Analysis of the Kinetic and Redox Properties of NADH Peroxidase C42S and C42A Mutants Lacking the Cysteine-Sulfenic Acid Redox Center†

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ABSTRACT: The flavoprotein NADH peroxidase from *Enterococcus faecalis* 10C1 has been shown to contain, in addition to FAD, an unusual cysteine-sulfenic acid (Cys-SOH) redox center. The non-flavin center cycles between reduced (Cys-SH) and oxidized (Cys-SOH) states, and the 2.16 Å crystal structure of the non-native cysteine-sulfonic acid (Cys-SO_3H) form of the wild-type peroxidase supports the proposed catalytic role of Cys42. In this study, we have employed a site-directed mutagenesis approach in which Cys42 is replaced with Ser and Ala, neither side chain of which is capable of redox activity. Reductive titrations of both C42S and C42A mutants lead directly to full FAD reduction with 1 equiv of either dithionite or NADH, consistent with elimination of the Cys-SOH center. Direct determinations of the redox potentials for the FAD/$FADH_2$ couples yield values of −219 and −197 mV, respectively, for C42S and C42A peroxidases, indicating that the presence of Cys42-SH in the two-electron-reduced wild-type enzyme lowers the flavin potential by approximately 100 mV. Anaerobic stopped-flow analyses of the reduction of C42S and C42A peroxidases by NADH demonstrate that in both cases flavin reduction is rapid; these results are confirmed by enzyme-monitored, steady-state kinetic analyses which, in addition, give turnover numbers approximately 0.04% that of wild-type enzyme. These results are entirely consistent with the role proposed for Cys42 in the catalytic redox cycle of wild-type NADH peroxidase and indirectly support its function as a peroxidatic center in the homologous NADH oxidase.

The NADH peroxidase from *Enterococcus faecalis* 10C1 is unique among flavoproteins in its ability to catalyze the direct reduction of $H_2O_2 \rightarrow 2H_2O$, in an NADH-dependent reaction (Poole & Claiborne, 1989b; Claiborne et al., 1992, 1993). Earlier studies in this laboratory (Poole & Claiborne, 1986) demonstrated that the peroxidase contained, in addition to FAD, a non-flavin redox center capable of accepting two electrons, and the spectral properties of the two-electron-reduced (EH_2)[1] form of the enzyme first suggested the possible involvement of a redox-active disulfide. Although the reduced enzyme was highly refractory to chemical modification with thiol reagents in the absence of denaturants, selective modification was observed with phenylmercuric acetate in the presence of 1.3 M urea (Poole & Claiborne, 1989a). Thiol titrations combined with peptide analyses of the [^{35}S]cysteine-labeled peroxidase, however, clearly demonstrated the presence of only a single half-cystine per subunit (FAD) (Poole & Claiborne, 1988), and this redox-active half-cystine was subsequently identified as an unusual stabilized cysteine-sulfenic acid (Cys-SOH) derivative of Cys42 (Poole & Claiborne, 1989b).

The sequence of the enterococcal *npr* (NADH peroxidase) gene has been reported (Ross & Claiborne, 1991), and the expression and characterization of the recombinant enzyme have previously been described (Parsonage et al., 1993). The steady-state kinetic mechanism involves the formation of an EH_2·NADH species which then reacts with H_2O_2 in a limiting type of ternary complex mechanism (Parsonage et al., 1993). Refined crystal structures of the non-native cysteine-sulfonic acid (Cys42-SO_3H) derivative of the wild-type peroxidase (Stehle et al., 1991), and of its complex with NADH (Stehle et al., 1993), support a proposed catalytic mechanism for the native enzyme (Poole & Claiborne, 1989b) in which NADH reduction of Cys42-SOH, as mediated by the flavin, leads to the formation of the EH_2 intermediate. The low pK_a of ≤4.5 for the nascent Cys42-SH is consistent with its role as the charge-transfer donor to oxidized FAD; this interaction is responsible for the characteristic visible absorption spectrum of the EH_2 species. A second mole of NADH rapidly binds to EH_2, but the flavin remains oxidized in the EH_2·NADH complex; this step represents one of several which distinguish the peroxidase mechanism from those of flavoprotein disulfide reductases such as lipoamide dehydrogenase, glutathione reductase, and trypanothione reductase (Williams, 1992). The chemical mechanism of peroxide reduction is thought to involve direct nucleophilic attack of the Cys42-S^- thiolate on the substrate in a general acid-catalyzed reaction (Poole & Claiborne, 1989b), and His10 represents a logical candidate for the active-site proton donor (Stehle et al., 1993).

The essential catalytic redox role ascribed to the Cys42-SOH center is supported by earlier studies of the inactivation of the native enzyme by excess H_2O_2 in the absence of NADH (Poole & Claiborne, 1989b); the loss of activity was attributed to irreversible oxidation of the sulfenic acid and loss of the non-flavin redox center. In the present study we have employed a site-directed mutagenesis approach with

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[1] Abbreviations: EH_2, two-electron-reduced enzyme; Cys-SOH, cysteine-sulfenic acid; IPTG, isopropyl β-D-thiogalactopyranoside; $E'°$, midpoint oxidation−reduction potential at pH 7; EH_4, four-electron-reduced enzyme.

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the recombinant peroxidase in which Cys42 has been replaced with Ser and Ala, in order to allow a detailed analysis of the kinetic, redox, and other physical properties of enzyme forms lacking the non-flavin redox center.

EXPERIMENTAL PROCEDURES

Materials. IPTG was purchased from 5Prime → 3Prime, Inc., agarose from FMC Bioproducts, and streptomycin sulfate from Sigma. Ultrapure ammonium sulfate was from ICN Biomedicals (Schwarz/Mann Biotech), NADH and NAD^+ were from Boehringer Mannheim, and hydrogen peroxide (30%) was from Mallinckrodt. All other chemicals, as purchased from sources previously described (Parsonage et al., 1993), were of the best grades available. DNA modification and restriction enzymes were obtained from Promega and were used as recommended by the manufacturer.

Mutagenesis and Expression. Escherichia coli XL1-Blue (Stratagene) and JM109DE3 were used as hosts, as previously described, and standard DNA isolation and manipulation protocols were employed (Ross & Claiborne, 1991, 1992). Single-stranded phagemid DNA was prepared by infection with helper phage M13K07 and was then used as a template for oligonucleotide-directed mutagenesis; oligonucleotides were synthesized by the DNA Synthesis Core Facility supported by the Comprehensive Cancer Center of Wake Forest University. Mutagenesis was carried out using the kit from Amersham except that T7 DNA polymerase (New England BioLabs) was used for initial elongation of the mutagenic primers (Parsonage et al., 1993).

For each of the mutants, the entire *Sph*I−*Sal*I fragment of the final pNPX9-derived expression plasmid (Parsonage et al., 1993) was sequenced using the chain termination method. Sequencing templates were either single-stranded phagemid or double-stranded plasmid DNA, and the Sequenase Version 2.0 (United States Biochemicals) protocol was employed. The resultant pC42S and pC42A plasmids were used to transform *E. coli* JM109DE3 as previously described (Parsonage et al., 1993). Mutant proteins were purified from approximately 25 g (wet weight) of recombinant *E. coli*, following the protocol developed for the wild-type recombinant NADH peroxidase (Parsonage et al., 1993).

Reductive Titrations. Anaerobic titrations and other general analytical methods followed protocols previously described (Poole & Claiborne, 1986); unless stated otherwise, all titrations were carried out in a standard buffer consisting of 50 mM potassium phosphate, pH 7.0, containing 0.6 mM EDTA. Fluorescence measurements were made with an SLM Aminco-Bowman Series 2 spectrofluorimeter. Flavin redox potentials for the C42S and C42A peroxidase mutants were determined by dithionite titrations in the presence of an appropriate reference dye of known potential, with benzyl viologen present to ensure rapid equilibration of reducing equivalents (Stankovich & Fox, 1983). Anthraquinone-2-sulfonate [$E'^\circ = -225$ mV (Massey, 1991); Aldrich] and anthraquinone-2,6-disulfonate [$E'^\circ = -184$ mV (Loach, 1976); Aldrich] were employed as reference dyes for the C42S and C42A mutants, respectively.

Enzyme-Monitored Steady-State Kinetic Analysis. Because of the low turnover numbers of the C42S and C42A mutants, a modified application of the enzyme-monitored turnover method (Gibson et al., 1964) was employed under anaerobic conditions, using a Hewlett-Packard 8452A diode array spectrophotometer. In these experiments, a buffered 0.9 mL solution containing H_2O_2 at the desired concentration was made anaerobic in a standard 1-cm path length anaerobic cuvette; NADH (50 μL) and enzyme (40−50 μL) were maintained in the side arms. NADH was tipped first to allow determination of the very slow rate of nonenzymatic breakdown of this substrate, monitored at 340 nm. Subsequently, the concentrated enzyme solution was added from the second side arm to give a final enzyme (FAD) concentration of approximately 10 μM. After a 20-s mixing period, complete spectral data were collected over the wavelength range 200−820 nm (scanning time = 1 s) at intervals of 5−30 s. Turnover numbers were determined directly from the initial linear rates of NADH oxidation.

Reduction Kinetics. The rapid anaerobic reduction of the C42S and C42A mutants by NADH was followed in an Applied Photophysics DX.17MV stopped-flow spectrophotometer thermostated at 5 °C. Rates were generally monitored at 444−450 nm and at 720−750 nm in order to determine whether transient species were involved. An anaerobic solution of protocatechuate dioxygenase and protocatechuic acid (Sigma) was used to scrub residual oxygen from the stopped-flow system prior to all anaerobic experiments, as described previously (Bull & Ballou, 1981; Claiborne & Massey, 1983). Enzyme solutions were prepared in specially-designed glass tonometers in order to allow anaerobiosis by alternating cycles of evacuation and oxygen-free nitrogen, essentially as described earlier (Claiborne & Massey, 1983). NADH solutions were prepared in glass syringes (Popper & Sons) and bubbled with oxygen-free nitrogen before use in anaerobic experiments. All kinetic data were analyzed with the software package provided by Applied Photophysics.

RESULTS

Mutagenesis and Expression. We adopted the mutagenesis protocol described initially by Eckstein and co-workers (Nakamaye & Eckstein, 1986), using the two oligonucleotides:

5′-TTC TTG TCT G*C*T GGC ATG CAG-3′

and:

5′-TC TTG TCT TC*T GGC ATG CAG-3′

to generate the desired C42A and C42S mutations, as denoted by asterisks, in the plasmid pNPR4 (Parsonage et al., 1993). These oligonucleotides correspond to the NADH peroxidase active-site sequence Phe39-Leu40-Ser41-Cys42-Gly43-Met44-Gln45. The mutated plasmids were digested with *Ssp*I and *Sph*I, and the resulting 159 bp fragments were transferred to pNPX9 via an intermediary pBluescript plasmid, as described for the generation of pNPX9 (Parsonage et al., 1993). The final plasmids, pC42A and pC42S, were sequenced from the *Sph*I site to the initiation codon. The mutant proteins were expressed in *E. coli* JM109DE3 on induction with IPTG and were purified using the procedure developed for wild-type recombinant NADH peroxidase (Parsonage et al., 1993). As purified, both mutant enzymes contained tightly-bound FAD, indicating that these substitutions for Cys42 caused no major changes in active-site structure. The C42A mutant was purified with an overall

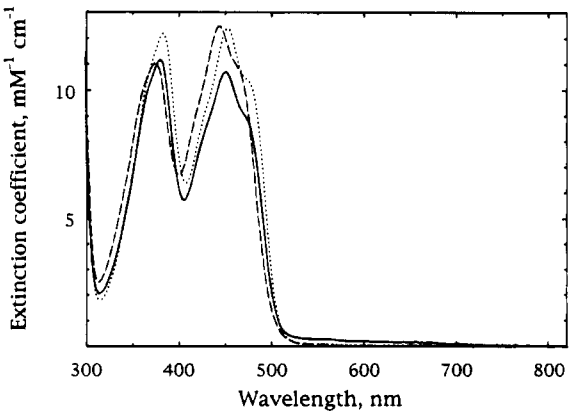

FIGURE 1: Comparison of the spectrum of oxidized wild-type NADH peroxidase (—) with those of C42A (•••) and C42S (- - -) mutant enzymes.

yield similar to that of wild-type enzyme (~140 mg), while the C42S protein was consistently obtained in lower yields ranging from 25 to 75 mg from 4 L of recombinant *E. coli*.

Spectral Properties. Figure 1 gives the visible absorption spectra of wild-type recombinant NADH peroxidase (λ_{max} = 380, 450 nm; ϵ_{450} = 10 900 M^{-1} cm^{-1}; Poole & Claiborne, 1986), the C42S mutant (λ_{max} = 374, 442 nm; ϵ_{442} = 12 700 M^{-1} cm^{-1}), and the C42A protein (λ_{max} = 384, 452 nm; ϵ_{452} = 12 600 M^{-1} cm^{-1}). The absorbance ratios at 280 and 450 nm for the purified enzymes are 7.8, 7.0, and 7.1, respectively, and the hyperchromic blue shift seen in the visible absorption spectrum of the C42S peroxidase resembles that seen with the C49S mutant (Cys49 represents the charge-transfer thiol) of *E. coli* lipoamide dehydrogenase (Hopkins et al., 1991). We have consistently observed a very low-extinction, long-wavelength absorbance band in the wild-type peroxidase extending to 700 nm (Poole & Claiborne, 1986, 1989b). Since irreversible oxidation of the Cys42-SOH redox center by H_2O_2 leads to loss of this feature, commensurate with enzyme inactivation, it has been attributed to a weak charge-transfer interaction between the electron-rich sulfenic acid oxygen and the oxidized flavin (Poole & Claiborne, 1989b). Although the absorbance differences in this region are small, the absence of this long-wavelength band in both the C42S and C42A mutants is consistent with this assignment. As noted previously, the wild-type peroxidase exhibits essentially no flavin fluorescence (quantum yield is 1−2% that of free FAD; Poole & Claiborne, 1986). The C42S and C42A proteins are fluorescent, however, with respective quantum yields 18% and 5−6% that of free FAD.

Reductive Titrations. Figure 2 gives the result of an anaerobic dithionite titration of the C42S protein. As expected, the flavin is reduced directly with 0.93 equiv of reductant per FAD, further confirming the absence of the Cys42-SOH redox center. There was no evidence for even transient flavin semiquinone stabilization at any stage of the reductive titration. The reduced enzyme spectrum (λ_{max} = 358 nm) is very similar to that of the reduced wild-type peroxidase and is consistent with stabilization of the N(1)-anionic form of the dihydroflavin (Poole & Claiborne, 1989a). Dithionite titration of the C42A mutant gave similar results corresponding to 1.1 equiv of reductant per FAD for full reduction.

NADH titrations of the wild-type enzyme are biphasic (Poole & Claiborne, 1986); the first component leads to the

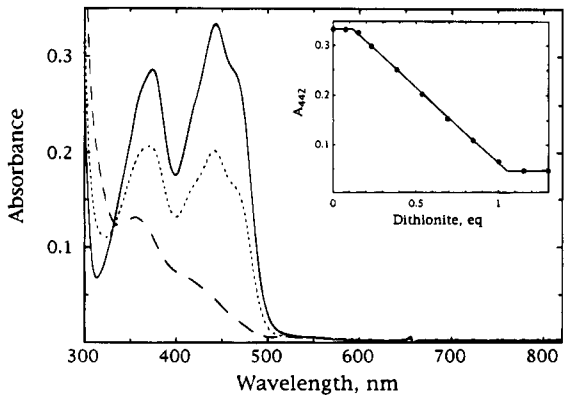

FIGURE 2: Dithionite titration of C42S peroxidase. Anaerobic C42S mutant (27.1 μM) was titrated with a standardized solution of dithionite. Spectra shown represent oxidized enzyme (—) and enzyme after addition of 0.42 (•••) and 1.03 (- - -) equiv of dithionite/FAD (equiv values corrected for the slight lag due to residual oxygen). *Inset*: Changes in absorbance at 442 nm plotted *versus* added dithionite. The end point corresponds to 0.93 equiv of dithionite/FAD.

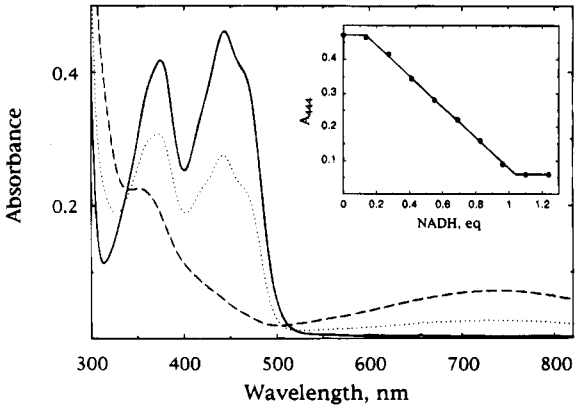

FIGURE 3: Anaerobic titration of C42S peroxidase with NADH. A 0.7 mL solution of 37 μM C42S mutant enzyme was made anaerobic prior to the addition of an oxygen-scrubbing system (0.02 unit of protocatechuate dioxygenase and 40 nmol of protocatechuic acid). Aliquots of an anaerobic 2.5 mM NADH solution were added from a titrating syringe. Spectra shown correspond to oxidized enzyme (—) and enzyme after addition of 0.41 (•••) and 0.96 (- - -) equiv of NADH/FAD. *Inset*: Absorbance changes at 444 nm *versus* added NADH. The end point corresponds to 0.91 equiv of NADH/FAD. All equiv values are corrected for the slight lag.

EH_2 species characterized by an increase in absorbance at 540 nm (0.8−0.9 equiv of NADH/FAD). Addition of a second equivalent of NADH gives the EH_2·NADH complex directly; the flavin is not reduced significantly even in the presence of a 20-fold excess of the pyridine nucleotide. In sharp contrast to the behavior of wild-type enzyme, NADH titrations of the C42S mutant (Figure 3) and of the C42A peroxidase lead to direct reduction of FAD with 1 equiv of reductant. The long-wavelength absorbance band centered at 740 nm is due to bound NAD^+, which gives rise to an $FADH_2 \rightarrow NAD^+$ charge-transfer interaction. This feature also contrasts directly with the behavior of wild-type peroxidase, since titration of the dithionite-reduced enzyme (four-electron-reduced EH_4 species) with NAD^+ leads to stoichiometric formation of the wild-type EH_2·NADH complex (Poole & Claiborne, 1986). In order to confirm the identity of the C42S $FADH_2$·NAD^+ complex, dithionite-reduced protein was titrated anaerobically with NAD^+ (Figure 4). This experiment gives rise to the same 740 nm

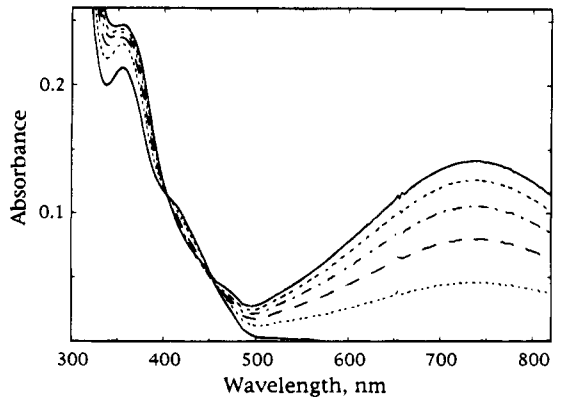

FIGURE 4: Binding of NAD^+ to dithionite-reduced C42S peroxidase. An anaerobic solution of 44.6 μM mutant peroxidase was reduced with dithionite. The titrating syringe was then exchanged for one containing an anaerobic 4.6 mM NAD^+ solution. Spectra shown, in order of increasing absorbance at 740 nm, represent the dithionite-reduced enzyme (—) and reduced enzyme plus 0.4 (•••), 0.85 (- - -), 1.3 (-•-), 1.93 (- - -), and 3.0 (—) equiv of NAD^+/FAD.

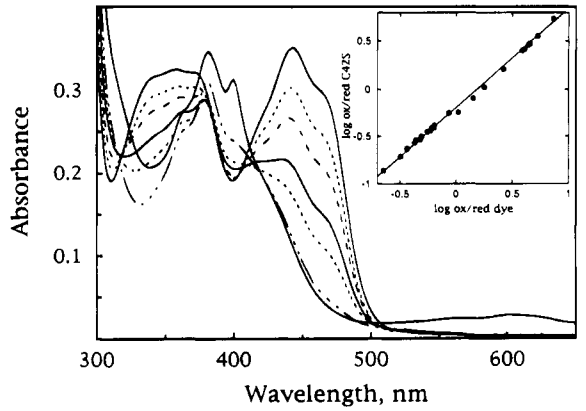

FIGURE 5: Determination of the redox potential of the FAD bound to C42S peroxidase. In an anaerobic cuvette, enzyme (28.8 μM), anthraquinone-2-sulfonate (25 μM), and benzyl viologen (2.5 μM), in 0.1 M potassium phosphate, pH 7.0, containing 0.6 mM EDTA, were titrated with dithionite. After each addition, absorbance was monitored, and the final spectra shown were recorded after no further absorbance changes were observed. *Inset*: log of the oxidized/reduced concentrations of the C42S mutant (monitored at 354 nm) *versus* log of the oxidized/reduced concentrations of anthraquinone-2-sulfonate (measured at 332 nm).

absorbing species and allows the determination of the dissociation constant for the reduced C42S·NAD^+ complex (K_d = 20 μM).

Redox Potentials. The results presented in Figures 3 and 4 indicate that the FAD/$FADH_2$ redox potential in the C42S mutant is higher than that in the EH_2 form of wild-type NADH peroxidase. Direct determination of this value was made by dithionite titration of the enzyme in the presence of the reference dye anthraquinone-2-sulfonate (E'° = −225 mV), with benzyl viologen present at low concentration to ensure rapid equilibration of reducing equivalents. The titration data are shown in Figure 5; reduction of the reference dye was monitored at 332 nm, an isosbestic wavelength for oxidized and reduced forms of the protein. Similarly, enzyme reduction was followed at 354 nm. Preliminary experiments had established that the redox potential for anthraquinone-2-sulfonate was within approximately 30 mV of that for the enzyme flavin. By calculating the concentrations of oxidized and reduced dye and enzyme at each titration point, the midpoint potential for the enzyme can be determined from the Nernst equation, given the known potential of the reference dye and the ratio of the concentrations of oxidized and reduced dye at the midpoint of enzyme reduction (Stankovich & Fox, 1983; Massey, 1991). Following this procedure a redox potential of −219 mV was determined for the FAD of the C42S mutant. A similar titration of the C42A protein using anthraquinone-2,6-disulfonate (E'° = −184 mV) led to a value of −197 mV for the redox potential of this mutant.

Catalytic Properties. Standard NADH peroxidase assays of the two mutants under aerobic conditions at pH 5.4 consistently gave initial rates only twice that of the background NADH breakdown rate under these conditions. In order to obtain reliable turnover numbers in the absence of oxygen (where background oxidase activity could be avoided), and under conditions where the spectral properties of the enzyme could be followed, we decided to employ a modified version of the enzyme-monitored turnover method (Gibson et al., 1964). Furthermore, since preliminary assays showed that the mutants had very poor catalytic activity, this method could be applied with a diode array spectrophotometer. The wild-type enzyme has a turnover number of 70 s^{-1} at pH 7.0, 25 °C.[2] When 10 μM C42S mutant was mixed with 0.25 mM NADH and 1 mM H_2O_2 at pH 7.0, 25 °C, under anaerobic conditions, the enzyme is rapidly reduced within the 20-s mixing time; there is a very slight continual decrease in absorbance at 442 nm over the next 22−23 min. As NADH is exhausted at the end of this time frame, there is relatively rapid reoxidation of the enzyme, reflecting the presence of an excess of oxidizing substrate. In order to perform these experiments over a more practical time frame, the C42S mutant (11.1 μM) was mixed with only 50 μM NADH in the presence of 5 mM H_2O_2. The results of such an experiment are shown in Figure 6. Again there is rapid reduction of the enzyme to a steady-state level corresponding to the fully-reduced form; this steady state is maintained for almost 2 min and corresponds to a linear NADH oxidation rate, as measured at 340 nm. A turnover number of 0.03 s^{-1} was calculated from this rate. In addition, although data at 442 nm are clearly consistent with a steady state of fully-reduced enzyme, the absorbance at 720 nm corresponding to the C42S $FADH_2$·NAD^+ complex continually increases. This observation is interpreted as representing binding of NAD^+ generated in turnover to the reduced enzyme. Once NADH is exhausted, the spectrum of the oxidized enzyme (monitored at both 442 and 720 nm) returns within 1.5 min. These rate data for enzyme reoxidation fit a single exponential corresponding to k_{obs} = 0.04 s^{-1}, in very good agreement with the steady-state turnover number of 0.03 s^{-1}, which is 0.04% that for wild-type enzyme at pH 7.0. Taken together, these data confirm that replacement of Cys42 with Ser leads to an essentially inactive enzyme. Similar kinetic analyses of the C42A mutant gave turnover numbers of 0.01−0.02 s^{-1}. When 10 μM C42A protein was mixed anaerobically with 0.5 mM NADH and 0.1 mM H_2O_2, the initial linear rate of NADH oxidation (measured at 366 nm) gave a turnover number of 0.01 s^{-1}; since NADH is present in excess, the enzyme is reduced once all H_2O_2 is consumed.

[2] E. J. Crane, D. Parsonage, and A. Claiborne, unpublished observations.

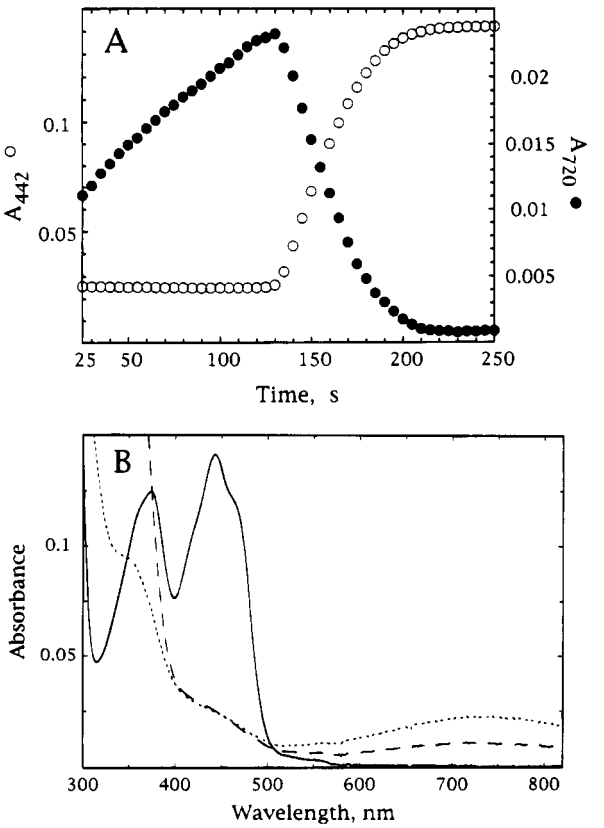

FIGURE 6: Enzyme-monitored kinetic analysis of the C42S mutant. The anaerobic cuvette contained 5 mM H_2O_2 in the standard buffer, while the two side arms contained enzyme and NADH to give final concentrations of 11.1 and 50 μM, respectively, after mixing. After mixing NADH from one side arm to establish the background rate of NADH breakdown, enzyme was tipped in and mixed thoroughly. The first spectrum was measured 25 s after mixing, and subsequent spectra were taken every 5 s. (A) Absorbance changes at 442 nm (○) and 720 nm (●) are plotted *versus* time. (B) Spectra shown correspond to reaction times of 25 s (- - -), 120 s (• • •), and 250 s (—).

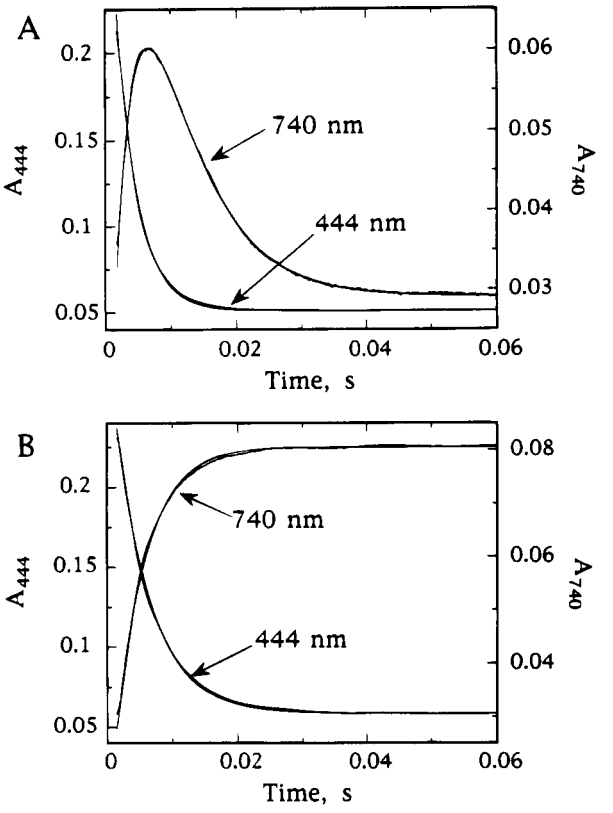

FIGURE 7: Anaerobic reduction of the C42S mutant by NADH. 20.3 μM C42S enzyme (final concentration after mixing) was mixed anaerobically at 5 °C in the stopped-flow spectrophotometer with 0.2 mM NADH (A) or with 0.2 mM NADH plus 1 mM NAD^+ (B). Each trace is the average of at least three measurements. Also shown are the calculated best-fit curves for each trace.

Assays for free H_2O_2 confirmed the reduction of at least 94% of this substrate during turnover.

The absence of Cys42-SOH (and other) redox centers, aside from FAD, in these two mutants implies that the very slow enzyme turnover observed is due to direct reduction of H_2O_2 by $FADH_2$. In order to test this possibility, solutions of free FAD were made anaerobic, titrated with dithionite, and mixed with H_2O_2 while still under anaerobic conditions. The reoxidation of $FADH_2$ was then followed in the diode array spectrophotometer. Reaction traces with either 1 or 10 mM H_2O_2 under pseudo-first-order conditions at pH 7.0, 25 °C, can be fit to single exponentials corresponding to observed rates of 0.01−0.02 s^{-1}. These rates agree well with the rates of 0.03−0.04 s^{-1} for turnover and enzyme $FADH_2$ reoxidation discussed previously.

Reduction Kinetics. When the mutant C42S peroxidase was mixed anaerobically at 5 °C in the stopped-flow spectrophotometer with a 10-fold excess of NADH, the enzyme was rapidly reduced (k_{obs} = 305 s^{-1} as monitored at 444 nm; Figure 7A). When reduction of the C42S mutant was monitored at 740 nm, traces were markedly biphasic and resulted in nonzero end points (Figure 7A). When fit to a double exponential, rates of 280 s^{-1} and 134 s^{-1} were obtained for the formation and decay, respectively, of the transient intermediate. Given the spectral properties of the C42S $FADH_2$•NAD^+ complex described earlier, we considered the possibility that the second phase in these traces at 740 nm represented dissociation of NAD^+ in an approach to equilibrium. When these experiments were repeated in the presence of excess NAD^+, the second phase at 740 nm disappeared, yielding an observed rate of 215 s^{-1} for the increase in A_{740} (Figure 7B). This rate agrees well with that of 185 s^{-1} obtained for reduction of the enzyme, in the presence of excess NAD^+, at 444 nm. Anaerobic stopped-flow measurements of the reduction of the C42A mutant by NADH (3-fold excess) at 5 °C gave a rapid rate of 508 s^{-1} for flavin reduction at 450 nm. At 750 nm the same marked biphasic behavior described earlier for the C42S mutant was observed, with k_{obs} = 461 s^{-1} and 96 s^{-1} for the formation and decay, respectively, of the transient intermediate.

DISCUSSION

The enterococcal NADH peroxidase and the homologous NADH oxidases ($2NADH + 2H^+ + O_2 \rightarrow 2NAD^+ + 2H_2O$) from *E. faecalis* (Ross & Claiborne, 1992) and *Serpulina hyodysenteriae*[3] (Stanton & Jensen, 1993) have been taken to represent a new class of flavoprotein peroxide reductases clearly related to and yet easily distinguished from other members of the disulfide reductase family (Claiborne et al., 1993). The utilization of the Cys-SOH redox center, as demonstrated for the *E. faecalis* NADH peroxidase and NADH oxidase, instead of the redox-active cystine disul-

[3] T. Stanton, personal communication.

fide employed by glutathione reductase and the disulfide reductases, represents the primary point of structural and mechanistic contrast between these flavoprotein groups. The chemical evidence for the Cys-SOH center in the peroxidase has recently been reviewed (Claiborne et al., 1993). Studies of active-site Cys-SOH stabilization in monoalkylated yeast glutathione reductase (Miller & Claiborne, 1991) have also provided a convenient spectral probe for investigating protein-sulfenic acid formation and reactivity and furthermore serve to provide indirect support for the identification of Cys42-SOH in the peroxidase. One disappointing aspect of the NADH peroxidase crystal structure concerns the unavoidable oxidation of Cys42-SOH to the sulfonic acid, Cys42-SO_3H; this modification appears to be a consequence of X-irradiation of the crystalline enzyme (Stehle et al., 1991, 1993). The OX1 oxygen of Cys42-SO_3H is hydrogen-bonded to both the peptide nitrogen of Cys42 and one of the imidazole nitrogens of His10. These hydrogen-bonding interactions have been taken as evidence supporting the hypothesis that OX1 most closely approximates the position of the authentic Cys42-SOH oxygen atom in the native peroxidase (Stehle et al., 1993). Similar active-site thiol oxidations to the sulfinic and sulfonic acid forms have been observed in the X-ray analyses of several thiol proteases (Baker, 1980; Kamphuis et al., 1984) and of the related enzyme dienelactone hydrolase (Pathak & Ollis, 1990). Recently, the crystal structure of an unexpectedly stable sulfenic acid (4,6-dimethoxy-1,3,5-triazine-2-sulfenic acid) was determined by X-ray analysis at 90 K (Tripolt et al., 1993), suggesting that cryo-crystallographic analysis of NADH peroxidase should be undertaken in an attempt to avoid and/or minimize oxidation of Cys42-SOH.

The peroxidase crystal structure (Stehle et al., 1991) shows that Cys42 and His10 lie at the bottom of the proposed hydrogen peroxide access channel; the rear end of the channel is sealed by a group of apolar residues from the complementary subunit, perhaps explaining the selective modification of Cys42-SH with phenylmercuric acetate only in the presence of 1.3 M urea (Poole & Claiborne, 1989a). Solvent access (the channel has an inner diameter of ~5 Å and is partially filled with fixed water molecules; Stehle et al., 1993), chemical reactivity, and other intrinsic factors combine to yield a kinetic bias of $\sim 7 \times 10^6$ in favor of Cys42-SOH formation (from Cys42-SH + H_2O_2) over further oxidation of Cys42-SOH by H_2O_2 (Poole & Claiborne, 1989b). The approach followed in this work, in which Cys42 has been replaced by Ser and Ala, was undertaken in part to overcome the refractile behavior of the peroxidase toward thiol reagents. In both cases the resulting mutant proteins were expressed and purified in high yields, and both mutants showed very tight FAD binding with no propensity toward coenzyme dissociation. In collaboration with Dr. Wim Hol of the University of Washington we have crystallized both C42S and C42A peroxidases, and the refined structures have been solved at resolutions of 2.0 Å.[4]

As expected, dithionite titrations of each protein are complete on addition of 0.9−1.1 equiv of reductant per FAD and lead directly to flavin reduction. This behavior is fully consistent with substitution of the Cys42-SOH redox center of the enzyme with Ser or Ala. More surprising, however, is the direct reduction observed with each of the mutant peroxidases by stoichiometric NADH. With the wild-type enzyme, NADH titrations follow two distinct phases. In the first phase NADH is oxidized (0.8−0.9 equiv per FAD) as the Cys42-SOH redox center is reduced to Cys42-SH, giving rise to the characteristic 540 nm charge-transfer absorbance. Addition of a second equivalent of NADH to the EH_2 species results in the formation of an EH_2·NADH complex (K_d = 0.3 μM) but does not result in flavin reduction (Poole & Claiborne, 1986). Dithionite titration data for the wild-type enzyme indicate that the redox potentials for the E/EH_2 (Cys42-SOH/Cys42-SH) and EH_2/EH_4 (FAD/$FADH_2$) couples are separated by ≥86 mV (Poole & Claiborne, 1989a), ensuring virtually complete Cys42-SOH reduction in the first phase. Redox potentials for the E/EH_2 couples in glutathione reductase and lipoamide dehydrogenase have been measured, giving values of −243 mV (Veine et al., 1994) and −280 mV (Matthews & Williams, 1976), respectively, at pH 7.0, 20−25 °C. Unlike disulfide reduction in these enzymes, however, reduction of Cys42-SOH in NADH peroxidase is effectively irreversible in the absence of H_2O_2, precluding direct determination of the corresponding redox potential. Recently, we have shown that titration of the EH_2 form of the peroxidase with NADPH, in the presence of $NADP^+$, does not lead to an EH_2·NADPH complex but instead reduces the peroxidase to the EH_4 state.[2] At pH 7.0, 23 °C, the EH_2/EH_4 potential obtained in this manner is −312 mV. Given the values of −219 and −197 mV determined for the Ser and Ala mutants, respectively, this indicates that the flavin potentials for the C42S and C42A peroxidases are considerably higher than that for the wild-type EH_2/EH_4 couple.

At present we attribute this dramatic shift of the peroxidase flavin potential to the charge-transfer interaction with the electron-rich Cys42 thiolate in the wild-type EH_2 form. Substitution of the sulfur with oxygen in the Ser mutant is sufficient to abolish this interaction with the flavin and leads to the increased potential observed. The strong interaction of the Cys42 thiolate with the EH_2 flavin, which contributes to an altered redox behavior that does not allow further reduction by bound NADH, is likely to serve as a critical factor distinguishing the catalytic redox behavior of the peroxidase ($H_2O_2 \rightarrow 2H_2O$) from that of the homologous NADH oxidase ($O_2 \rightarrow 2H_2O$). The flavin potentials of the EH_2 forms ($E'°$ for the NADH oxidase EH_2/EH_4 couple is −296 mV)[2] have been directly implicated in the distinction between their respective two-electron and four-electron redox chemistries (Ahmed & Claiborne, 1989, 1992). In the case of wild-type and Cys42 mutant peroxidases it should also be pointed out that each of the corresponding two-electron-reduced proteins (wild-type EH_2, C42S or C42A $FADH_2$) binds the complementary form of NAD(H) with high affinity. The reduced NADH oxidase also binds NAD^+ very tightly (Ahmed & Claiborne, 1989), and preliminary kinetic data support roles in catalysis for both the peroxidase EH_2·NADH and oxidase EH_4·NAD^+ complexes.[2]

The combination of stopped-flow and enzyme-monitored steady-state kinetic data presented in this work for the C42S and C42A mutants clearly shows that the replacement of Cys42 leads to enzyme(s) incapable of any significant peroxidatic function. Very poor turnover numbers approximately 0.04% that of the wild-type enzyme are observed and compare very favorably to rates measured independently for the reduction of H_2O_2 by free $FADH_2$. These observa-

[4] S. S. Mande, D. Parsonage, A. Claiborne, and W. G. J. Hol, manuscript in preparation.

tions fully support the primary catalytic role proposed for the Cys42 thiolate, in serving as the nucleophile against the electrophilic H_2O_2 substrate (Poole & Claiborne, 1989b). The sulfur atom is thought to attack peroxide oxygen in a general acid (possibly protonated His10)-catalyzed reaction that leads to the formation of Cys42-SOH and the elimination of one H_2O. The electron-rich Cys42-S^- is therefore required both in the regulation of the redox behavior of the EH_2 flavin, preventing over-reduction to EH_4, and in the heterolytic cleavage of the -O-O- bond, analogous to the disulfide reduction catalyzed by glutathione reductase. The active-site Cys42 thiolate of the peroxidase, which serves both as charge-transfer donor to FAD and as nucleophile against H_2O_2, combines elements of the roles played together by the charge-transfer and interchange thiols in glutathione reductase (Williams, 1992).

Preliminary stopped-flow studies of the NADH peroxidase mechanism show that a step(s) in the conversion of oxidized enzyme to EH_2·NADH is rate-limiting for turnover, with a value of approximately 20−25 s^{-1} at pH 7.0, 5 °C.[2] Under identical conditions, the C42S and C42A mutants are reduced at rates of 290 s^{-1} and 480 s^{-1}. Flavin reduction, although presumed to represent an obligatory component of the reduction of Cys42-SOH by NADH, has not been observed with the wild-type enzyme. If there is indeed a discrete ($FADH_2$, -SOH) EH_2 intermediate on the catalytic pathway, this would suggest that intramolecular electron transfer to Cys42-SOH is considerably faster than hydride transfer to FAD. A second distinct possibility in which the hydride transfer from NADH and Cys42-SOH reduction follow a concerted mechanism involving the flavin as an essential component cannot, however, be discounted.

In summary, this work demonstrates that replacement of Cys42 by Ser or Ala in the enterococcal NADH peroxidase virtually eliminates peroxidatic activity. Parallel investigations of active-site mutants of the enterococcal NADH oxidase, designed to test the proposed role of Cys42 as a peroxidatic center in that enzyme, are presently underway.

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