Mechanism of Superoxide and Hydrogen Peroxide Formation by Fumarate Reductase, Succinate Dehydrogenase, and Aspartate Oxidase*

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Oxidative stress is created in aerobic organisms when molecular oxygen chemically oxidizes redox enzymes, forming superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Prior work identified several flavoenzymes from *Escherichia coli* that tend to autoxidize. Of these, fumarate reductase (Frd) is notable both for its high turnover number and for its production of substantial O$_2^-$ in addition to H$_2$O$_2$. We have sought to identify characteristics of Frd that predispose it to this behavior. The ability of excess succinate to block autoxidation and the inhibitory effect of lowering the flavin potential indicate that all detectable autoxidation occurs from its FAD site, rather than from iron-sulfur clusters or bound quinones. The flavin adenine dinucleotide (FAD) moiety of Frd is unusually solvent-exposed, as evidenced by its ability to bind sulfite, and this may make it more likely to react adventitiously with O$_2^-$. The autoxidizing species is apparently fully reduced flavin rather than flavosemiquinone, since treatments that more fully reduce the enzyme do not slow its turnover number. They do, however, switch the major product from O$_2^-$ to H$_2$O$_2$. A similar effect is achieved by lowering the potential of the proximal [2Fe-2S] cluster. These data suggest that Frd releases O$_2^-$ into bulk solution if this cluster is available to sequester the semiquinone electron; otherwise, that electron is rapidly transferred to the nascent superoxide, and H$_2$O$_2$ is the product that leaves the active site. This model is supported by the behavior of “aspartate oxidase” (aspartate:fumarate oxidoreductase), an Frd homologue that lacks Fe-S clusters. Its dihydroflavin also reacts avidly with oxygen, and H$_2$O$_2$ is the predominant product. In contrast, succinate dehydrogenase, with high potential clusters, generates O$_2^-$ exclusively. The identities of enzyme autoxidation products are significant because O$_2^-$ and H$_2$O$_2$ typically grow poorly or die, indicating that these species are formed in potentially toxic doses inside living cells (12, 13). Similar toxicity occurs in wild-type organisms when they are exposed to higher-than-usual levels of oxygen, evidently because these species are formed at elevated rates. Despite this progress, the mechanisms of O$_2^-$ and H$_2$O$_2$ formation are less well understood.

Molecular oxygen is a triplet species that can only accept electrons one at a time from potential donors (14). This restriction ensures that oxygen does not spontaneously oxidize most reduced biomolecules, such as NAD(P)H, which are obligate two-electron donors. Instead, enzymes that are competent univalently convert electron donors are the most likely effectors of inadvertent oxygen reduction. Such enzymes are prominent in electron transport chains. Accordingly, studies in *Escherichia coli* determined that NADH dehydrogenase II, succinate dehydrogenase, sulfite reductase, and fumarate reductase each formed O$_2^-$ and H$_2$O$_2$ when the reduced enzymes were exposed to oxygen (15, 16). These autoxidizing enzymes contain flavins and either iron-sulfur clusters or quinones, all of which are competent at univalent redox reactions. However, in each case the flavin appeared to be the primary site of electron transfer to oxygen. This trend has been noted in other autoxidizing enzymes as well. This may be due to the solvent accessibility of the flavins, which are situated at the protein surface in order to interact with soluble substrates. In contrast, the iron-sulfur clusters are typically buried within polypeptide, and quinones may be sequestered in hydrophobic regions of the protein-membrane interface where O$_2^-$ formation is disfavored.

Interestingly, the rates at which flavoenzymes leak electrons to oxygen vary over several orders of magnitude (15, 17–19), indicating that additional factors must govern turnover number. We are hopeful that some of these factors might be revealed by study of members of the succinate dehydrogenase/fumarate reductase family (Fig. 1). These enzymes are structurally and functionally similar to one another (20–22), and each autoxidizes, albeit at different rates. Succinate dehydrogenase (Sdh), a primary respiratory dehydrogenase, catalyzes electron transfer from succinate to membrane-bound quinone. Fumarate reductase (Frd) catalyzes the opposite reaction in its service as a terminal oxidase during the anaerobic growth of some bacteria and eukarya. The physical structure of the *E. coli* fumarate reductase has been determined (23), and the pathways of electron movement through both it and succinate dehydrogenase have been well studied (20–22). A notable distinction between the enzymes is that the iron-sulfur clusters are poised at higher potentials in Sdh than in Frd. This arrangement favors electron flow away from the flavin and to...
ward the quinone-binding site in Sdh, and toward the flavin in Frd, and is consonant with the physiological directions of the enzymes. However, Frd and Sdh are sufficiently similar that each can functionally substitute for the other in E. coli (24, 25).

Aspartate oxidase is a newly recognized member of the succinate:fumarate oxidoreductase family (26, 27). It catalyzes the first step in the nicotinamide biosynthetic pathway, oxidizing aspartate to iminoadipate. Aspartate oxidase has a flavoprotein structure homologous to those of Frd and Sdh, but it lacks their iron-sulfur clusters and membrane attachment subunits (28–30). It has conventionally been considered to use oxygen as its natural electron acceptor, forming H2O2 as a stoichiometric product, but recent in vitro studies (27) revealed that it can use fumarate as well. This observation raises the possibility that fumarate is its natural substrate in vivo and that the reported formation of H2O2 occurs in vitro because the enzyme adventitiously transfers electrons to oxygen, in the manner of Frd and Sdh.

An earlier study (16) found that Frd adventitiously generates O2− at a higher rate in vitro than any other native metabolic enzyme yet characterized. Indeed, overproduction of Frd debilitated superoxide dismutase-defective E. coli when it was exposed to oxygen, and lack of the enzyme was protective. In contrast, Sdh generated considerably less O2−. It was speculated that the ability of the reduced flavins to react with oxygen might derive from partial flavosemiquinone character, which would be created when one electron from the reduced flavin delocalized onto the adjacent iron-sulfur clusters. Alternatively, it is also possible that a dihydroflavin is the autoxidizing species. The issue is of interest, because if the latter were true, then in principle many simple flavoproteins could contribute to the formation of endogenous O2−.

The degree and type of endogenous oxidative stress that a cell experiences depend upon the rates at which its enzymes autoxidize and upon whether O2− or H2O2 is the predominant product. This study examined the Frd/Sdh family to explore reasons that its members react so rapidly with oxygen. We also sought to identify the factors that govern whether O2− or H2O2 is formed.

MATERIALS AND METHODS

Chemicals—Most chemicals were purchased from Sigma or Fisher. Amplex Red was from Molecular Probes, and catalase was from Roche Molecular Biochemicals. In vitro experiments used house-deionized water that was further purified with a Labconco Water Pro PS system.

Cell Growth and Media—Membrane vesicles containing Frd were prepared from cells grown either in LB medium supplemented with 0.2% glucose or in minimal A medium (31) supplemented with 0.65% casamino acids and either 40 mM glucose, 40 mM glucose plus 40 mM fumarate, or 40 mM glycerol plus 40 mM fumarate. Standard antibiotic concentrations were used for plasmid maintenance and P1 transductions (31). Media for anaerobic cultures were degassed by autoclaving and then moved into a Coy anaerobic chamber (5% H2, 10% CO2, and 85% N2) and equilibrated for at least 36 h before inoculation.

Sdh-containing membranes were prepared from strain KM3 (Δfrd-ABCD ubiA240 menA601 katG14 pGSI3 expressing schD/CADB) grown on LB plus 0.2% glucose. To allow robust growth of the Sch/minus-deficient strain, the culture was grown for 4 generations anaerobically and then, to enhance expression of Sdh, aerated for a final generation of growth before harvesting at anA600 = 0.6.

Strains and Genetic Techniques—P1-mediated transduction and plasmid transformation were conducted by standard methods (31). For strains bearing the ubiA240 menA601 alleles, 1 mM p-hydroxybenzoic acid was added to the medium (16) during strain construction to prevent reversion. The katG17::Tn10 and katG14 alleles were confirmed by the absence of the hydroperoxidase I/catalase band on an activity gel (32). The sdhC4::kan− allele was verified by loss of succinate:plumagin oxidoreductase activity from the membranes of cells grown aerobically in LB medium. The Δfrd/ABCD18 null allele was screened by loss of benzyl viologen:ferrireductase activity in membranes of cells grown anaerobically on glucose/fumarate medium.

Construction of Aspartate Oxidase-Expressing Plasmid pETNADB—The nadB gene encoding l-aspartate oxidase was amplified by PCR from a crude cell lysate of E. coli strain MG1655. The primers were 5′-GCTATACAGCAAAACATTTAGA-3′ and 5′-AGGGCTCGATGTGTATTTAGAT-3′. The PCR products were ligated into a Promega pGEM-T PCR vector, and the plasmid was transformed into E. coli NK6004 (nadB::Tn10) to verify complementation of the nicotinamide acid autotrophy. An Xba1-Xhol fragment containing the gene was then ligated into pET28a (Novagen). In strain BL21(DE3) (Novagen) this construct allowed expression of the full coding region of the nadB gene, followed at the C terminus by the amino acids Leu-Glu-His.

Enzyme Preparations—Cells expressing Frd or Sdh were lysed by French press, and membrane vesicles were isolated as described previously (16), except that cells grown in minimal A media were generally harvested in early stationary phase (A600 0.5–0.7) rather than in mid-exponential phase. For Frd purification, membranes were prepared from strain KM32 (sdhC4::kan− katG14, pH 3, encoding wild-type Frd) and prepared by Triton X-100 extraction (33). SDS-PAGE gel analysis indicated that these preparations were ~95–98% pure (data not shown). Further purification by glycerol gradient (33) resulted in some depletion of the protein bands that correspond to FrdC and FrdD (see “Results”); therefore, Frd was prepared without this step.

For purification of the His-tagged l-aspartate oxidase, BL21(DE3) + pETNADB was grown to anA600 of 0.3 and then induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested at anA600 of 1.0 and resuspended in 20 mM Tris-HCl, 5 mM imidazole buffer, pH 7.9, that contained 0.5 mM NaCl and 20 mM μFAD. The cells were lysed by two passages through a French press. The lysate (~5 ml) was then eluted from a His-Bind column (2.5 ml, Novagen) according to the manufacturer’s instructions. Fractions were collected and assayed for l-aspartate oxidase (37). Homogeneity of the protein was confirmed by SDS-PAGE (data not shown).

Enzyme Assays—Unless otherwise noted assays were conducted at 37 °C. Conversion of l-aspartate to oxaloacetate (l-aspartate oxidase activity) was performed as described by Tedeschi et al. (27). One μM l-aspartate was used unless otherwise indicated.

Assays to be conducted in partially or fully anaerobic conditions were set up in the Coy anaerobic chamber. Aerobic buffer was brought into the chamber in air-tight syringes, and the amount needed to reach the desired oxygen concentration was mixed with aerobic buffer and other reaction components in sealable cuvettes. After mixing and sealing, the cuvettes were removed from the chamber to the spectrophotometer. Time lapse between the start of reactions and the first time point was 20 s or less.

Addition of Superoxide—Superoxide generation was assayed by the standard cytochrome c reduction method (34, 35), using 10 μM cytochrome c. Twenty units/ml CuZn-superoxide dismutase was added in a parallel reaction to quantify any superoxide-independent, direct electron transfer from the superoxide-generating enzyme to cytochrome c; this rate was subtracted from the overall rate to yield the rate of superoxide formation. For the enzyme systems considered in this paper, the amount of direct electron transfer to cytochrome c was always less.
that the enzyme substrates used in this study did not interfere with detection of superoxide at the concentrations used. Unless otherwise noted, assays were conducted in air-saturated 50 mM potassium phosphate, pH 7.8, buffer at 37 °C. Reaction time was generally 3–5 min.

Assay of Hydrogen Peroxide—Horseradish peroxidase-based assays of hydrogen peroxide were employed with either the chromogenic substrate o-dianisidine (36) or the fluorogenic Ampex Red (37) as the indicator. Air-saturated 50 mM potassium phosphate, pH 7.8, was used as the reaction buffer. The H2O2-generating reactions were run, and 1-ml samples were removed and added to 30 μl of 600 mM fumarate (unless already present) to halt autoxidation of the enzyme. The o-dianisidine method, samples were then mixed with 100 μl of a mixture containing 150 μM o-dianisidine and 60 μg/ml type II horseradish peroxidase; after a 1-min incubation, the sample absorbance was read at 460 nm. For the Ampex Red method, after the addition of fumarate, the 1-ml samples were mixed with 0.5 ml of 45 μg/ml Ampex Red and 0.5 ml of 20 μg/ml horseradish peroxidase, and after 1 min fluorescence was read in a Shimadzu RF-mini 150 fluorometer using a 460 nm excitation filter and 520 nm emission filter. The absorbance and fluorescence values were converted into H2O2 content using standard curves. At the concentrations that were used, the enzyme substrates did not interfere with detection of H2O2.

Energy of Activation—The sample chamber of the spectrophotometer was enclosed within a thermally controlled water jacket. Reaction components were heated or chilled to the reaction temperature before assembly of the reaction mixture. The reaction rate versus temperature curves were biphasic, with a slowly responsive component at lower temperatures and a highly responsive component at higher temperatures. Membrane phase effects (38) and oxaloacetate present in the enzyme preparations (39) likely explain this behavior. The linear portion of this curve (generally between 25 and 50 °C) was used to determine the energy of activation using the Arrhenius equation.

Sulfite Binding Measurements—Three ml of solubilized Frd (12.5 μm) was placed in a cuvette and warmed to 20 °C, and an initial UV-visible spectrum was obtained. Small additions (<1 μl) of 10–500 μM sulfite were made to the sample, and the sample was allowed to equilibrate until the spectrum remained constant (15–30 min). After scanning, further additions of sulfite were then made in the same manner, until no further spectroscopic response was apparent. The total time for the experiment was ~5 h, during which time the Frd showed little (<5%) loss of activity. A binding constant was calculated (40), using the assumptions that the decrease in absorbance value at 460 nm (the wavelength with the strongest response to sulfite) was proportional to sulfite binding to the enzyme and that the absorbance value at 20 mM SO3− represented a fully titrated enzyme.

Covalent and Noncovalent Flavin Measurements—Covalent FAD attached to membrane-bound Frd and Sdh and to solubilized Frd was measured as described by Singer et al. (41). The histidyl-FAD cofactors in Frd and Sdh represent the only sources of covalent flavin in E. coli cell membranes; hence, in sdh mutants the covalent flavin content of the membranes represents their Frd content and vice versa. Noncovalent FAD content was measured by fluorescence as described (42).

RESULTS

Fumarate Reductase Rapidly Generates Both Superoxide and Hydrogen Peroxide—A previous report (16) demonstrated that E. coli fumarate reductase generates abundant superoxide when the reduced enzyme is exposed to oxygen. It was assumed that the electron transfer was an adventitious event, since molecular oxygen is not present during the normal function of the enzyme as a terminal reductase in the anaerobic electron transport chain. To verify this, the rate of superoxide production was measured as a function of the concentration of dissolved oxygen (Fig. 2, circles). For these experiments, membrane vesicles were prepared from a strain with mutations that blocked the synthesis of succinate dehydrogenase, to avoid interference from this Frd homologue, and of respiratory quinones, so that electrons supplied by succinate could not move beyond Frd to other respiratory chain components. The first-order dependence upon oxygen concentration confirmed that superoxide formation is a chemical event and indicates that no long lived complex between the enzyme and oxygen is formed.
Malonate is an inhibitor of Frd that also binds at the flavin site. When 100 μM malonate was included in the reactions, the malonate inhibited both $O_2^-$ and H$_2$O$_2$ generation when succinate concentrations were low but had no detectable effect when succinate concentrations exceeded 5 mM (data not shown). This lack of further effect at high succinate concentrations affirms the idea that excess succinate inhibits autoxidation by occupying the flavin active site, making malonate addition redundant.

A Noncovalently Attached Flavin Mutant of Frd Makes Little Superoxide—To confirm that the flavin is the site of autoxidation, we examined the rate of superoxide production by a mutant Frd in which the electron density is shifted from FAD toward the iron-sulfur clusters. The flavin of the FrdA H44C mutant has a low reduction potential because it lacks the covalent histidyl linkage to FAD (42). It has lower activity as a fumarate reductase. Because this enzyme is not reducible with succinate, we prepared the enzyme in vesicles from strain KM31 ($\Delta$frdABCD)18 sdhC4::kan' katG14 pFRDA H44C) containing α-glycerophosphosphate dehydrogenase, and we reduced the enzyme via the quinone pool, using α-glycerol phosphate as a respiratory substrate. The rate of superoxide generation in membranes from these cells was ~22 (~5%) that of membranes from isogenic cells expressing wild-type Frd. The diminished rate of autoxidation supports the idea that the flavin is the site of electron transfer to oxygen, and it indicates that the redox potential of the flavin is an important determinant of its autoxidizability.

Turnover Numbers—The amount of Frd present in vesicles was determined by measurement of the covalent FAD content of the membranes. At the peak $O_2^-$ generation rate, the turnover based on covalent flavin content was 310 O$_2$/[H]/min. This is lower than the indirect estimate given in the previous report (16) but is substantially higher than that of other native flavoenzymes. The peak oxidation rate, considering both $O_2^-$ and H$_2$O$_2$, was 430 electrons/min. Xanthine oxidase, a damaged form of xanthine dehydrogenase, produces superoxide at a similar rate (18).

Frd purified from membranes had similar turnover numbers and dependence on succinate concentration, indicating that it is the sole participant in this autoxidation process and that the autoxidation does not depend upon electronic or physical interaction with any other membrane component.
and H2O2 generation are expressed as fractions of the enzyme with 20, 50, 150, and 500 μM and 20 mM sulfite. Inset, difference spectra of the enzyme without sulfite, minus the spectrum of the enzyme + 20 mM sulfite. Abs, absorbance.

ing the conclusion that SO₃⁻ binds at the FAD moiety on the enzyme.

Thus Frd shares with the flavin oxidases both a reactivity with oxygen and a high degree of flavin exposure. The apparent ability of the SO₃⁻ molecule to access the N(5) of the flavin lends credence to the idea that the nearby carbon(4a) of the flavin is also solvent-accessible. This site is typically the point of O₂ interaction with reduced flavin (46), and its exposure is thought to be a typical feature of flavin oxidases. In contrast, in "flavin dehydrogenases" the distal benzyl ring of the flavin, rather than the isoalloxazine, is typically solvent-exposed (44). Thus the ability of Frd to rapidly transfer electrons to oxygen is likely to derive from a combination of the high electron density of its flavin and the accessibility of the isoalloxazine ring to dissolved oxygen.

Superoxide Is Optimally Formed When Frd Is Partly Reduced—The succinate concentrations affected the relative yields of superoxide and H₂O₂. This pattern is clearest if the rates of O₂⁻ and H₂O₂ generation are expressed as fractions of the total autoxidation at a given succinate concentration (Fig. 4B). Below 1 mM succinate, O₂⁻ accounted for as much as 80% of the autoxidized electrons, while near 1 mM a distinct shift to predominantly (~60%) H₂O₂ formation occurred. This profile suggested that the product of autoxidation depended on the degree of reduction of fumarate reductase, with higher concentrations of succinate more effectively reducing the enzyme during turnover and yielding the more heavily reduced oxidation product.

Because the concentrated enzyme rapidly depletes dissolved oxygen, we were unable to test this idea by monitoring the redox status of autoxidizing enzyme. As an alternative approach, we tested whether the rate of enzyme reduction by menaquinol, rather than by succinate, also determined whether superoxide or H₂O₂ was the primary product. Inverted vesicles were incubated with a range of α-glycerol phosphate concentrations, and the rates of O₂⁻ and H₂O₂ generation were measured. The addition of saturating amounts (5 mM) of malonate inhibited 90% of the production of superoxide and H₂O₂, thereby confirming that Frd was responsible. At high concentrations of α-glycerol phosphate, O₂⁻ generation diminished and was replaced by H₂O₂ generation (Fig. 6). This pattern resembled the changeover seen with succinate-reduced enzyme. A distinction was that the overall autoxidation was not suppressed by α-glycerol phosphate, since unlike succinate it did not sterically occlude the flavin. This result supports the conclusion that superoxide is optimally generated when the enzyme is incompletely reduced.

Evidence That Dihydroflavin, Rather Than Flavosemiquinone, Is the Initial Species That Reacts with Molecular Oxygen—Our original idea was that Frd reacts so readily with oxygen because the species is an enzyme-stabilized flavosemiquinone. However, the previous results suggested that H₂O₂ was rapidly generated by the putatively four-electron-reduced enzyme, indicating that the dihydroflavin must also be capable of relatively efficient autoxidation. In fact, the overall rate of autoxidation when H₂O₂ was the predominant product was not substantially lower than when superoxide was predominant (Fig. 4B), indicating that the rate-limiting event was likely to be the same in both cases.

We therefore measured the energy of activation of superoxide and H₂O₂ production. They were equivalent (50 ± 4.2 kJ/mol) within the error of the measurements. Solution pH had no specific effect upon superoxide formation, even though flavosemiquinone autoxidation is often favored by deprotonation. Furthermore, we were unable to detect a stabilized semiquinone by EPR or when the solubilized Frd was photoreduced (47, 48). Leger et al. (45) have also noted a lack of flavosemiquinone character in Frd. These results suggest that molecular oxygen initiates autoxidation by abstracting an electron from the dihydroflavin form of FAD. The resemblance noted above between Frd and flavin oxidases reduces the need to invoke flavosemiquinone character in order to rationalize its high rate of oxygen reduction.

Why Does Partial Enzyme Reduction Favor Superoxide Formation?—Quantum mechanical principles require that electrons be transferred one at a time from dihydroflavins to triplet oxygen, forming superoxide as an initial product. In flavin oxidase reactions a spin inversion allows recombination of the superoxide and flavosemiquinone radicals and the generation of a C4(a)-peroxyl adduct followed by release of H₂O₂ (44). Therefore, whereas the rate of the first electron transfer determines autoxidation rate, it is the behavior of the second electron that determines product identity. With most flavin oxidases the second electron follows the first onto oxygen because the reacting species is an enzyme-stabilized flavosemiquinone. However, the previous results suggested that H₂O₂ was rapidly generated by the putatively four-electron-reduced enzyme, indicating that the dihydroflavin must also be capable of relatively efficient autoxidation. In fact, the overall rate of autoxidation when H₂O₂ was the predominant product was not substantially lower than when superoxide was predominant (Fig. 4B), indicating that the rate-limiting event was likely to be the same in both cases.

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An explanation was suggested by the observation that H₂O₂...
is the predominant product of the more fully reduced Frd. In the more oxidized enzyme, the "second" electron could plausibly delocalize from the relatively unstable semiquinone onto adjacent vacant iron-sulfur clusters, thereby diminishing the semiquinone content and frequency of electron transfer to $O_2^-$. In contrast, the clusters of the more reduced enzyme would already be occupied, forcing the unpaired electron to remain on the active-site flavin and enhancing the likelihood of peroxyl adduct formation.

To test this idea, the autoxidation behavior of the FrdB C62S mutant was examined. In this mutant the [2Fe-2S] cluster has a strongly lowered redox potential (−200 versus −35 mV) (49). We reasoned that if $O_2^-$ formation in Frd predominated as a result of electron transfer from the flavosemiquinone to a higher potential [Fe-S] center, then this mutant would show a diminished capacity to generate $O_2^-$ but still be capable of $H_2O_2$ generation. This was in fact the case. $H_2O_2$ was the predominant product of the more fully reduced Frd. In the more oxidized enzyme, the "second" electron could plausibly delocalize from the relatively unstable semiquinone onto adjacent vacant iron-sulfur clusters, thereby diminishing the semiquinone content and frequency of electron transfer to $O_2^-$. In contrast, the clusters of the more reduced enzyme would already be occupied, forcing the unpaired electron to remain on the active-site flavin and enhancing the likelihood of peroxyl adduct formation.

**Aspartate Oxidase Is an Frd Homologue Whose Reduction of Oxygen Is Adventitious**—The *E. coli* t-aspartate oxidase, encoded by *nadB*, bears considerable sequence similarity to the flavoprotein subunit of fumarate reductase and succinate dehydrogenase (28) and might therefore provide insight into their reactions with oxygen. We suspected that t-aspartate oxidase, like its homologue, Frd, was likely to use fumarate as its physiological electron acceptor and that its *in vitro* reaction with oxygen was adventitious. Indeed, sulfite-binding experiments have shown that its flavin is highly solvent-exposed (27) and therefore likely to react with dissolved oxygen, as in the case of Frd.

Purified aspartate oxidase oxidized 10 mM t-aspartate more rapidly in the presence of 0.5 mM fumarate than in the presence of oxygen alone. Moreover, fumarate appeared to be the preferred acceptor, since its presence inhibited oxygen reduction (H$_2$O$_2$ formation) by ~90%. Furthermore, whereas fumarate reduction exhibited Michaelis-Menten-like saturation kinetics (data not shown), the rate of oxygen reduction was first-order in oxygen concentration (Fig. 2, triangles).

We sought to determine whether oxygen was the obligate electron acceptor for t-aspartate oxidase *in vivo*. Wild-type strains grew anaerobically without supplements of nicotinic acid, the product of the *nad* pathway, thus confirming that an oxygen-independent biosynthetic pathway exists. Furthermore, the *nadB* mutant failed to grow both aerobically and anaerobically unless nicotinic acid was supplied (data not shown), indicating that t-aspartate oxidase participates in the oxygen-independent pathway. Thus t-aspartate oxidase must use an electron acceptor other than oxygen. Given the preference of the enzyme for fumarate in *in vitro*, it seems likely that this is the physiological substrate both anaerobically and aerobically. Its reaction with oxygen is presumably adventitious.

**Aspartate Oxidase Generates H$_2$O$_2$ as the Predominant Autoxidation Product**—t-Aspartate oxidase lacks the iron-sulfur clusters of its structural homologues; thus, it afforded the opportunity to test further the hypothesis that the clusters of Frd are critical to its preferential formation of superoxide. Assays revealed that aspartate oxidase generates some superoxide but that it accounted for only 11–15% of the $H_2O_2$ generation (Fig. 8). This proportion was not noticeably dependent on t-aspartate concentration. This result was consistent with the hypothesis that in the absence of a second electron-accepting site near the autoxidizable flavin, H$_2$O$_2$ will predominate as the product. Furthermore, the production of some $O_2^-$ by the enzyme implied that the fully reduced flavin of this enzyme is capable of monovalent oxidation. Hence, $O_2^-$ generation does not occur only from flavosemiquinoid enzyme forms.

The energy of activation for hydrogen peroxide formation by t-aspartate oxidase was ~50 ± 6.9 kJ/mol (data not shown), similar to that of Frd, suggesting that autoxidation occurs by a similar chemical mechanism. In the case of aspartate oxidase, the flavin species involved is obviously fully reduced. Hence, these results provide further support for the idea that the oxygen-reactive species in Frd is a fully reduced flavin as well. A residual technical concern was the possibility that H$_2$O$_2$ generation from t-aspartate oxidase was mediated by exogenous FAD. Some flavoproteins transfer electrons from bound to dissociated flavins, and the latter react with oxygen to form reduced oxygen species (15). Because the flavin of t-aspartate oxidase binds weakly to the enzyme, 20 μM exogenous FAD must be added to ensure maximal oxidase activity. However,
several lines of evidence argued that the oxygen reduction that occurred in the preceding experiments was not the result of spurious autoxidation of free flavin. First, riboflavin was unable to substitute for FAD in supporting aspartate:oxygen activity. Second, aspartate oxidase was unable to reduce effectively free FAD under anaerobic conditions at a rate commensurate with the observed aerobic H2O2 generation rate (data not shown). Finally, the cyclic reduction and oxidation of free flavin would be expected to be monovalent under our conditions, generating a large amount of superoxide, which was inconsistent with our observations showing mostly H2O2 generation.

Succinate Dehydrogenase Makes Only Superoxide as Its Autoxidation Product—A previous study (16) indicated that Sdh, like Frd, generates superoxide when reduced with succinate, although at a lesser rate. We were interested to compare their autoxidation rates and the identities of the products. Again, the rate of superoxide production was proportional to oxygen concentration (Fig. 2, squares). In contrast to Frd, however, all the H2O2 that was detected could be accounted for by the dismutation of O2 (Fig. 9). Specifically, Sdh makes only O2 and no H2O2 upon flavin autoxidation, even at high concentrations of succinate. The membranes used for these experiments did not exhibit detectable catalase activity.

A maximum turnover number of 13 molecules of O2 generation/min for Sdh was determined by covariant flavin analysis, consistent with that reported previously (16). Interestingly, the energy of activation for O2 production was measured as 150 kJ/mol (data not shown), far higher than for Frd and aspartate oxidase. This fact, and the failure to generate H2O2, indicates that despite their similar architectures, the electronic differences between Sdh and Frd cause quite different autoxidation behaviors.

**DISCUSSION**

*Flavin as the Site of Autoxidation*—Earlier studies (15, 16) of redox enzymes from *E. coli* implicated flavins rather than other electron-carrying cofactors such as iron-sulfur clusters or quinones, as the component responsible for their autoxidation. This study has reached the same conclusion for Frd, since its flavin needs to be reduced and sterically exposed for autoxidation to occur. Furthermore, the l-aspartate oxidase, an Frd homologue that autoxidizes at a rate similar to that of Frd, lacks redox cofactors other than its flavin.

Reduced Frd may be unusually disposed to autoxidation because electrons migrate toward its FAD. In the FrdA H44C mutant, which bears a non-covalently attached flavin with a low redox potential, O2 generation is lowered, presumably due to the decreased electron occupancy of the flavin. Thus it seems likely that in enzymes with multiple redox centers, the relative preference of electrons to sit on the flavin is a major determinant of their tendency to autoxidize. The cofactor redox potentials of Sdh appear to be arranged to withdraw electrons away from the flavin, and this may largely explain its comparatively low rate of autoxidation.

The ability of Frd and l-aspartate oxidase to bind sulfite suggests that the flavins of these flavin dehydrogenase-class enzymes might be exposed to solvent in a manner more typical of flavin oxidases. This character probably contributes to their oxidizability. It remains to be seen whether other flavoenzymes that reduce organic metabolites might also share these properties. It is possible that some, like l-aspartate oxidase, have as a result been misidentified as oxidases.

Interestingly, although quinones and iron-sulfur clusters are competent univalent electron donors, those of Frd and Sdh did not transfer electrons to oxygen at perceptible rates. The clearest evidence of this was the ability of excess succinate to fully suppress superoxide and H2O2 formation. Presumably the clusters did not react with oxygen because they were buried within polypeptide. We observed that when anchor subunits were lost during a glyceral gradient step in the purification, autoxidation became partially resistant to excess succinate. A likely explanation is that the loss of the C and D subunits exposed the [3Fe-4S] cluster and enabled electron transfer directly from it to oxygen. Similarly, although it has been speculated that quinones bound to Sdh and Frd could be sites of autoxidation, we have found no evidence for that. The absence of quinone autoxidation is presumably due either to shielding during electron transfer or the hydrophobicity of the local environment, which would inhibit the generation of anionic superoxide.

*Dihydroflavin Is the Primary Species Responsible for Autoxidation*—Our initial hypothesis for the rapid autoxidation of Frd invoked a flavin semiquinone as the donor of a single electron to oxygen. Our data, however, implicate dihydroflavin as the species that initially transfers an electron to molecular oxygen. This is most clear in the case of aspartate oxidase, which lacks other redox centers and cannot generate a semiquinone species. In fact, we and others (45, 50) have observed little semiquinone character in reduced Frd as well. Furthermore, in our studies of Frd the autoxidation of fully reduced enzyme and half-reduced enzyme can be distinguished by the identity of the predominant oxidation products, H2O2 and O2, respectively. The data indicate that fully reduced Frd autoxidizes as rapidly as does the half-reduced enzyme. The similar energies of activation imply that a common step limits the two reactions. Presumably, this common step is the initial one-electron transfer from the dihydroflavin to oxygen. Both the turnover number and energy of activation for Frd autoxidation are very similar to those of aspartate oxidase. The implication is that the redox moieties of enzymes need not be configured to generate semiquinone character in order to create substantial adventitious oxidation. Indeed, NADH dehydrogenase II from *E. coli* autoxidized at a high rate even if this flavoprotein was isolated from mutants that lacked ubiquinone, its only other known redox moiety (15).

Sdh may present a contrasting case. This enzyme produced no detectable direct H2O2, and the energy of activation for
superoxide formation was substantially different from those of Frd or l-aspartate oxidase. Hence, it is plausible that a different flavin species, such as a semiquinone, was responsible for its autoxidation. Consistent with this, Leger et al. (45) detected substantial flavosemiquinone character in bovine Sdh.

Formation of Superoxide Versus Hydrogen Peroxide—The autoxidation processes of Frd, Sdh, and aspartate oxidase enzymes differ most noticeably in the relative yields of O$_2^*$ and H$_2$O$_2$. While Sdh and l-aspartate oxidase make predominantly O$_2^*$ and H$_2$O$_2$, respectively, Frd makes a mixture that depends on the reductant concentration. The predisposition to monovalent versus divalent autoxidation was previously examined for xanthine oxidase, another flavometaalloenzyme that generates a mixture of superoxide and peroxide. In xanthine oxidase a fully reduced flavin is the principal autoxidizing species, and the oxidation state of a pair of iron-sulfur clusters near the flavin determines the autoxidation product that is formed (18, 51, 52). After transfer of a first electron from the reduced flavin to oxygen, the second electron will quickly leave the unstable flavosemiquinone which remains, transferring to the [Fe-S] cluster if it is unoccupied, or onto the nascent superoxide if the cluster is already reduced. Hence, when the enzyme is highly reduced (as in the case of saturating xanthine concentrations), H$_2$O$_2$ predominates as the product. This is the same situation seen in E. coli Frd, and a similar model seems to apply. The [2Fe-2S] cluster nearest the flavin in Frd has a midpoint potential (measured by protein film voltammetry) of ~35 mV; the FAD 0–1 electron semiquinone potential is ~−45 mV (at pH 7) (45). Within the experimental uncertainty, these measured potentials suggest that an electron could shift from the flavin semiquinone to the [2Fe-2S] cluster. The FrdA C62S mutant, in which the iron-sulfur cluster nearest the flavin is missing, accepts an electron, generates a preponderance of H$_2$O$_2$ because the flavin semiquinone to the [2Fe-2S] cluster. The FrdA C62S mutant, in which the iron-sulfur cluster nearest the flavin is missing, accepts an electron, generates a preponderance of H$_2$O$_2$ because the flavin semiquinone to the [2Fe-2S] cluster. The FrdA C62S mutant, in which the iron-sulfur cluster nearest the flavin is missing, accepts an electron, generates a preponderance of H$_2$O$_2$ because the flavin semiquinone to the [2Fe-2S] cluster.

Autoxidation of Succinate Versus Fumarate—Oxidoreductases

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