Quinone Reductase Reaction Catalyzed by *Streptococcus faecalis* NADH Peroxidase†

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ABSTRACT: NADH peroxidase is a flavoenzyme having a single redox-active thiol, Cys42, that cycles between sulfenate and thiol forms in the NADH-dependent reduction of hydrogen peroxide. NADH peroxidase catalyzes the NADH-dependent reduction of quinones with turnover numbers between 1.2 and 3.9 s^{-1}, per mole of FAD, at pH 7.5. The bimolecular rate constants for quinone reduction, *V/K*, ranged from 4.3×10^3 to 6.0×10^5 M^{-1} s^{-1} for 14 quinones whose redox potentials varied between −0.41 and 0.09 V. The logarithms of the *V/K* values for these quinones are hyperbolically dependent on their single-electron reduction potentials (E_7^1). One-electron reduction of benzoquinone accounts for about 50% of the total electron transfer catalyzed by NADH peroxidase at pH 7, with the remainder of the reduction being catalyzed by a two-electron (hydride) transfer. Cys42 can be irreversibly oxidized to the sulfonate by hydrogen peroxide, with inactivation of the peroxidatic activity of the enzyme. The residual quinone reductase activity of NADH peroxidase which has undergone oxidative inactivation of the active site Cys42 indicates that this residue is not involved in the reduction of the quinones. Product inhibition studies suggest the possibility of overlap of the pyridine nucleotide and quinone binding sites in the reduced enzyme at low pH values. The pH dependence of the maximum velocity of naphthoquinone reduction shows that deprotonation of an enzymic group, exhibiting a p*K* value of *ca.* 6.2, decreases the maximal velocity. Primary deuterium kinetic isotope effects on *V* and *V/K* for quinone-dependent NADH oxidation increase upon protonation of a group, exhibiting a p*K* value of 6.4. These data are most consistent with a change in the rate-limiting step and the mechanism of quinone reduction as the pH is lowered. We suggest that these data can be accommodated in a model in which the ionization of the tightly-bound FAD affects the enzymes' affinity for nucleotides, and in which two-electron reduction of quinones occurs in the non-nucleotide-liganded enzyme. The data suggest that at neutral pH, where NADH binding is tight, quinones must occupy a distinct site and be reduced by long-range electron transfer.

NADH peroxidase (EC 1.11.1.1) isolated from *Streptococcus faecalis* 10C1 (ATCC 11700) is the only flavoprotein which is known to be capable of reducing hydrogen peroxide to water, and it is the only non-heme-, non-selenium-containing peroxidase. *Streptococcus faecalis*, like other lactic acid bacteria, lacks heme-containing proteins involved in electron transport and oxidative metabolism, and relies on flavoproteins such as NADH oxidase (Schmidt et al., 1986) and NADH peroxidase (Dolin, 1977) for the regeneration of NAD^+ and the decomposition of injurious hydrogen peroxide, produced during aerobic metabolism.

The streptococcal NADH peroxidase is a unique member of the class of flavoprotein reductases which includes lipoamide dehydrogenase and glutathione, thioredoxin, mercuric, and trypanothione reductases (Williams, 1992). These enzymes all show the same stereospecificity for hydride transfer between their respective pyridine nucleotide substrates and FAD (Stoll & Blanchard, 1991; Pai et al., 1988), exhibit steady-state ping-pong mechanisms with the exception of thioredoxin reductase, and can be prepared as stable, two-electron reduced forms with a spectroscopic charge-transfer signature due to the interactions of an active site enzymic thiol with FAD. A unique property of the peroxidase is that this thiol is not formed by reduction of an enzymic redox-active disulfide, as is the case for other flavoprotein reductases, but rather by the reduction of the sulfenic acid form of Cys42 (Poole & Claiborne, 1989). This unusual oxidation state for sulfur has precedent in the literature of the selenium-containing glutathione peroxidase, where the selenic acid (-SeOH) has been proposed as an intermediate in the decomposition of hydrogen peroxide (Flohe et al., 1972). A refined 2.16-Å structure of *S. faecalis* NADH peroxidase (Stehle et al., 1991) revealed that the overall chain fold of the peroxidase was similar to that for other flavoprotein reductases. Further, the redox-active Cys42 of the peroxidase is superimposible with the charge-transfer thiol provided by Cys63 of human glutathione reductase, and the FAD coenzymes are bound in similar regions and in similar conformations.

It has been demonstrated that many flavoprotein reductases can reduce a wide variety of nonphysiological substrates, including 2,4,6-trinitrobenzenesulfonate (TNBS; Carlberg & Mannervik, 1986), ferricyanide (Dubler & Anderson, 1981), and quinones and nitrofurans (Cenas et al., 1991; Bironaite et al., 1991; Cenas et al., 1994). The latter two classes of substrates are reduced by flavoprotein reductases via a combination of one- and two-electron processes, and subsequently generate reduced and reactive oxygen species via reaction of the reduced quinones and nitro compounds with

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molecular oxygen. These enzyme-mediated reductions convert the normally protective functions of these enzymes into potentially toxic ones (Henderson et al., 1988; Mau & Powis, 1992). The obvious structural and mechanistic similarities between NADH peroxidase and other flavoprotein reductases, which have demonstrated quinone-reducing abilities, encouraged us to perform the studies reported here.

MATERIALS AND METHODS

NADH peroxidase was purified to homogeneity from *S. faecalis* 10C1 as described previously (Poole & Claiborne, 1986) with minor adaptations. The concentration of enzyme was determined spectrophotometrically using $\epsilon_{450} = 10.9$ mM^{-1} cm^{-1} at pH 7.0 for the absorbance of enzyme-bound FAD. Pyridine nucleotides and their derivatives, cytochrome *c*, quinones,, and buffer compounds were purchased from Sigma and Aldrich. 1,4-Benzoquinone was purified by sublimation.

Steady-State Kinetic Studies. Reaction rates were determined by the decrease in NADH absorbance at 340 nm ($\epsilon_{340} = 6.2$ mM^{-1} cm^{-1}) on a Gilford 260 spectrophotometer equipped with thermospacers and connected to a circulating water bath. Assays were performed in 0.1 M Hepes, pH 7.5, containing 0.1 M acetate and 0.3 mM EDTA, at 25 ± 0.1 °C. For the determination of steady-state values of *V* and *V/K* of quinones, 5−7 concentrations of the quinone substrate were used. The maximal concentrations of quinone substrates were limited by their water solubilities, and quinones were dissolved in acetonitrile at concentrations which permitted the final concentration of organic solvent to be less than 1% of the total reaction volume. The benzosemiquinone-mediated reduction of cytochrome *c* (40 μM) was monitored at 550 nm ($\Delta\epsilon_{550} = 20$ mM^{-1} cm^{-1}). Reduction of hydrogen peroxide was measured by the decrease in NADH absorbacne at 340 nm in 0.1 M Hepes, pH 7.5, containing 0.1 M sodium acetate and 0.3 mM EDTA in the presence of 0.1 mM hydrogen peroxide. Initial velocity steady-state kinetic data were analyzed by Lineweaver−Burk analysis and fitted to eq 1,

$$v = VA/(K + A) \quad (1)$$

using the FORTRAN programs of Cleland (1979). Data for competitive and uncompetitive inhibition were fitted to eqs 2 and 3, respectively.

$$v = VA/(K(1 + I/K_{is}) + A) \quad (2)$$

$$v = VA/(K + A(1 + I/K_{ii})) \quad (3)$$

Inactivation of the Enzyme with H_2O_2. A 6.8 μM solution of NADH peroxidase in 0.1 M Hepes, pH 7.5, containing 0.1 M acetate and 0.3 mM EDTA was inactivated during a 4-h incubation with 10 mM H_2O_2 solution. Excess H_2O_2 was removed by applying the reaction mixture to a 1 × 20 cm G-25 column. Hydrogen peroxide and quinone-reducing activities were measured as described above.

pH Profiles. The following buffers, at 100 mM concentrations and containing 0.1 M acetate and 0.3 mM EDTA, were prepared and used at the stated pH values to allow for overlap: acetate (4.5−5.6), Bis-Tris (5.8−6.9), MES (6.0−6.6), PIPES (6.6−7.4), and HEPES (7.0−8.2). pH values were measured using a Radiometer PHM 84 pH meter equipped with a combined microelectrode. The kinetic parameters *V* and *V/K* for the substrate were determined at each pH, and their log values were plotted against pH, determined by insertion of a combined microelectrode into the cuvette after the initial velocity assays were carried out. Data for pH profiles that increased from a pH-independent value at high pH values to a higher pH-independent value at lower pH values were fitted to eq 4:

$$\log y = \log((y_L + y_H(K/[H^+]))/(1 + K/[H^+])) \quad (4)$$

where *y* is the parameter whose pH dependence is being determined, y_L is the low-pH-independent value, y_H is the high-pH-independent value of the parameter, and *K* is the dissociation constant for the group whose ionization is responsible for the pH-dependent values.

pH Dependence of the Kinetic Isotope Effect. (4*S*)-[4-^{1}H]- and (4*S*)-[4-^{2}H]NADH were prepared and purified as described previously (Stoll & Blanchard, 1988). Initial velocities were measured at various nucleotide concentrations for both protio- and deuterionucleotides at a fixed, saturating concentration of naphthoquinone, at pH values between 4.8 and 8.2. Primary deuterium kinetic isotope effects on the oxidation of NADH were calculated using eq 5,

$$y = VA/(K + A)/(1 + F_i(E_V)) \quad (5)$$

which assumes equivalent kinetic isotope effects on *V* and *V/K*, E_V, and where F_i is the fraction of isotopic substitution (=0.98). The pH dependenced of the kinetic isotope effects were fitted to eq 4.

RESULTS

Steady-State Reduction of Quinones. The NADH peroxidase catalyzed reduction of various quinones follows a steady-state ping-pong kinetic mechanism, as determined by the series of parallel lines observed when reciprocal velocities were plotted against reciprocal quinone concentrations at various fixed NADH concentrations (data not shown). The addition of bovine liver catalase (10 μg/mL) to reaction mixtures had no effect on the rate of quinone reduction. The steady-state kinetic parameters for reduction of quinones by NADH peroxidase are shown in Table 1. The calculated values of *V/K* for quinone substrates were independent of the concentration of NADH used, as expected for a ping-pong kinetic mechanism, and varied from 6 × 10^5 to 2 × 10^3 M^{-1} s^{-1}. Both the obligate one-electron acceptor, ferricyanide, and the two-electron acceptor, PQQ (methoxatin), exhibited low substrate activity.

To confirm these results and quantitate the percentage of one- and two-electron reduction of quinones by NADH peroxidase, we determined the rate of quinone reduction in the presence of cytochrome *c* and measured the one-electron reduction of cytochrome *c* at 550 nm. This method (Iyanagi & Yamazaki, 1969, 1970; Nakamura & Yamazaki, 1972) is based on the demonstration that at pH values < 7.2 the rate of cytochrome *c* reduction by benzohydroquinone, BQH_2, formed by the two-electron reduction of benzoquinone, is very slow, but that benzosemiquinone, $BQ^{\bullet-}$, formed by the one-electron reduction of benzoquinone, reduces cytochrome *c* at a high rate. The dismutation reaction between quinone and dihydroquinone, potentially formed, cannot be responsible for the observed rates of cytochrome *c* reduction, since

Table 1: Dependence of the Kinetic Parameters of Reduction of Quinones by NADH Peroxidase on Their Single-Electron Reduction Potentials[a]

no.	quinone	E^1 (V[b])	K_m (μM)	V_{max} (s^{-1})	V_{max}/K_m (M^{-1} s^{-1})
1	1,4-benzoquinone	0.09	4.1 ± 1.1	2.4 ± 0.5	(6.0 ± 1.8) × 10^5
2	2-methyl-1,4-benzoquinone	0.01	4.2 ± 1.9	2.0 ± 0.2	(4.9 ± 0.2) × 10^5
3	2,3-dichloro-1,4-naphthoquinone	−0.03	14 ± 4.4	2.9 ± 0.7	(2.0 ± 0.6) × 10^5
4	2,5-dimethyl-1,4-benzoquinone	−0.08	205 ± 82	3.9 ± 1.5	(5.7 ± 0.6) × 10^4
5	5-hydroxy-1,4-naphthoquinone	−0.09	10 ± 4	3.4 ± 0.8	(3.4 ± 0.9) × 10^5
6	5,8-dihydroxy-1,4-naphthoquinone	−0.11	7.7 ± 1.5	2.4 ± 0.2	(3.0 ± 0.5) × 10^5
7	9,10-phenanthrenquinone	−0.15	270 ± 80	2.0 ± 0.6	(6.3 ± 0.9) × 10^4
8	1.4-naphthoquinone	−0.15	13 ± 3	3.4 ± 0.3	(2.7 ± 0.4) × 10^5
9	2-methyl-5-hydroxy-1,4-naphthoquinone	−0.16	25 ± 7	2.5 ± 0.3	(1.0 ± 0.1) × 10^5
10	2-methyl-1,4-naphthoquinone	−0.2	19 ± 9	1.8 ± 0.3	(9.5 ± 3.1) × 10^4
11	tetramethyl-1,4-benzoquinone	−0.26	136 ± 40	1.2 ± 0.3	(9.0 ± 0.2) × 10^3
12	adriamycin	−0.33	40 ± 15	1.2 ± 0.3	(3.5 ± 0.1) × 10^3
13	9,10-antraquinone-2-sulfonate	−0.38	970 ± 60	2.6 ± 1.2	(2.7 ± 0.1) × 10^3
14	2-hydroxy-1,4-naphthoquinone	−0.415	100 ± 20	0.29 ± 0.03	(2.3 ± 0.3) × 10^3
15	PQQ		180 ± 70	0.38 ± 0.1	(2.1 ± 0.2) × 10^3
16	ferricyanide	0.41	114 ± 30	3.6 ± 0.6	(3.2 ± 0.4) × 10^3

[a] All data were obtained in 0.1 M Hepes containing 0.1 M acetate and 0.3 mM EDTA, pH 7.5. [b] From O'Brien (1991).

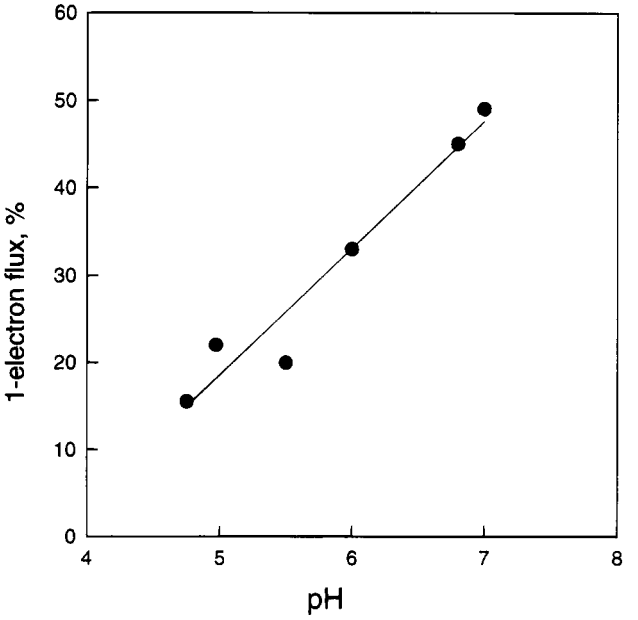


FIGURE 1: pH dependence of one-electron 1,4-benzoquinone reduction.

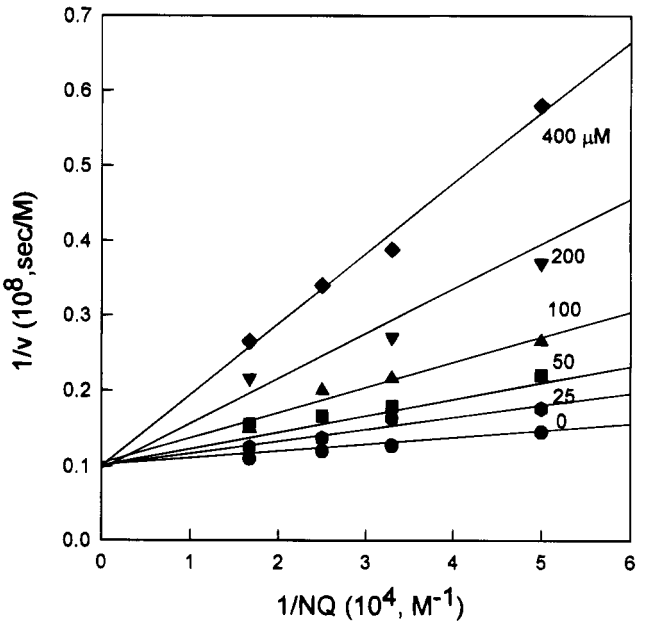


FIGURE 2: 3-Aminopyridine adenine dinucleotide inhibition versus 1,4-naphthoquinone at pH 5.5. The fit of the data yields a calculated K_{is} = 36 ± 8 μM.

the equilibrium constant for semiquinone formation at these pH values is too low. At the high concentrations of cytochrome *c* used in these experiments (40 μM), the percentage of single-electron flux is expressed as the ratio between the rate of cytochrome *c* reduction and twice the rate of enzymatic NADH oxidation in the presence of quinone. At pH 7.0, approximately 50% of the reducing equivalents transferred from NADH into the enzyme, via hydride transfer, are subsequently transferred to the quinone via single-electron transfer during its peroxidase-catalyzed reduction. The percentage of quinone reduction proceeding via one-electron reduction is independent of quinone concentration, but it is dependent on pH (Figure 1).

NADH peroxidase was inactivated using hydrogen peroxide, which causes the conversion of the Cys42 sulfenic acid to the corresponding sulfonic acid, with the irreversible loss of ability to reduce hydrogen peroxide (Poole & Claiborne, 1989). Incubation of NADH peroxidase with 10 mM hydrogen peroxide at pH 7.5 led to the loss of >97% of the peroxidatic activity in 4 h. Peroxide-inactivated enzyme retained approximately 70% of its original quinone reductase activity, with both the maximal velocity and the *V/K* value for naphthoquinone decreasing approximately 30%.

Product Inhibition Studies. When NAD^+ was used as a product inhibitor in the NADH peroxidase catalyzed naphthoquinone reductase reaction at pH 7.5, no inhibition or activation was observed. At pH 5.5, however, the naphthoquinone reductase reaction was effectively inhibited by both NAD^+ and the redox-inactive pyridine nucleotide analog 3-aminopyridine adenine dinucleotide (3-APAD^+). 3-APAD^+ exhibited linear, competitive inhibition versus naphthoquinone with a K_{is} of 36 ± 8 μM (Figure 2). The quinone reductase reaction is dependent on the NAD^+/NADH ratio at pH 5.5, and at the highest NAD^+/NADH ratio tested, the naphthoquinone reduction rate was found to be approximately 4.5 times slower than the rate in the absence of NAD^+ (Figure 3).

pH Dependence of the Kinetic Parameters and Kinetic Isotope Effects. The pH dependence of the quinone reductase

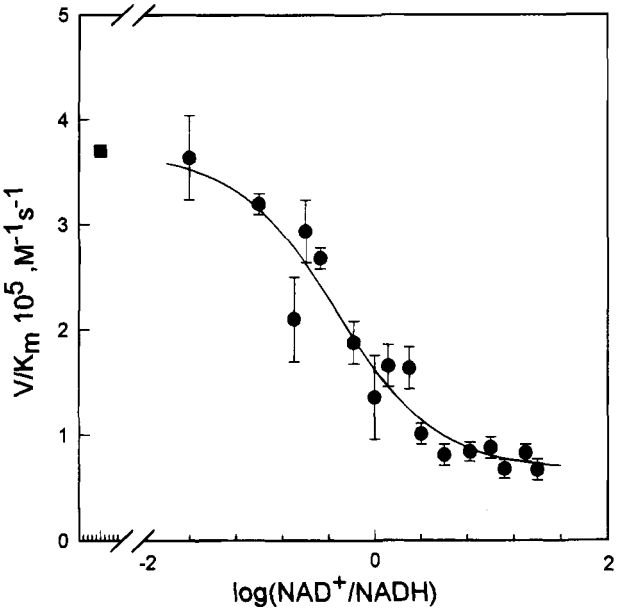


FIGURE 3: Dependence of $V/K_{1,4\text{-naphthoquinone}}$ on the $NAD^+/NADH$ ratio at pH 5.5. The points represent experimental data, and the curve is a fit of the data to eq 4. The point plotted as a square is the value in the absence of added NAD^-.

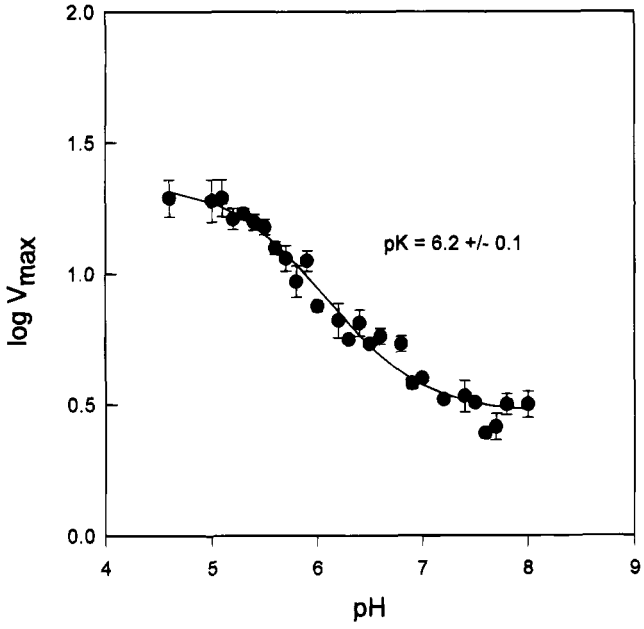


FIGURE 4: pH dependence of the V_{max} of 1,4-naphthoquinone reduction. The points represent experimental data, and the curve is a fit of the data to eq 4, which yields a calculated $pK = 6.21 \pm 0.08$.

activity of NADH peroxidase was examined using NADH as a variable substrate at high concentrations of 1,4-naphthoquinone (100 μM, $K_m = 12 \pm 3$ μM) at pH values between 4.8 and 8.2. Naphthosemiquinone in aqueous solutions exhibits a pK value of 4.8 (Swallow, 1982), so the pH-dependent changes in the activity observed are considered to be due to the ionization behavior of the residues in the protein. The maximum velocity was highest at low pH, and it decreased almost 10-fold at higher pH values (Figure 4), as a group exhibiting a pK value of 6.2 ± 0.1 was deprotonated. The V/K value for naphthoquinone is pH-independent in the pH range investigated. The measurements of kinetic parameters for quinone reduction at higher pH

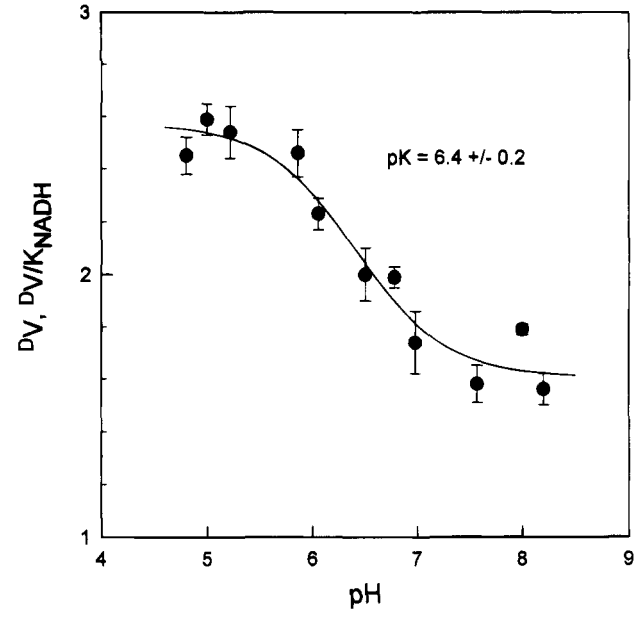


FIGURE 5: pH dependence of the primary deuterium kinetic isotope effect on 1,4-naphthoquinone reduction. Equivalent isotope effects were observed on V and V/K_{NADH}. The points represent experimental data, and the curve is a fit of the data to eq 4, which yields a calculated $pK = 6.4 \pm 0.2$.

values were complicated by the high disproportionation rate between the dihydroquinone and the quinone.

The primary deuterium kinetic isotope effects on V and V/K_{NADH} were determined over the pH range from 4.8 to 8.2. There was no statistically significant change in the observed isotope effect obtained at various concentrations of naphthoquinone. As the pH decreases, both $^DV/K$ and ^DV increase, reaching a common limiting pH-independent value of 2.6 at pH 5.0 (Figure 5). A pK value of 6.4 ± 0.2 was determined from the fit of these data to eq 4, reflecting the pK value of the group whose protonation increases the kinetic isotope effect.

DISCUSSION

Quinones find widespread use as antitumor, antifungal, and antiparasitic drugs, as well as antibiotics. The formation of active metabolites of quinone-containing drugs, often by reductive activation, underscores the importance of understanding the detailed mechanism of bioreduction and the effects of the reduced forms of the drug on cellular function. Hydroquinones have been shown to be able to alkylate essential proteins and inactivate enzymes, either directly or following reduction (Mau & Powis, 1992; Henderson et al., 1988). The bioreductive activation of quinones by both one- and two-electron processes can also result in redox cycling with the formation of reduced oxygen species, such as superoxide and hydrogen peroxide. Most cells have evolved sophisticated enzymatic defense systems to prevent the intracellular accumulation of these reactive oxygen species. Cultures of *S. faecalis* generate large amounts of superoxide and peroxide, but lack heme-containing catalases and peroxidases to protect against intracellular oxidative damage. The flavoprotein NADH peroxidase, which catalyzes the two-electron reduction of hydrogen peroxide to water, maintains the intracellular levels of peroxide at low levels.

The ability of functionally and structurally related flavoprotein reductases to catalytically reduce quinones has been

investigated (Cenas et al., 1989, 1994). We demonstrate here that NADH peroxidase is catalytically capable of reducing quinones, as has been reported for both *Trypanosoma congolense* trypanothione reductase and yeast glutathione reductase. Bimolecular rate constants for quinone reduction by NADH peroxidase (V/K ~6.0 × 10^5 to 2.7 × 10^3 M^{-1} s^{-1}) are approximately 1 order of magnitude greater than those of either glutathione reductase (2.4 × 10^4 to 3.6 × 10^2 M^{-1} s^{-1}) or trypanothione reductase (2.9 × 10^4 to 2.4 × 10^2 M^{-1} s^{-1}). This is true in spite of the higher maximal velocities exhibited by trypanothione reductase and glutathione reductase catalyzing the reduction of their cognate disulfide substrates (122 and 240 s^{-1}, respectively; Fairlamb & Cerami, 1992; Cenas et al., 1989), compared to the NADH peroxidase catalyzed reduction of H_2O_2 (37 s^{-1} at pH 7.5; Parsonage et al., 1993). The maximal velocities of the flavoprotein reductase catalyzed reduction of quinones in the absence of added oxidized nucleotide are all approximately the same: 3−5 s^{-1} (Cenas et al., 1989, 1994; Table 1). This suggests that the rate-limiting chemical steps involved in the reduction of the quinones by flavoprotein reductases may be the same.

The ping-pong kinetic mechanism observed for quinone reduction by NADH peroxidase suggests a possible mechanism for reduction involving an initial Michael-type addition of the thiolate form of Cys42 of the reduced enzyme to the quinone, followed by electron transfer from the flavin to the Michael adduct. This mechanism, and the site of quinone reduction catalyzed by NADH peroxidase, has been investigated by oxidative modification of Cys42, the active site residue involved in reversible electron storage and peroxide reduction. Conversion of the Cys42 sulfenic acid to the corresponding sulfonic acid, by incubation with H_2O_2, resulted in nearly complete loss of hydrogen peroxide reducing activity; however, the modified enzyme retained 70% of its original catalytic ability to reduce quinone substrates. Similar studies involving the reductive alkylation of the catalytic site dithiols of trypanothione reductase (Cenas et al., 1994) have demonstrated specific effects on the substrate disulfide reduction activity with little loss of their quinone reductase activity. These results suggest that quinone reduction can be catalyzed without the catalytic involvement of the sulfenic acid of NADH peroxidase involved in peroxide reduction.

Since both quinones and flavins undergo facile one- and two-electron reduction, it was important to distinguish between these two potential mechanisms by which NADH peroxidase catalyzes quinone reduction. The hyperbolic dependence of the V/K values of quinones on their single-electron quinone/semiquinone reduction potentials (E_7^1; Figure 6) is characteristic of an outer sphere single-electron transfer reaction (Marcus & Suttin, 1985). Theory predicts that the slope of the linear portion (between −0.4 and −0.2 V) of the curve should be ~9.2 V^{-1}, which is close to our experimentally obtained value of 8.5 V^{-1}. Data presented above demonstrate that at pH 7.0 NADH peroxidase reduces quinones via both one- and two-electron mechanisms with *ca.* 50% of the reducing equivalents supplied from NADH being subsequently transferred in a one-electron step. This percentage of one-electron reduction is comparable to that observed with trypanothione reductase (40%), but is much greater than that observed with glutathione reductase (3.6%). Further, the observed correlation between the V/K value of

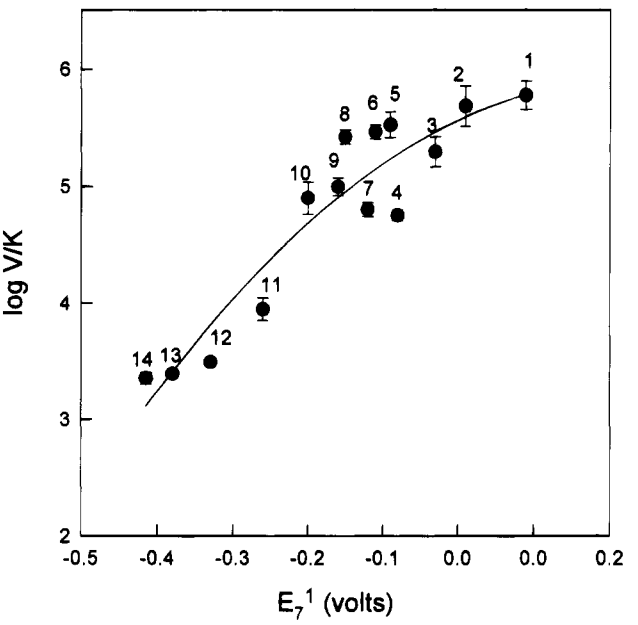
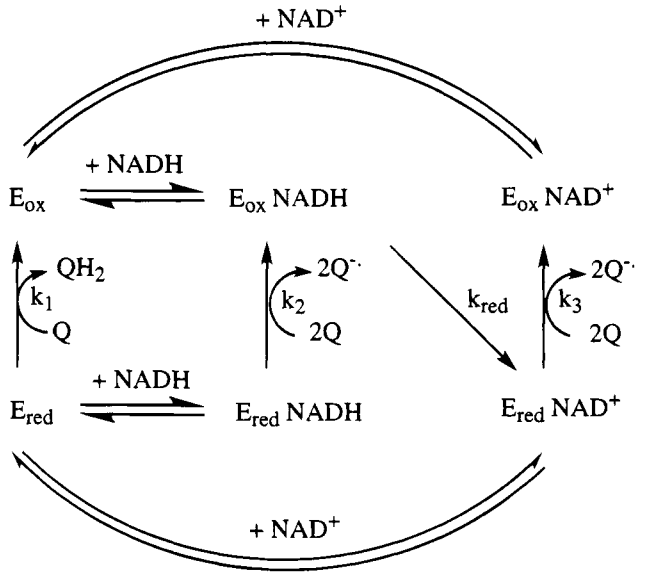


FIGURE 6: Dependence of the steady-state V/K values of quinone reduction by NADH peroxidase on their single-electron reduction potentials (E_7^1): 1,4-benzoquinone (1), 2-methyl-1,4-benzoquinone (2), 2,3-dichloro-1,4-naphthoquinone (3), 2,5-dimethyl-1,4-benzoquinone (4), 5-hydroxy-1,4-naphthoquinone (5), 5,8-dihydroxy-1,4-naphthoquinone (6), 9,10-phenanthrenquinone (7), 1,4-naphthoquinone (8), 2-methyl-5-hydroxy-1,4-naphthoquinone (9), 2-methyl-1,4-naphthoquinone (10), tetramethyl-1,4-benzoquinone (11), adriamycin (12), 9,10-anthraquinone-2-sulfonate (13), and 2-hydroxy-1,4-naphthoquinone (14). All rate constants were determined as described in the text at pH 7.5.

a quinone and its single-electron reduction potential implies that a significant factor determining the reactivity of the series of uncharged quinones studied here is their single-electron redox potential. Similar correlations have been reported for both glutathione and trypanothione reductase (Cenas et al., 1989, 1994). These results are also supported by *in vivo* results correlating the antitumor activity of 12 1,4-naphthoquinone derivatives with their one-electron redox potential (Hodnett et al., 1983). The ability to predict the cytotoxic effects of quinone-containing drugs from their oxidation−reduction potentials has been discussed elsewhere (O'Brien, 1991).

We investigated the pH dependence of the maximal velocity and V/K values of naphthoquinone to identify groups whose ionization behavior would influence these kinetic parameters and for comparison with similar studies on the peroxide-reducing reaction (Stoll & Blanchard, 1991). In contrast to the pH dependence of $V/K_{H_2O_2}$, $V/K_{naphthoquinone}$ was pH-independent between pH 4.8 and 8.2, indicating that the ionization state of enzyme groups has no effect on the oxidative half-reaction rate in this pH range, since this substrate has no ionizable groups in this pH range which would influence binding. Peroxide reduction has been proposed to occur in a small pocket adjacent to FAD, Cys42, and His10, the general acid responsible for proton transfer to the leaving hydroxide ion product generated during peroxide bond cleavage (Stehle et al., 1993; Stoll & Blanchard, 1991). Quinone reduction does not occur at this site, and the pH independence of $V/K_{naphthoquinone}$ suggests that reduction is not accompanied by general acid assisted protonation. In contrast, the V pH profile for the naphthoquinone reductase reaction shows a clear pH-independent

Scheme 1: Proposed Minimal Kinetic Scheme for Quinone Reduction Catalyzed by NADH Peroxidase



maximum at low pH and a pH-independent lower value at high pH. This profile is reminiscent of that observed for the pH dependence of the maximum velocity of hydrogen peroxide reduction. The pK value of 6.2 determined for the group whose protonation effects the quinone reductase reaction may be compared to the pK value of 7.2 obtained for peroxidatic reaction (Stoll & Blanchard, 1991).

In an attempt to further define the nature of the rate-limiting step of the quinone reductase reaction, we determined the dependence of the primary kinetic isotope effect on the pH. The primary deuterium kinetic isotope effects on both V/K_{NADH} and maximum velocity are small (~1.5) and pH-independent at high pH values. Both values increase to a higher, pH-independent value (~2.5) as a group exhibiting a pK value of 6.4 ± 0.2 is protonated. This behavior is qualitatively similar to the pH dependence of the kinetic isotope effects observed for NADH peroxidase catalyzing the reductive cleavage of H_2O_2, although in that case the low pH value of ^DV was 7.2, and $^DV/K_{NADH}$ increased only modestly (Stoll & Blanchard, 1991). It is likely that protonation of this group observed in both the V and ^DV pH profiles causes the oxidative half-reaction rate to increase, since both V and ^DV increase as the pH is lowered. However, ^DV never approaches the value observed for peroxide reduction, suggesting that the oxidative half-reaction involving quinone reduction remains partially rate-limiting even at the lowest pH values tested. The lower turnover numbers for quinone reduction versus hydrogen peroxide reduction (Parsonnage et al., 1993) support this idea. $^DV/K_{NADH}$ increases as the pH values decrease, suggesting that NADH reduces the enzyme faster than it dissociates from the binary complex (Cleland, 1982) at neutral and higher pH values, but dissociates more rapidly at lower pH values.

The data presented here allow us to propose a minimal kinetic scheme for quinone reduction catalyzed by NADH peroxidase which can account for these observations (Scheme 1). Oxidized enzyme binds NADH tightly in a stacked orientation between the isoalloxazine ring of FAD and the aromatic side chain of Tyr159 (Stehle et al., 1993). This step is followed by 4S hydride ion transfer to the N5 position of the flavin, forming NAD^+ and reduced FAD. Subsequent electron transfer from the transiently reduced flavin to the Cys42 sulfenate generates the oxidized flavin and thiolate form of Cys42, via a C4a-thiol−FAD adduct. The distribution of electron density between the flavin and Cys42 thiolate will be influenced by nucleotide binding and the ionization state of His10 (Reitveld et al., 1994). These steps of the reductive half-reaction are subsumed in the rate constant denoted k_{red} in Scheme 1.

The demonstration that oxidative inactivation of the Cys42 sulfenic acid has only a modest attenuating effect on the quinone reductase activity suggests that quinone reduction does not involve this residue. At low pH values, two-electron transfer is the preferred reductive pathway, presumably by direct hydride transfer from N5 of $FADH_2$ to the quinone bound at the nucleotide binding pocket on the *re* face of the isoalloxazine ring of FAD (k_1 in Scheme 1). The possibility of binding planar, aromatic electron acceptors, such as TNBS, in the pyridine nucleotide pocket has been proposed previously for glutathione reductase (Pai et al., 1988), and high concentrations of added NAD^+ or 3-$APAD^+$ inhibit quinone reduction competitively at low pH values (Figure 2). The increased magnitude of $^DV/K_{NADH}$ at low pH also suggests that nucleotide binding is weakened at low pH values. The EH_2−NAD^+ enzyme complex can be formed at low pH values (Poole & Claiborne, 1986) and is capable of reducing quinones, but in a one-electron reaction (k_3 in Scheme 1) and at a 5-fold lower rate than direct hydride ion transfer from EH_2 (Figure 3).

At neutral pH, NAD^+ is weakly bound and dissociates to allow for the binding of NADH to form the EH_2−NADH complex (Stoll & Blanchard, 1988). Both the peroxidatic and the quinone reductase reaction are only weakly inhibited by NAD^+ at neutral pH values. This suggests that at neutral pH values, and in the absence of added NAD^+, the 50% of quinone reduction occurring via one-electron reduction occurs from the EH_2−NADH complex at a third site, via long-range, single-electron transfer (k_2 in Scheme 1). This third site could be similar to a previously described site on the crystallographic 2-fold axis in human erythrocyte glutathione reductase where safranin and menadione bind (Karplus et al., 1989).

Due to the different sites, and mechanisms, of peroxide and quinone reduction on NADH peroxidase, the qualitatively similar pH dependencies of V and ^DV for the two reactions may represent the ionization behavior of either the same group or different groups. In the case of quinone reduction, the pH dependence of the percentage of single-electron reduction of quinones, the maximum velocity, and the magnitude of the primary deuterium kinetic isotope effects on $^DV/K_{NADH}$ can be interpreted in terms of the pH-dependent formation and stability of free and nucleotide-liganded reduced enzyme forms (e.g., EH_2, EH_2−NADH, EH_2−NAD^+). One possibility for the identity of the group influencing all these parameters is the bound flavin whose ionization may influence adjacent nucleotide binding, since the reported pK value of 1,5-dihydroflavin, $FADH_2$, is 6.2−6.4 (Hemmerich et al., 1971). Transient flavin reduction to form $FADH_2$ or $FADH^-$ is rarely observed for any native flavoprotein reductase, even in rapid reaction experiments [for an exception, see Sahlman et al. (1986)]. The stable, two-electron-reduced form of NADH peroxidase is likely to be a mixture of the Cys42 thiolate and oxidized flavin (E−S^-−FAD) in equilibrium with a small amount of sulfenic

acid and reduced flavin (E−SOH−$FADH_2$), and under appropriate conditions the equilibrium will be shifted toward the reduced flavin species. The protonation state of His10 may have an effect on this equilibrium, since in the related glutathione reductase mutagenic conversion of His439 (functionally equivalent to His10) to an alanine residue has been shown to have an effect on the rate of electron transfer from the reduced flavin to the enzymic disulfide (Reitveld et al., 1994). At low pH values, the reduced enzyme composed partially of sulfenic acid and protonated, reduced flavin ($FADH_2$) can transfer a hydride ion to the quinone substrate bound in the unoccupied nucleotide pocket. Added NAD^+ will prevent quinone binding in the nucleotide pocket and change both the equilibrium electronic distribution and the mechanism of reduction from a two-electron to a one-electron reduction. At higher pH values, the formation of the unprotonated, reduced flavin ($FADH^-$) enhances NADH binding, thus favoring a slower, sequential one-electron transfer from the EH_2−NADH complex to quinones bound at a third site. This is in agreement with our data, where a considerable increase (~20 fold) in the V_{max} is observed at low pH values, reflecting the relatively rapid hydride-transfer step in the oxidative, quinone-reducing half-reaction. The reductive half-reaction becomes more rate-limiting, accounting for the increased magnitude of ^DV at low pH values.

Streptococcus, growing aerobically and utilizing glycerol as a carbon source, oxidizes glycerol 3-phosphate via a flavin-linked oxidase (Claiborne, 1986), generating the glycolytic intermediate dihydroxyacetone phosphate and hydrogen peroxide. Potentially high, and injurious, levels of intracellular hydrogen peroxide are reduced by the catalytic action of NADH peroxidase. We have shown here that the relative *V/K* values of high-potential quinones, such as 1,4-benzoquinone, are within an order of magnitude of the *V/K* values for the physiological substrate hydrogen peroxide (6×10^5 vs *ca.* 4×10^6 M^{-1} sec^{-1}; Parsonnage et al., 1993). The efficient peroxidase-catalyzed reduction of quinones may effectively subvert the *in vivo* function of the peroxidase in *Streptococcus*, and related bacteria, by both competing for reducing equivalents and generating high fluxes of one- and two-electron-reduced forms of quinones. The subsequent reaction of these reduced quinones with molecular oxygen generates increasing intracellular levels of reactive oxygen species, including superoxide and hydrogen peroxide. The increased oxidative stress may inhibit either the growth or the viability (Olenick & Hahn, 1974) of quinone-treated cells.

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