Heterogeneity among the Flavin-containing NADH Peroxidases of Group D Streptococci

ANALYSIS OF THE ENZYME FROM STREPTOCOCCUS FAECALIS ATCC 9790*

Holly Miller, Leslie B. Poole, and Al Claiborne
From the Department of Biochemistry, Wake Forest University Medical Center, Winston-Salem, North Carolina 27103

NADH peroxidase (1-4) is a unique flavoprotein which appears to serve two functions in the Gram-positive streptococci. First, it eliminates potentially toxic hydrogen peroxide under aerobic growth conditions and represents the only as yet identified enzymatic defense available against H2O2-mediated oxidative stress. Second, the enzyme presents an additional mechanism for regeneration of the oxidized pyridine nucleotide essential to the strictly fermentative metabolism of this organism. It has previously been observed that NADH peroxidase activity is more frequently associated with the group D Streptococcus faecalis (5), although some strains of the oral pathogen Streptococcus mutans (6) possess this adaptive enzyme as well. The Gram-positive streptococci are well over 1 billion years ago (7-9), prior to the accumulation of oxygen in the atmosphere. These relatively primitive facultative anaerobes resemble a number of obligate anaerobes, such as Clostridium and Peptococcus, which also lack hemoglobin containing proteins (including catalase and the cytochromes) and are thought to reflect very closely the metabolic capacities of the earliest bacterial species (9).

There are several striking parallels which can be drawn between the streptococcal peroxidase and the flavoprotein disulfide reductases (10) such as glutathione reductase. Their redox-active components are similar, in that each contains 1 mol of FAD and one redox-active cysteinyl derivative. In the disulfide reductases this derivative is a protein disulfide; in the peroxidase an unusual stabilized cysteine-sulfenic acid (Cys-SOH) has been identified as the nonflavin redox center (4). With the exception of thioredoxin reductase (11), each of these flavoenzymes yields a spectroscopically distinct intermediate (two-electron reduced NADH peroxidase, EH2) on two-electron reduction; the charge-transfer absorbance at long wavelength is due to the interaction between a nascent cysteine thiolate and the oxidized flavin. We have recently shown (3) that the single cysteine residue in the peroxidase polypeptide follows a putative ββα super-secondary structural element; this allows alignment with the corresponding residues in the glutathione reductase N2-terminal sequence. These enzymes also show the same stereospecificity of hydride transfer with their respective pyridine nucleotide substrates (12, 13), and their kinetic mechanisms appear similar (12, 14). Yet the sequence of the active-site cysteinyl peptide from the peroxidase (2) bears no clear relationship to the corresponding peptide which is so highly conserved in all but one of the disulfide reductases (15, 16).

These and other observations have raised questions about the evolution of the streptococcal peroxidase gene. Interest in this regard has been heightened with the recent observation (17, 18) that the streptococcal NADH oxidase, a flavoenzyme capable of reducing O2 to 2H2O, is related to the NADH peroxidase. The development of these two gene products thus appears to parallel, to a certain extent, the evolutionary history of lipoamide dehydrogenase and glutathione reductase (8, 15, 19). Since the streptococci appear to lack the high levels of glutathione found in Escherichia coli (8, 19), the NADH peroxidase may have arisen in an even more urgent response to the accumulation of oxygen in the environment. The genes encoding lipoamide dehydrogenase and glutathione reductase appear to have evolved relatively slowly (19); comparisons (20) of the mercuric reductase genes from the Gram-positive Bacillus sp. and from the Tn501 transposon found in the Gram-negative Pseudomonas aeruginosa, which indicate approximately 40% identity in predicted amino acid sequences, provide evidence for a relatively slow rate of evolution for this

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§ Present address: Dept. of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

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disulfide reductase as well. The immunological cross-reactivity observed (21) for all mercuric reductases from Gram-negative bacteria (with only one exception (22)) lends further support to this conclusion.

In order to begin to trace the evolutionary history of the streptococcal NADH peroxidase we have isolated the enzyme from S. faecalis ATCC 9790 (Enterococcus hirae (23)). This strain was chosen to facilitate cloning efforts with the enzyme as well (24). The analysis presented in this report indicates that considerable heterogeneity exists among the flavoprotein peroxidases of group D streptococci with respect to immunological and chemical properties.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pheny1-Sepharose CL-4B was from Pharmacia LKB Biotechnology, Inc. Ethyl hydroperoxide (5% aqueous) was from Polysciences, Inc., Warrington, PA. Methyl methanethiosulfonate was from Aldrich and iodo[L-14C]acetamide (57 mCi/mmol) was from Amersham Corp. The Protoblot AP & Stem was from Promega Biotec, Madison, WI. Freeze-dried cultures of S. faecalis ATCC 9790 were purchased from American Type Culture Collection, Rockville, MD. In the purified S. faecalis 8043 NADH peroxidase was obtained from Boehringer Mannheim. All other chemicals, as purchased from sources previously described (1-4), were of the highest reagent grade.

**Methods**—General procedures for NADH peroxidase assays and reductive titrations, and for SDS-polyacrylamide gel electrophoresis and Western analyses, followed protocols previously established in this laboratory (1, 17). Polyclonal antisera were raised by subcutaneous and intramuscular injections of 150 μg of purified ATCC 9790 NADH peroxidase, together with complete Freund's adjuvant, into two rabbits. Booster injections with incomplete adjuvant were given 2 weeks later. Preimmune serum was taken prior to immunization, and rabbits were bled 4 weeks after the initial injections to obtain the antiperoxidase sera. The purified antisera were prepared by using the homogenous ATCC 9790 peroxidase, bound to nitrocellulose, as an immunoaffinity matrix (11, 22).

**Thiol Titrations and Reductive Alkylation**—5,5'-Dithiobis(2-nitrobenzoate) titrations of the oxidized and NADH-reduced peroxidase, under anaerobic denaturing conditions, followed the protocol described for the ATCC 11700 enzyme (2). Two slightly different protocols were employed in reductive alkylations (26) of the ATCC 9790 NADH peroxidase. In the first labeling experiment 30 nmol of protein, in 0.5 M Tris-HCl, pH 8.5, containing 5 mM EDTA, was incubated with 440 nmol of dithiothreitol in the sidearm of an anaerobic cuvette. The total volume was 70 μl. After anaerobiosis was achieved by repeated cycles of evacuation and equilibration with oxygen-free nitrogen, the contents of the sidearm were mixed with 0.5 ml of 8.3 μm guanidine HCl in 0.5 M Tris-HCl, pH 8.5, containing 5.4 mM EDTA and 50 μCi of iodo[1-14C]acetamide. Following a 1-h incubation in the dark at 20 °C the reaction was quenched by adding 50 μl of glacial acetic acid (final pH ~4). The alkylated protein was then dialyzed in the dark at 4 °C, first against 5% (v/v) glacial acetic acid and then exhaustively against 0.1% 2-mercaptoethanol.

In the second alkylation experiment 60 nmol of protein in the 0.5 M Tris-HCl, pH 8.5 buffer, was incubated with 6 μmol of dithiothreitol and 26 μmol of methylamine, in a total volume of 0.1 ml. The protein was then anaerobically mixed with 0.8 ml of 8.3 μm guanidine HCl (alkaline buffer at pH 8.5) containing 50 μCi of labeled iodacetamide. After 30 min the reaction was quenched and the protein was dialyzed against 5% glacial acetic acid as before except that 0.1% 2-mercaptoethanol was present in all dialyses. The protocols employed for digestion with TPCK-treated trypsin, reversed-phase HPLC purification of peptides, and sequence analysis have been described previously (2, 3).

**Purification of the ATCC 9790 NADH Peroxidase**—All steps of the enzyme purification procedure were performed at 4 °C. Tris buffer pH values and ammonium sulfate concentrations (expressed as percent saturation) refer to a standard temperature of 25 °C. Unless otherwise indicated all column and dialysis buffers contained 0.6 M EDTA. Details of the growth of S. faecalis ATCC 9790 were essentially as described previously for S. faecalis 10C1 (1) except that cultures were harvested 6 h after inoculation, yielding 20-25 g (wet weight) per 6 liters of culture.

140 g of frozen cell paste was thawed, suspended in Tris buffer plus 1 mM diithiothreitol, and homogenized with a Gaulin homogenizer. The supernatant obtained after centrifugation at 13,700 × g was applied to a phenyl Sepharose CL-4B column (2.5 × 40 cm) previously equilibrated in 50 mM potassium phosphate, pH 6.8, containing 35% ammonium sulfate. The column was washed with 38% ammonium sulfate in the same buffer, and the peroxidase was eluted by decreasing the ammonium sulfate concentration to zero in the same buffer through a 2-liter linear gradient. The pool of active fractions was dialyzed against 3 × 4 liters of 20 mM phosphate, pH 6.5, containing 0.1 M NaCl.

The dialyzed pool was applied to a column of DE52 (5 × 30 cm) equilibrated in 20 mM phosphate, pH 6.5, with 0.1 M NaCl. After washing the column with the same buffer, the peroxidase was eluted by running a 1.5-liter linear gradient from 0.1 to 0.4 M NaCl in the 20 mM phosphate buffer, and the active fractions were pooled and concentrated to about 15 ml in an Amicon concentrator with a YM-30 membrane. The concentrated sample was loaded onto a column (4 × 85 cm) of Sephacryl S-200 Superfine equilibrated in 10 mM phosphate, pH 6.8, and eluted with 1.2 liters of the same buffer. Fractions containing a specific activity >20 units/mg (1 unit oxidizes 1 pmol of NADH/min at 25 °C) were pooled and dialyzed against 3 × 2 liters of 10 mM phosphate, pH 7.0, without EDTA. The dialyzed sample was applied to a column (2.5 × 20 cm) of Bio Gel HTP equilibrated in 10 mM phosphate, pH 7.0, without EDTA. The column was washed with the same buffer, and the peroxidase was eluted by increasing the phosphate concentration to 0.2 M in a 1-1 linear gradient. The pure peroxidase fractions exhibiting a specific activity of 80 units/mg or more were pooled, concentrated by ultrafiltration with a YM-30 membrane, dialyzed against 50 mM phosphate, pH 7.0, and stored in 0.6-ml aliquots at −20 °C.

**RESULTS**

**Enzyme Purification**—The results of our purification scheme for the ATCC 9790 NADH peroxidase are given in Table 1. Approximately 7 mg of pure enzyme were obtained in an overall 16% yield from 140 g (wet weight) of S. faecalis. There are important differences between this protocol and that previously established for the ATCC 11700 enzyme (1). At 55% ammonium sulfate, over 60% of the ATCC 9790 peroxidase is precipitated, whereas the S. faecalis 10C1 enzyme is precipitated in the supernatant under these conditions. In order to provide better resolution by reversed-phase ammonium sulfate chromatography, we chose a phenyl-Sepharose matrix and eluted the enzyme with a gradient of 30 to 0% ammonium sulfate. The ion-exchange chromatography step was also modified slightly to accommodate differences in behavior observed with the ATCC 9790 peroxidase. SDS-polyacrylamide gel electrophoresis analysis of the purified enzyme (Fig. 1) gives an apparent subunit molecular weight of 50,000 and shows that there are no intersubunit disulfides.

The specific activity of the purified ATCC 9790 NADH peroxidase is ~60% that of the S. faecalis 10C1 enzyme as assayed under identical conditions. The spectral properties of the ATCC 9790 enzyme are very similar to those of the previously purified flavoprotein peroxidase (1). Absorbance ratios are 7.5, 1.03, and 1.0 at 290, 378, and 450 nm, respectively.

**Tryptic Maps and Immunochemical Analyses**—The intact ATCC 9790 enzyme was submitted for NH2-terminal sequence analysis; the first 17 residues are Met-Glu-Lys-Lys-Lys-Val-Ile-Ile-Val-Gly-Ala-Ala-His-Gly-Gly-Ohn. In order to further assess similarity of the overall structure between the two peroxidases the enzymes were subjected to parallel trypsin digestions without prior modification. The tryptic maps in Fig. 2 show very few major peptide peaks in the...
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TABLE 1

| Step                              | Volume | Total Protein | Total Activity | Specific Activity | Purification | Yield |
|-----------------------------------|--------|---------------|----------------|-------------------|--------------|-------|
| I. Crude extract                  | 1120   | 7605          | 3955           | 0.52              | 1.0          | 100   |
| II. MnCl₂ supernatant             | 1140   | 6555          | 3845           | 0.59              | 1.1          | 98    |
| III. 35% (NH₄)₂SO₄ supernatant    | 2420   | 6408          | 3845           | 0.6               | 1.2          | 97    |
| IV. Reversed-phase (NH₄)₂SO₄ column | 975   | 1765          | 2753           | 1.56              | 3.0          | 70    |
| V. DEAE-cellulose column          | 348    | 487           | 974            | 2.0               | 3.9          | 25    |
| VI. Sephacryl S-200 column        | 33     | 50            | 1285           | 25.7              | 49.4         | 33    |
| VII. Hydroxylapatite column       | 28.6   | 6.7           | 576            | 86                | 165          | 15    |

common. The unmodified cysteinyl peptide of the *S. faecalis* 10C1 peroxidase has been shown to elute at ~100 min in this HPLC system, based on experiments with the ³⁵S-labeled enzyme (4).

The differences in overall structure between the two peroxidases were reinforced by the results of the Western analysis shown in Fig. 3. Polyclonal rabbit antiserum to the purified ATCC 9790 enzyme detect the protein with good sensitivity (below 2 ng) in both crude extracts and in purified form. In addition, these affinity-purified antiserum also react strongly with a commercially available NADH peroxidase preparation. However, they do not recognize the purified ATCC 11700 peroxidase when present at even 10-fold higher levels. In separate experiments not shown we have also determined that polyclonal rabbit antiserum against the *S. faecalis* 10C1 enzyme react very poorly, if at all, with the ATCC 9790 protein. We conclude that antigenic epitopes are not strongly conserved between the two enzymes.

Active-site Peptide Isolation and Sequence—5,5'-Dithiobis(2-nitrobenzoate) titration of the purified ATCC 9790 peroxidase under anaerobic conditions in the presence of 4 M guanidine HCl demonstrated the absence of free sulfhydryls. Anaerobic reduction of the native enzyme with 1 eq/FAD of NADH, followed by denaturation with 4 M guanidine HCl and 5,5'-dithiobis(2-nitrobenzoate) titration, resulted in a value of 0.9 thiols/FAD in the two-electron reduced enzyme. These data are consistent with the presence of a single redox-active cysteinyl derivative/FAD in the oxidized peroxidase. Previous attempts to alkylate the nascent EH₂ thiol with iodoacetamide or N-ethylmaleimide under non-denaturing conditions were unsuccessful with the *S. faecalis* 10C1 enzyme (3). Methyl methanethiosulfonate is a relatively small, neutral reagent which reacts rapidly with most enzyme thiols (27). A 30-min incubation of the dithionite-generated EH₂ intermediate of the ATCC 9790 peroxidase with 2 mM methyl methanethiosulfonate, however, led to no loss of either charge-transfer absorbance or of enzyme activity. We conclude that the active-site thiol of the peroxidase EH₂ form is also inaccessible to this reagent.

In order to allow purification and sequencing of the active-site cysteinyl peptide, the ATCC 9790 peroxidase was incubated with iodo[¹³⁵]Iodacetamide for 1 h under reducing conditions in the presence of 7.3 M guanidine HCl (see "Experimental Procedures"). Following acidification to pH 4 and exhaustive dialysis the alkylated protein sample was digested

FIG. 1. SDS-polyacrylamide gel electrophoresis of *S. faecalis* (ATCC 9790) NADH peroxidase at various stages of purification. A 10% acrylamide gel was run with samples pretreated by heating for 5 min in sample buffer containing 2% SDS with (lanes 2-8) or without (lane 9) 5% 2-mercaptoethanol. The gel was electrophoresed at 25 mA in the presence of 0.1% SDS. Lanes 2-7, pooled samples from Steps I-VI of the purification; lanes 8 and 9, purified enzyme pretreated in the presence and absence, respectively, of 2-mercaptoethanol. Lanes 1 and 10 contain molecular weight standards, as indicated.

FIG. 2. Comparative tryptic maps of the ATCC 9790 and ATCC 11700 NADH peroxidases. A, 20 nmol of tryptic digest of the ATCC 11700 enzyme; B, 4.5 nmol of a parallel tryptic digest of the ATCC 9790 enzyme. Conditions for digestions and HPLC analyses are found under "Experimental Procedures." Peptide absorbance at 206 nm is plotted versus retention time.
with a modified protocol (see "Experimental Procedures"). Although we were not able to separate the radiolabeled peak from one major contaminating peptide, sequence and radiolabel analyses indicate that tryptic peptide T-1 results from a secondary cleavage of the T-2 peptide after the NH2-terminal Met residue. In addition, tryptic peptide T-2 was also isolated as a result of this second alkylation experiment and its sequence confirmed that observed previously. No attempt was made to analyze any of the smaller radioactive peaks identified in the chromatogram given in Fig. 4.

**Reductive Titrations**—Dithionite titration of the NADH peroxidase requires 2 eq/FAD of reductant, as shown in Fig. 5, and proceeds through a characteristic two-electron reduced intermediate (EH2) with charge-transfer absorbance at 540 nm. Comparison of experimental and theoretical curves for this titration, however, indicates that only 69% of the enzyme appears as the EH2 species on half-reduction. The corresponding formation constant K can be calculated from the relationship:

$$K = \frac{[EH2]^2}{[E][EH]}$$

The value of 20 determined for this enzyme is considerably lower than that for the ATCC 11700 enzyme (K = 780 under identical conditions (3)) and indicates a significant increase in the extent of disproportionation of EH2 to oxidized (E) and fully reduced (EH2) enzyme at equilibrium. From the formation constant one can estimate that the separation in potentials (E2 − E1), where E1 is the midpoint potential for redox couple E/ EH2, and E2 is the midpoint potential for redox couple EH2/EH4, between the nonflavin redox center and the flavin is 39 mV. The redox potentials of the two centers are considerably closer in this enzyme than in the S. faecalis 10C1 enzyme, where E2 − E1 is ≥86 mV (3). The enhanced disproportionation in the present case also explains why the absorbance at 450 nm for the EH2 intermediate is only 87% of that observed with the ATCC 11700 enzyme at half-reduction; this difference is attributable to the 15% of the total enzyme present as EH2 at equilibrium. The altered redox behavior of the ATCC 9790 peroxidase is somewhat similar to that of the S. faecalis 10C1 enzyme in the presence of low concentrations of urea (3) which dramatically decreases the rate of disproportionation of E and EH2.
The $\text{EH}_2$ spectra of the dithionite- and NADH-reduced $S$. faecalis 10C1 peroxidase are virtually identical, although oxidized pyridine nucleotide appears to remain tightly bound in the latter case (1, 3). With the ATCC 9790 peroxidase, however, the $A_{340}$ and $A_{450}$ values of the NADH-titrated enzyme (Fig. 6) are ~20% higher than those of dithionite-generated $\text{EH}_2$. These observations provide further support for a specific, high-affinity interaction between the two-electron reduced peroxidase and NAD$^+$. We conclude that the presence of NAD$^+$ leads to preferential stabilization of $\text{EH}_2$.

$$E + \text{EH}_2 \rightarrow 2\text{EH}_2$$

$$\text{EH}_2 + \text{NAD}^+ \rightarrow \text{EH}_2\cdot \text{NAD}^+$$

The subsequent addition of a second eq/FAD of NADH under anaerobic conditions (Fig. 6) yields an $\text{EH}_2\cdot \text{NAD}^+$ complex similar to that previously observed (1). Recent studies with the ATCC 11700 enzyme indicate that the higher-extinction charge-transfer band of $\text{EH}_2\cdot \text{NAD}^+$ is primarily due to an NADH→FAD interaction (4); the very low $K_d$ ($\approx 10^{-8}$ M) for this complex suggests a probable role in catalysis, as described recently (4).

**Inhibition by Ethyl Hydroperoxide**—As discussed previously, the proposed mechanism for the streptococcal NADH peroxidase, involving a reducible cysteine-sulfenic acid (4), is very similar to that proposed for glutathione peroxidase, which is thought to shuttle between selenolate (Cys-Se$^-$) and selenenic acid (Cys-SeOH) redox states in catalysis (28). The latter enzyme is also known to catalyze the reductions of alkyl hydroperoxides at rates comparable to those of hydrogen peroxide reduction (28). There are, however, major differences in active-site structures for the two enzymes. The 2-Å x-ray structure of the selenoenzyme shows the essential Cys-Se$^-$ residue to be readily accessible from solvent channels (28). Chemical modification studies of the streptococcal peroxidase, on the other hand, indicate a very restricted access to solvent for the reduced active-site cysteine (3). In order to extend the analysis of the flavoprotein peroxidase with respect to its specificity for peroxide substrates, we investigated ethyl hydroperoxide as a substrate with the ATCC 9790 enzyme. Whereas initial rates of NADH oxidation by the streptococcal peroxidase under standard assay conditions (1.3 mM H$_2$O$_2$, pH 5.4) are linear for several minutes (1), substitution of ethyl hydroperoxide at the same concentration resulted in nonlinear progress curves as followed at 340 nm. Fig. 7 gives an example of such an initial velocity pattern with 5.3 mM ethyl hydroperoxide. The limiting rate is virtually identical to the nonenzymic rate of NADH breakdown observed under the mildly acidic conditions of the assay. Approximately 30% of the NADH present initially has been oxidized during the course of enzyme inhibition, but the $K_m$(NADH) for the peroxidase determined at pH 7.5 is only 2.5 $\mu$M (12); the concentration of NADH remaining under the conditions of the assay at complete inhibition is on the order of 0.1 mM. As shown in Fig. 7, the rate of inhibition is essentially first-order and corresponds to an apparent rate of 0.07 min$^{-1}$ at 5.3 mM ethyl hydroperoxide. The observed rate increases to 0.15 min$^{-1}$ at 12.4 mM peroxide; however, a thorough analysis of the rate of inhibition versus [CH$_3$CH$_2$OOH] has not been performed. We have, however, shown that preincubation of enzyme with 12.4 mM ethyl hydroperoxide for 15 min does not alter the progress curve obtained when NADH is added subsequently. Furthermore the NADH peroxidase is stable for incubation times of at least 45 min at pH 5.4. Ethanol, the anticipated product of ethyl hydroperoxide reduction, does not inhibit the enzyme under standard assay conditions. Although we have recently reported that H$_2$O$_2$ incubations with the ATCC 11700 peroxidase in the absence of NADH lead to inactivation attributed to irreversible oxidation of the cysteinyl redox center (4), parallel incubations of the enzyme with 20 mM ethyl hydroperoxide lead to <10% inactivation over 30 min. Treatment with 20 mM H$_2$O$_2$ under identical conditions gives 98% inactive peroxidase.

**DISCUSSION**

The analysis presented in this report suggests that considerable heterogeneity exists among the streptococcal NADH peroxidases. The comparative tryptic maps of the ATCC 9790 and ATCC 11700 enzymes indicate extensive differences between the respective polypeptides. The immunochemical analysis, which shows essentially no cross-reactivity between
the two peroxidases, demonstrates that antigenic epitopes are not conserved in the two proteins. In contrast, previous reports have shown that all mercuric reductases from Gram-negative sources (21), with the exception of the enzyme from *Thiobacillus ferroxidans* (22), are immunochemically cross-reactive. The strong cross-reaction observed between the ATCC 9790 NADH peroxidase and a commercially available preparation from *S. faecalis* ATCC 8043 is consistent with the results of recent molecular and chemotaxonomic analyses since these strains are now both designated as *E. hirae* (23, 29); this species does not include the ATCC 9790 strain which has been reclassified as *Enterococcus faecalis* (29).

The chromosomal mer*A* gene of the Gram-positive *Bacillus* sp. (20) encodes a polypeptide which exhibits only 40% amino acid sequence identity when compared to the Gram-negative *Tn*501 (*P. aeruginosa*) mercuric reductase, and does not appear to cross-react with the anti-*Tn*501 mercuric reductase sera (22). Despite the heterogeneity observed among mercuric reductase sequences from Gram-negative and Gram-positive bacteria Laddaga et al. (30) have shown that the percentage identity in those amino acid sequences corresponding to active-site positions and to FAD and NADPH contacts is greater than 90%. The NH$_2$-terminal sequence of the ATCC 11700 NADH peroxidase (3) contains a putative redox-active disulfide which is likely to represent either an FAD or NADH binding site. Alignment with the corresponding segment of the ATCC 9790 enzyme (Table II) shows that this motif is conserved, although only 7 of 14 residues in the overlap region are identical. The NH$_2$-terminal segments of the ATCC 11700 peroxidase and the NADH oxidase of this strain also show nearly 50% identity over a 15-residue overlap. The active-site cysteinyl peptide sequences given for the two peroxidases are identical in 8 of 12 positions within the indicated overlap; again, a similar comparison can be made for the active-site sequences of the ATCC 11700 NADH peroxidase and NADH oxidase. These limited sequence comparisons, confined to predicted FAD or NADH binding regions and to active-site positions, suggest that the two peroxidase genes have undergone considerable divergence relative to the genes encoding mercuric reductase (30) and glutathione reductase (31). With respect to the active-site peptides, however, different structural requirements and stringencies must be met in the latter two cases to accommodate the redox-active disulfides in the respective oxidized enzymes. Furthermore, the conformation about the disulfide in glutathione reductase is somewhat unique; the main chain in this region is subject to mechanical strain which could be of functional importance (32). As the peroxidases do not contain disulfides at their active centers, more flexibility in amino acid replacement may have been tolerated over time.

The results of this analysis also serve to further distinguish the streptococcal NADH peroxidases from the flavin-dependent alkyl hydroperoxide reductase recently purified from *Salmonella typhimurium* (33). The latter enzyme appears to exist as a heterodimer (apparent subunit molecular mass values of 57 and 22 kDa); the larger subunit contains 1 mol of FAD. The enzyme is specific for alkyl hydroperoxides; hydrogen peroxide appears to inactivate the alkyl hydroperoxide reductase during turnover with NADPH. The 22-kDa subunit is thought to contain one redox-active disulfide which represents the site of alkyl hydroperoxide reduction. Aside from the differences in quaternary structure and in the nature of the nonflavin redox centers we have also shown that ethyl hydroperoxide actually inhibits the streptococcal peroxidase in turnover. Furthermore, the NADH peroxidase is specific for its pyridine nucleotide substrate whereas the *S. typhimurium* enzyme uses either NADH or NADPH. In addition, NH$_2$-terminal sequence comparisons (Table II) show no clear relationship between the two flavin-dependent peroxidases. These structural and mechanistic distinctions serve to further underscore the parallels which exist between the enteric streptococci and the enteric Gram-negative bacteria, in terms of their flavin-linked evolutionary responses to oxygen accumulation.

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