oxidation of NADH can be monitored accurately. The reaction traces at all wavelengths studied, from 350–550 nm, can be fitted accurately with three exponentials. At the concentration of NADH illustrated in Fig. 1, (50 μM), the k_{obs} values are 55 ± 2, 55 ± 2, and ~0.35 s⁻¹. At 450 nm, the first phase is exemplified by a lag, with at most 5% of the total absorbance decrease associated with it, followed by most of the absorbance change in the next phase. The last phase is associated with approximately 4% of the total change. It must be emphasized that a good fit to the data requires two phases of almost equal rate but different amplitude. The traces in the 450 nm region are distinctly different from those at 350 nm. At this wavelength the absorbances of oxidized and reduced enzyme are close to isosbestic, so the oxidation of NADH can be monitored. The reaction traces are fitted by the same rate constants as those at other wavelengths, but now there is no lag in the absorbance decrease, and the third slow phase is much more distinct. The absorbance changes are associated with the oxidation of 1.0 equivalent of NADH/mol of flavin in each of the first two phases and another 0.6 equivalent in the final slow phase. Thus it is clear that the three phases observed represent the successive reduction of the enzyme to the EH2, EH4, and EH6 forms. The observed rate constant for the final phase is independent of NADH concentration; those for the two fast phases are quite dependent and reach a limiting value of 200±10 s⁻¹, with a K_d for binding of NADH of ~1.2 × 10⁻⁴ M, as shown by the double reciprocal plot in the inset of Fig. 1. The pathway of electron transfer in the reductive half-reaction is envisaged in Scheme 1 and involves rate-limiting reduction of the enzyme flavin by NADH followed by very rapid electron transfer to the disulfide Cys²³⁷-Cys³⁴⁰. The oxidized flavin so
regenerated undergoes a second rapid reduction by NADH to generate the EH$_3$ state. The final reduction step is slow, presumably because of slow dithiol-disulfide interchange between the Cys$^{337}$-Cys$^{340}$ dithiol and the Cys$^{128}$-Cys$^{131}$ disulfide (see “Discussion” for further details).

The Reductive Half-reaction of C337S/C340S NADH Oxidase—Fig. 2 shows the traces at 350, 450, and 550 nm for the reduction of C337S/C340S NADH oxidase with 100 $\mu$M NADH. Analysis of the traces at these and 10 other wavelengths in the range 350–650 nm reveals a triphasic process at all wavelengths. The reduction rate of the first phase reaches a maximum value at saturating NADH concentration. A limiting rate of 285 s$^{-1}$ and a $K_p$ of 1.1 $\times$ 10$^{-4}$ M for binding of NADH was estimated from a double-reciprocal plot (1/$k_{\text{obs}}$ versus 1/[NADH]) (results not shown). On the other hand, the rates of the second and third phases were completely independent of NADH concentrations at all wavelengths and were 8 and 0.2 s$^{-1}$, respectively.

The major absorbance change at all wavelengths was that associated with the fast NADH concentration-dependent phase. The absorbance decrease at 350 nm corresponds to consumption of 1.03 equivalent of NADH/mol of enzyme-bound flavin. These results clearly indicate that only the flavin is reduced in the C337S/C340S mutant, in keeping with previously reported titration behavior with either dithionite or NADH. Approximate fits to the experimental data can be obtained from the traces at 350 and 450 nm, and $k_{\text{red}}$ and $k_{\text{red}}$ are independent of NADH concentration.

![Fig. 2. Time course for the reduction of mutant C337S/C340S NADH oxidase by NADH. 11.3 $\mu$M mutant C337S/C340S NADH oxidase was mixed with various concentrations of NADH (100 $\mu$M in this figure) at pH 7.0, 25 °C under anaerobic conditions (concentrations given are after mixing). The traces shown are for the changes at 350, 450, and 550 nm (optical path length, 2 cm). Inset, $k_{\text{red}}$, is the maximal value of the reduction rate at saturating NADH concentration obtained from the traces at 350 and 450 nm, and $k_{obs}$ and $k_{red}$ are independent of NADH concentration.](image1)

![Fig. 3. Time course for the oxidation of reduced wild-type NADH oxidase by oxygen. Reduced NADH oxidase (final concentration, 7.5 $\mu$M) was mixed with various concentrations of oxygen at pH 7.0, 25 °C. The trace shown was obtained at a final oxygen concentrations of 125 $\mu$M. Inset, direct plot of the observed rate constants as a function of oxygen concentration.](image2)
The above stopped-flow results agree with the results of oxidase reaction were not different from those of the wild type and the mutant enzyme obtained from the trace at 450 nm is close to that expected consumption of 260 μM NADH (equivalent to the theoretical value for the first 2 min, after which it was consumed in the primary turnover sequence. In the turnover experiments in which there was an excess concentration of NADH over that of oxygen, such as that in the lower part of Fig. 5, consumption of NADH continued over a period of minutes, accompanied by the accumulation of the neutral semiquinone form of the enzyme, and in some cases full return to the spectrum of oxidized enzyme. This was due to a slow secondary reaction of reduced enzyme with H₂O₂ produced in the primary turnover sequence. In the experiment with 400 μM NADH, analysis of the data at 340 nm showed the expected consumption of 260 μM NADH (equivalent to the oxygen concentration) within a few seconds, followed by the remaining 140 μM over a period of 10 min. During this time the neutral semiquinone of the enzyme accumulated to approximately 50% of its theoretical value for the first 2 min, after which it was converted to oxidized enzyme, concomitant with the consumption of the last 30 μM NADH. In a similar experiment, at a total concentration of 600 μM NADH, shown in the lower trace of Fig. 5, 260 μM NADH was again consumed rapidly, followed by another 255 μM in the next 10 min, with
the enzyme stabilized largely in the neutral semiquinone form. These results imply the following:

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\begin{align*}
\text{very fast} & \quad \text{EFI}_{\text{ox}} + \text{NADH} \rightarrow \text{EFI}_{\text{red}}\text{H}_2 + \text{NAD} \\
\text{fast} & \quad \text{EFI}_{\text{red}}\text{H}_2 + \text{O}_2 \rightarrow \text{EFI}_{\text{ox}} + \text{H}_2\text{O}_2 \\
\text{very slow} & \quad \text{EFI}_{\text{red}}\text{H}_2 + \text{H}_2\text{O}_2 \rightarrow \text{EFI}_{\text{ox}} + 2\text{H}_2\text{O} \\
\text{slow} & \quad \text{EFI}_{\text{ox}} + \text{EFI}_{\text{red}}\text{H}_2 \rightarrow 2\text{EFHLH}
\end{align*}
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**REACTIONS 1–4**

Equilibrium Formation of Neutral Semiquinone from Mixtures of Oxidized and Reduced Enzyme—Previous reductive titration experiments of wild-type (16) or mutant forms (17) of the NADH oxidase with NADH had shown the intermediate accumulation of the neutral flavin semiquinone form of the enzyme. Since it is generally accepted that reduction of flavin by NADH proceeds via a 2-electron hydride transfer mechanism, the formation of flavin semiquinone in such experiments is most logically explained by intermolecular electron transfer between reduced and oxidized enzyme, as formulated in the previous section. Direct evidence for this process with the C337SC340S double mutant was obtained by experiments such as those illustrated in Fig. 6, in which approximately equal concentrations of reduced and oxidized enzyme were achieved by reaction of oxidized enzyme in the stopped-flow spectrophotometer under anaerobic conditions with a 0.5 equivalent of NADH. Reduction of the flavin by the limited concentration of NADH was complete within 100 ms (data not shown) and was followed by the slow formation of the neutral semiquinone form of the enzyme. Although this intermolecular equilibration of reducing equivalents between oxidized and reduced flavin forms for the enzyme is slow on a stopped-flow time scale, it should be emphasized that it is unusually fast for this particular enzyme, since similar experiments carried out with other flavoproteins (e.g., glucose oxidase, d-amino acid oxidase, and L-lactate oxidase) require hours to days to achieve equilibrium.1

**DISCUSSION**

The *A. xylanus* NADH oxidase is thought to function *in vivo* to regenerate NAD from NADH produced in the aerobic pathway, and formation of hydrogen peroxide was observed in the isolated NADH oxidase reaction (5). However, in the presence of the 22-kDa component (AhpC) of *Salmonella* alkyl hydroperoxide reductase, the enzyme shows strong scavenging activity for hydrogen peroxide and alkyl hydroperoxide, with *V*max values of 185 and 170 s⁻¹, respectively (8). Although several enzymes that show scavenging activity for hydrogen peroxide and alkyl hydroperoxides have been purified and characterized from bacterial and mammalian sources (7, 21–28), none of these enzymes has been reported to show such high turnover numbers for both hydrogen peroxide and alkyl hydroperoxide.

The complete reduction of the NADH oxidase by dithionite required 6 electron equivalents/mol of enzyme-bound flavin (16). Such behavior indicated the presence of redox centers in addition to the FAD, and these were shown to be two disulfides (17). To provide information about the relationship between such high turnover numbers and the three redox centers of the enzyme, the reductive half-reactions of the wild-type and the mutant (C337S/C340S) NADH oxidase were performed. In the wild-type enzyme, rate constants for the first and the second phases in which the enzyme-bound FAD is reduced showed very similar values to each other (*k*red1 = *k*red2 = 200 s⁻¹), and two equivalents of NADH were consumed. However, the rate constant (*k*red3 = 0.3 s⁻¹) for the third phase, in which the EH4 state is reduced finally to the EH6 level, was very slow compared with those of the first two steps. In contrast, in mutant enzyme, the third phase observed in wild-type enzyme was not found, and a single equivalent of NADH was consumed to reduce the enzyme-bound FAD. Previous titration studies had shown that 2 electron equivalents were required to reduce the mutant enzyme-bound FAD (17), whereas the complete reduction of the wild-type enzyme required 6 electron equivalents (16). The above reductive half-reaction experiments and the stoichiometry of NADH consumption are consistent with the titration results, indicating that electrons from FADH2 must pass through the first disulfide (Cys337 and Cys340) to reduce the second disulfide, which is slowly reduced in the third phase, as shown in Scheme 1. The values of *k*red1 and *k*red2 are a little higher than the *V*max values for NADH-linked reduction of hydrogen peroxide and alkyl hydroperoxide in the presence of saturating concentrations of AhpC (8), but the value of *k*red3 is too low to be involved in *k*cat (0.3 versus 185 s⁻¹). The above results all suggest that in the alkyl hydroperoxide reductase reaction of the NADH oxidase, electrons are transferred from the first reduced disulfide (Cys337-Cys340) in the EH2 state to the disulfide of AhpC. The efficiency of electron transfer between the reduced flavin and the Cys337-Cys340 of the enzyme and thiol-disulfide interchange with the disulfide of AhpC must be very great, occurring considerably faster than the limiting rate of flavin reduction (*k*red = 200 s⁻¹). The value of *k*cat determined for *E. coli* thioredoxin reductase (29, 30), which catalyzes the interchange with the disulfide of thioredoxin, is lower than the *k*cat of the NADH oxidase (22 versus 185 s⁻¹), emphasizing that the efficiency of electron transfer with NADH oxidase is remarkably high.

In the AhpC-coupled reactions the single mutant enzymes lacking Cys337 or Cys340 showed very little peroxide reductase activity for hydrogen peroxide or alkyl hydroperoxide, and the

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1 V. Massey, unpublished observations.
double mutant enzyme lacking both cysteines showed no peroxide reductase activity for either peroxide (8). In contrast, for the NADH oxidase activity, the \( K_m \) values for NADH and oxygen, and the \( V_{max} \) values for these mutant enzymes were not different from those for the wild-type enzyme (17). To provide information about the reaction mechanism of the NADH-oxygen reductase activity, the oxidative half-reactions were performed with the wild-type and the mutant (C337S/C340S) enzymes. Three sequential second-order reaction rates were determined in the oxidative half-reaction for wild-type enzyme and is faster than the maximal turnover rate of the mutant enzyme, which is involved in the oxidation of one of which is derived from semiquinone. The first phase rate of the mutant enzyme, which is involved in the oxidation of reduced FAD (from EH2 to E), is closest to the fastest rate of the wild-type enzyme. In contrast, in the mutant enzyme, analysis of the oxidative half-reaction showed two second-order reaction rates, one of which is derived from semiquinone. The first phase rate in limiting conditions of NADH or oxygen concentration are consistent with the reduced oxidation and oxidation rates and are consistent with the involvement of both the EH\(_2\) and EH\(_4\) forms of the enzyme in the NADH-oxygen reductase activity, clearly possible because of the rapidity of the first two reduction steps (Scheme 1).

Although hydrogen peroxide is the primary product in the NADH oxidase reaction (5), the stoichiometry in the oxidation of NADH under conditions of limiting oxygen concentration show that the NADH oxidase formed in the primary reaction is reduced slowly to water at the expense of further NADH oxidation. The reaction is very slow and requires NADH in excess and anaerobic conditions to compete with the NADH oxidase reaction. Large amounts of hydrogen peroxide are presumed to be produced in aerobic growing cells of \( A. \) \( xylanus \), which lack catalase and a respiratory chain (2), because the NADH oxidase regenerates NAD from NADH formed in the aerobic pathway. Whereas the NADH oxidase flavoprotein thus has weak peroxide reductase activity, the scavenging activity for hydrogen peroxide must be high for cells to survive under aerobic conditions. \( A. \) \( xylanus \) NADH oxidase showed extremely high scavenging activity for hydrogen peroxide in the presence of \( S. \) \( typhimurium \) AhpC (8) and so is thought to be part of a functional alkyl hydroperoxide reductase system also involving an analogous \( A. \) \( xylanus \) AhpC component, since a partial open reading frame homologous to the \( Salmonella \) AhpC structural gene has been found up-stream of the \( Amphibacillus \) NADH oxidase structural gene (31).

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