The Identification of Primary Sites of Superoxide and Hydrogen Peroxide Formation in the Aerobic Respiratory Chain and Sulfite Reductase Complex of Escherichia coli*

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The fitness of organisms depends upon the rate at which they generate superoxide (O$_2^\cdot$) and hydrogen peroxide (H$_2$O$_2$) as toxic by-products of aerobic metabolism. In Escherichia coli these oxidants arise primarily from the autoxidation of components of its respiratory chain. Inverted vesicles that were incubated with NADH generated O$_2^\cdot$ and H$_2$O$_2$ at accelerated rates either when treated with cyanide or when devoid of quinones, implicating an NADH dehydrogenase as their source. Null mutations in the gene encoding NADH dehydrogenase II averted autoxidation of vesicles, and its overproduction accelerated it. Thus NADH dehydrogenase II but not NADH dehydrogenase I, respiratory quinones, or cytochrome oxidases formed substantial O$_2^\cdot$ and H$_2$O$_2$. NADH dehydrogenase II that was purified from both wild-type and quinone-deficient cells generated ~130 H$_2$O$_2$ and 15 O$_2^\cdot$ min$^{-1}$ by autoxidation of its reduced FAD cofactor.

Sulfite reductase is a second autoxidizable electron transport chain of _E. coli_, containing FAD, FMN, [4Fe-4S], and siroheme moieties. Purified flavoprotein that contained only the FAD and FMN cofactors had about the same oxidation turnover number as did the holoenzyme, 7 min$^{-1}$ FAD$^{-1}$. Oxidase activity was largely lost upon FMN removal. Thus the autoxidation of sulfite reductase, like that of the respiratory chain, occurs primarily by autoxidation of an exposed flavin cofactor. Great variability in the oxidation turnover numbers of these and other flavoproteins suggests that endogenous oxidants will be predominantly formed by a few oxidizable enzymes. Thus the degree of oxidative stress in a cell may depend upon the titer of such enzymes and accordingly may vary with growth conditions and among different cell types. Furthermore, the chemical nature of these reactions was manifested by their acceleration at high temperatures and oxygen concentrations. Thus these environmental parameters may also directly affect the O$_2^\cdot$ and H$_2$O$_2$ loads that organisms must bear.

The discovery of superoxide dismutase (SOD)$^1$ in 1969 (1) was the first indication that aerobic organisms are threatened by superoxide (O$_2^\cdot$). SOD catalyzes the dismutation of superoxide (Reaction 1) and, in combination with catalase (Reaction 2), helps clear the cell of reactive oxygen species.

\[
O_2^\cdot + O_2^\cdot + 2H^+ \rightarrow O_2 + H_2O_2
\]

**REACTION 1**

\[
2H_2O_2 \rightarrow O_2 + 2H_2O
\]

**REACTION 2**

SOD was found to be virtually ubiquitous among aerobic organisms, suggesting that O$_2^\cdot$ might be an unavoidable by-product of metabolism in air. This idea was extended to develop the hypothesis that oxygen toxicity might be generally mediated by intracellular O$_2^\cdot$ (2). The molecular details that underpin this idea (the intracellular sources and targets of O$_2^\cdot$) were unresolved.

Within the past 10 years many details of oxygen toxicity have been revealed. In 1986 Carlioz and Touati (3) reported the properties of a mutant strain of *Escherichia coli* that lacked both of its cytosolic isozymes of SOD. The mutant grew normally in the absence of oxygen; however, in aerobic medium it exhibited requirements for branched chain, aromatic, and sulfur-containing amino acids, an inability to grow on non-fermentable carbon sources, and a high rate of spontaneous mutagenesis (4). These same traits were elicited when SOD-proficient wild-type strains were exposed to hyperbaric oxygen (5), suggesting that in these conditions O$_2^\cdot$ formation must be accelerated enough to overwhelm the cellular defenses. Therefore these observations supported the original model of oxygen toxicity.

The root cause of the branched chain auxotrophy was tracked to the ability of O$_2^\cdot$ to inactivate dihydroxy-acid dehydratase, an enzyme midway in this pathway (6). O$_2^\cdot$ does so by oxidizing and destabilizing the [4Fe-4S] cluster that acts as a Lewis acid during catalysis (7, 8). Iron dissociates from the oxidized cluster, causing a complete loss of activity. The requirement for fermentable carbon sources apparently stems from similar damage to aconitase and fumarase, which also belong to the [4Fe-4S] dehydratase class (9, 10). The high rate of mutagenesis is linked to the same damage: the iron that is lost from destabilized clusters floats freely into the cytosol where it catalyzes DNA oxidation by H$_2$O$_2$ (11–13). Thus to date all the well understood deficits of SOD mutants arise from this single type of lesion. In higher organisms O$_2^\cdot$ toxicity is linked to similar damage to mitochondrial aconitase (14).

Less clear is the mechanism by which O$_2^\cdot$ is generated in cells in the first place. Molecular oxygen is actually a poor chemical oxidant, because its triplet state constrains it to accept one electron at a time from potential donors. Because biological electron carriers such as NAD(P)/H resist the loss of a single electron, and the oxidizing potential of the O$_2$/O$_2^\cdot$ couple is low (~0.18 V using 1 M O$_2$ as standard state), most electron traffic is unaffected by the presence of oxygen. However, complex
electron transport chains include redox moieties such as flavins, quinones, and metal centers that excel at univalent electron transfers and are therefore plausible electron donors for oxygen. In the seminal experiments that led to the discovery of SOD, xanthine oxidase served as an enzymic source of $O_2^{-}$. Xanthine oxidase is actually a damaged form of xanthine dehydrogenase and has lost the ability to bind NAD$^{+}$; as a consequence, electrons accumulate on its flavin, iron-sulfur cluster, and molybdopterin cofactors. In the absence of the native substrate, the electrons are transferred at a moderate rate from the flavin to dissolved oxygen, and an admixture of $H_2O_2$ and $O_2$ is produced (15–17). This example provides evidence that adventitious $O_2$ (and $H_2O_2$) production can occur quite rapidly but that the physical and electronic structures of proteins (from one growth condition to another or one organism to another) might substantially change the degree of endogenous oxidative stress.

**MATERIALS AND METHODS**

**Chemicals and Enzymes**—Bovine erythrocyte copper, zinc superoxide dismutase, horseradish peroxidase, *E. coli* manganese superoxide dismutase, NADH, NADPH, deaminase, acetylpyridine adenine dinucleotide (AcPyNAD$^+$), acetylpyridine adenine dinucleotide phosphate (AcPyNADP$^+$), plumbagin, potassium ferricyanide, ubiquinone-1, FAD, FMN, chloromercuriphenyl sulfonic acid, cytochrome c, ADP, $H_2O_2$, fereone, $o$-dianisidine, scopoletin, dichlorofluorescein, hydroxylamine, $a$-glycerol phosphate, fumaric acid, 4-hydroxybenzoate, djenkolic acid, chloramphenicol, kanamycin, and tetracycline were purchased from Sigma. Catalase was from Boehringer Mannheim, ferric chloride was from G. Frederich Smith Chemical, and potassium cyanide and ferrous chloride were from Aldrich. Deionized house water was further purified with a Labconco Water Pro PS system.

**Strains**—Strains used in this study are listed in Table I. Mutant strains were constructed by P1 transduction. All comparisons in this work were between congenic strains. Mutant *for* alleles were co-transduced with a linked tetracycline-resistant Tn$10$; inheritance of the *for* allele was demonstrated by the inability of mutants to grow anaerobically on glycerol/nitrate medium (25). In a *ubi men* (quinoneless) background this screen cannot work, so the allele from putative transductants was screened by enzymatic assay for NADH:plumbagin oxidoreductase activity after vesicles were incubated at pH 7.8 to inactivate the NADH dehydrogenase I complex (28). The *nuo* mutant allele was selected by screening for growth on glycerol/nitrate medium (28), or minimal A supplemented with 1% casamino acids and 0.5 mM tryptophan. Standard antibiotic concentrations were used for plasmid maintenance and P1 transductions (29). Media for anaerobic cultures were degassed by autoclaving and equilibrated in an anaerobic chamber (10% H$_2$, 5% CO$_2$, 85% N$_2$) for at least 24 h before inoculation.

**Table I. Strains and plasmids**

| Strains     | Genotype                                      | Source |
|-------------|-----------------------------------------------|--------|
| AN387       | F$^{-}$ rpsL gal                             | 52     |
| AN386       | As AN387 plus menA401                        | 52     |
| JI224       | As AN387 plus zjd::Tn10 ΔfrdABC/Δ18          | P1(GV141) × AN387 |
| AN384       | As AN387 plus ubiA420 menA401                | 52     |
| IY13        | F$^{-}$ thi his ilo trp rpsL                 | 53     |
| IY12        | As IY13 plus ndh nuo                        | 53     |
| KM44        | As AN384 plus Δfrd::Sp$^+$ zjd::Tn10 plus pohd$^+$ | P1(RZ8457) × AN384 plus pMW |
| JI341       | As UM1 plus ndh cam                         | 54     |
| JI342       | As UM1 plus zjd::Tn10 nuo                    | P1(MWC215) × UM1 |
| JI303       | As AN384 plus zjd::znu-Tn10                 | P1(MWC190) × UM1 |
| SLC1        | As AN384 plus ndh cam                        | P1(MWC190) × AN384 |
| KM50        | As GK100 plus Δfrd::Sp$^+$ zjd::Tn10 plus pohd$^+$ | P1(RZ8457) × GK100 plus pMW |
| JI132/pJRS102| (sodA::Mad PR13/25 (sodB-kan)Δ2 thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY galK2 rpsL supE44 ara-14 xyl-15 mitl-1 tss-3 | JI132 plus pJRS102 |
| DHI5/pcySdD | supE44 lacU169 (80lacZDM15) hsdR17 recA1 endA1 gyra96 thi-1 relA1 fbiB deoC1 pRS25 rbsR rec938 cat | Bob Gennis |
| GV141       | zjd::Tn10 ΔfrdABC/Δ18 adhC4 araD139 ΔargF-lacU169 rpsL150 relA1 fbiB deoC1 pRS25 rbsR rec938 cat | Patricia Kiley |
| RZ8457      | Δfrd::Sp$^+$ zjd::Tn10                      | 54     |
| MWC215      | thi rpsL ndh cam                            | Bob Gennis |
| MWC190      | thi rpsL nuo zjd::Tn10                      | Bob Gennis |
| UM1         | katE katG14 lacY rpsL thi-1                 | 41     |
| G1K00       | AcylAB-cam' AcyABC/Δ456::kan thi rpsL gal   | Bob Gennis |
| pMW01       | pBR322 plus ndh$^+$ insert                  | 31     |
| pJRS012     | pBR322 plus cys/JHG                         | Nick Kredich |
| pcySdD      | pBR322 plus cysJHG                          | 31     |
For purification of sulfite reductase, the MgSO₄·7H₂O and (NH₄)₂SO₄ present in minimal A salts were replaced with (per liter) 0.08 g of MgCl₂ and 0.8 g of NH₄Cl, and cultures were supplemented with 0.5 mM each amino acid except methionine and cystine. For preparation of holoenzyme 0.25 g of djenkolic acid was provided as sole sulfur source (30); for preparation of active enzyme, start cultures were grown to 0.6 absorbance units at 600 nm. MgSO₄·7H₂O were diluted 1:100 into sulfur-free medium (31). The difference in protocols reflects our evolving strategies to induce maximal expression of the sulfite-reductase operon.

**Enzyme Purification—** Respiratory vesicles were customarily prepared as described previously (19). When we sought to avoid the inactivation of NADH dehydrogenase I, cells were washed, lysed, and centrifuged at 15,000 rpm for 30 min. The final growth period in air was monitored to direct reduction of the ferric chelate, and this flux was subtracted in the assay of H₂O₂. The rate of NADH dehydrogenase activity was quantitated by monitoring NADH oxidation in the presence of 100 μM plumbagin and 3 mM cyanide (21).

**Sulfite reductase flavoprotein (subunit B) was purified from DH5α/pJRS102.** This plasmid (34) includes cysG, which encodes an enzyme needed to generate mature siroheme for sulfite reductase and nitrite reductase, and causes abundant overproduction of sulfite reductase in sulfur-limited cells. A 2.6-g cell pellet was recovered from 4.5 liters of 0.2% glucose to an absorbance of 1.0. The incubation was carried out for 18 h incubation with 1 mM chloromercuriphenylsulfonic acid at 4°C (31); the FAD content of treated enzyme was below the detection limit, and ~90% of FAD was retained.

**NADH dehydrogenase II was purified from both quinone-proficient and -deficient strains.** The wild-type enzyme was purified from KM50, an ndh-overexpressing strain which is quinone-proficient but lacks cytochrome oxidases a and d. Preliminary preparations had revealed that trace amounts of cytochrome oxidase activity interfered slightly with assays of NADH oxidase activity during purification. Because cytochrome oxidase mutants grow poorly in air, KM50 was grown in 10 liters of anaerobic LB, 0.2% glucose to an absorbance of 0.3 and then shifted into air to a final absorbance of 0.45. The final growth period in air diminished the rate of its direct reduction. Five ml of 0.62 mM cytochrome oxidase in 50 mM MES buffer, pH 6.0, was added to scavenge the residual NADH before detection by the horseradish peroxidase-based assay. Diacetyldichlorodihydrofluorescein was used as horseradish peroxidase substrate.

**Assay of H₂O₂ Formation—** The production of H₂O₂ by the aerobic respiratory enzyme in the absence of active NADH dehydrogenase I was measured by the standard cytochrome c method (1). This method did not work when NADH dehydrogenase I was active, because that enzyme can directly reduce cytochrome c. Acetylation of cytochrome c (35) diminished the rate of its direct reduction.

**Assay of O₂ Formation—** The production of O₂ by the aerobic respiratory enzyme in the absence of active NADH dehydrogenase I was measured by the standard cytochrome c method (1). This method did not work when NADH dehydrogenase I was active, because that enzyme can directly reduce cytochrome c. Acetylation of cytochrome c (35) diminished the rate of its direct reduction. Five ml of 0.62 mM cytochrome c was treated with 125 mM acetic anhydride for 30 min on ice, dialyzed against two changes of 1 liter of 50 mM KPi, pH 7.0, and frozen at −70°C. 1.5 μM acetylated cytochrome c then replaced 10 μM native cytochrome c in the standard assay of O₂ production by xanthine oxidase and NADH dehydrogenase II was quantitatively detected by the acetylated cytochrome c.

Acetylated cytochrome c was still reduced too rapidly by sulfite reductase. We used an assay of O₂ production by that enzyme. Epinephrine, tetranitromethane, luciferase, and nitro blue tetrazolium assays (reviewed in Ref. 35) also failed because of direct interactions between sulfite reductase and the detector molecules. Therefore, O₂ was detected by its ability to reduce Fe³⁺. Reactions contained 5.2 mM ADP, 210 μM FeCl₃, 800 units of catalase, enzyme, and enzyme substrate in 50 mM MES buffer, pH 7.8. All stock solutions were prepared in 3.5 mM buffer, with the exception of the enzymes, which were in 50 mM Tris, pH 7.8. The ADP stock solution was adjusted to pH 7.8. Control reactions included 30 units of SOD. Reactions were initiated by the addition of enzyme substrate and aliquoted to cuvettes that were each continuously monitored for absorbance at 562. At defined 1-min intervals, ferene was added to consecutive cuvettes to a 900 μM final concentration, and the jump in absorbance was recorded. This increase was used to calculate the amount of Fe⁴⁺ that had accumulated by that time, using ε₅₅₂ = 27.5 at 562 nm for the Fe⁴⁺-ferene complex. In control experiments this system efficiently detected the O₂ that was generated by xanthine oxidase, xaminate reductase, and NADH dehydrogenase II. Catalase was included to prevent re-oxidation of Fe⁴⁺ by H₂O₂. The rate of NADH dehydrogenase II was also measured in the same reaction mixture (without ferene addition) so that O₂ yields could be compared with enzyme turnover. The oxidation rates of some enzymes were affected by the presence of ferric-ADP. Some of this effect was due to direct reduction of the ferric chelate, and this flux was subtracted in calculations of the oxygenase activity of the enzymes.

**Assay of H₂O₂ Formation—** H₂O₂ production was measured using horseradish peroxidase. H₂O₂ was generated in high yield by quinoneless respiratory chains and could be conveniently assayed using diaminoside as the dye (36). Because NADH is not consumed by quinoneless membranes and interferes with horseradish peroxidase-based assays by competing with the dye, respiration-proficient vesicles were added to scavenge the residual NADH before detection by the horseradish peroxidase system. The H₂O₂ yield by the oxidizing vesicles was scant compared with that from the quinoneless membranes, which were present in higher concentration. The initial reaction contained in a total volume of 4.2 ml: 30 μM NADH, quinoneless vesicles containing 0.2 units of NADH dehydrogenase, and 30 units of SOD. At time points over 8 min, 800-ml aliquots were removed, and 0.02 units of NADH oxidase activity were added as UM1 membranes to scavenge rapidly the residual NADH. Then the sample was filtered and assayed as above.

**NADH dehydrogenase II was extracted and purified as above.** Respiratory vesicles were customarily prepared in 50 mM MES buffer, pH 6.0, containing 10% glycerol (26). When we sought to avoid the inactivation of the sulfite-reductase operon.

**Respiratory vesicles were customarily prepared as described previously (19). When we sought to avoid the inactivation of NADH dehydrogenase I, cells were washed, lysed, and centrifuged at 15,000 rpm for 30 min.** The final growth period in air was monitored to direct reduction of the ferric chelate, and this flux was subtracted in the assay of H₂O₂. The rate of NADH dehydrogenase activity was quantitated by monitoring NADH oxidation in the presence of 100 μM plumbagin and 3 mM cyanide (21).

**Cytochrome oxidase activities of vesicles and of purified proteins was measured by** the dianisidine assay. Therefore the fluorescent substrates, diacetyldichlorodihydrofluorescein, and amplex red were used as horseradish peroxidase substrates. Diacetylchlorodi- hydrofluorescein was synthesized by standard methods (37). Vesicles were incubated with different amounts of NADH up to 300 μM, in each case until the NADH was exhausted, and the H₂O₂ that was generated was quantitated by published methods (38–40). By so doing we determined the H₂O₂ yield as a function of the amount of NADH that was oxidized. With all three fluorescent substrates, we observed a high yield of H₂O₂ when as little as 10 μM NADH was added; above 25 μM NADH, however, the ratio of H₂O₂ produced per NADH oxidized was consistent, and that is the value cited in this work. Whereas it is formally possible that H₂O₂ formation is unusually rapid at low NADH concentrations, we suspect an horseradish peroxidase-based artifact, since the H₂O₂ yields for these doses were constant over the period of dye development.

The basis of the artifact remains unclear.

**Enzyme Assays—** NADPH oxidase activities of vesicles and of purified enzymes were determined spectrophotometrically at 340 nm. NADH dehydrogenase activity was quantitated by monitoring NADH oxidation in the presence of 100 μM plumbagin and 3 μM cyanide (21).
and H2O2 per 10,000 that we detected above escaped from formation was also observed in vesicles from mutants produced by most aerobically grown cells (21). Detection because it directly reduces exogenous cytochrome production actually increased (Table II, line 1). The source, they did not test the autoxidizable component of the anaerobic chain, but that enzyme is not normally prevent oxygen exchange with a gas phase. The reactions in the sealed cuvettes were then monitored at 340 nm. The temperature dependence of reactions was determined by using a thermostatted spectrophotometer and equilibrating the buffer temperature before initiating the reaction.

### RESULTS

**NADH dehydrogenase II Is the Primary Site of O2 and H2O2 Formation in the Aerobic Respiratory Chain** —The first goal of this study was to identify the autoxidizable members of the aerobic electron transport chain. In a previous investigation fumarate reductase was identified to be an autoxidizable component of the anaerobic chain, but that enzyme is not normally synthesized in air and was shown not to be responsible for the O2 produced by most aerobically grown cells (21).

Membrane vesicles were prepared from cells that had been grown both aerobically and anaerobically on minimal glucose medium. These vesicles contained intact respiratory chains but were prepared at neutral pH, which inactivates NADH dehydrogenase I, one of two respiratory NADH dehydrogenases in E. coli. That was useful, because NADH dehydrogenase I has a cytochrome c reductase activity that interferes with the standard O2 assay (see below). In these vesicles NADH dehydrogenase II was intact, and the chain oxidized NADH efficiently. As was observed previously, substantial O2 was formed (0.9 nmol min⁻¹ O2/unit of NADH dehydrogenase; Table II, line 1). The precise yield per electron flux varied slightly among preparations but was consistently within the range of 3–5 O2 per 10,000 electrons, also in agreement with a previous report (19). O2 was not formed from vesicles that lacked both of the NADH dehydrogenases, confirming that the O2 evolved from the respiratory chain (Table II, line 1 versus 7).

Experiments verified that fumarate reductase was not a significant source of O2 in aerobic, NADH-reduced chains. First, fumarate, a potent inhibitor of Frd autodestruction (21), failed to abate the NADH-driven O2 production (data not shown). Second, the absence of either menaquinone, which delivers electrons to Frd, nor of Frd itself slowed O2 formation by NADH-reduced cells (Table II, lines 1–3). In contrast, both of these mutations blocked the formation of O2 during the respiration of α-glycerol phosphate, a substrate that efficiently directs electrons to Frd (lines 4–6). The vesicles used in the experiments of Table II were derived from cells grown on aerobic casamino acids medium, which is known to permit substantial synthesis of Frd; vesicles from glucose medium showed no dependence at all of O2 production upon Frd (not shown). Thus the O2 that was generated during the oxidation of NADH evolved from some site on the respiratory chain other than fumarate reductase.

Superoxide is a formal intermediate during the reduction of oxygen at cytochrome oxidase. However, free O2 is not formed in significant quantity, since oxygen remains tightly bound during its four-electron reduction to water (42). To test whether the substoichiometric O2 that we detected above escaped from cytochrome oxidase, cyanide was provided to block the binding of oxygen to that enzyme. NADH oxidation was 98% inhibited, but O2 production actually increased (Table II, line 1). The increase presumably occurred because electrons were backed up on an autoxidizable component of the chain upstream of the cytochrome oxidase.

Rapid O2 formation was also observed in vesicles from mutants that lacked both ubiquinone and menaquinone (Fig. 1A). In these vesicles the electrons from NADH could proceed no further than NADH dehydrogenase II itself. The high rate of O2 production exceeded that which was measured with quinone-proficient vesicles, presumably because NADH dehydrogenase II remained highly reduced, as if cyanide were present (compare Table II, lines 1 and 8). Using a ubiA high Ks mutant that generates some ubiquinone if its substrate, 4-hydroxybenzoate, is provided exogenously (43), we demonstrated that O2 production and respiratory capacity were inversely related (Fig. 2).

E. coli contains two abundant NADH dehydrogenases: NADH dehydrogenase I, a large proton-translocating complex analogous to the mammalian enzyme, and NADH dehydrogenase II, a non-proton-translocating single subunit enzyme important in maintaining a redox-balanced dinucleotide pool. Whereas the previous experiments pinpointed NADH dehydrogenase II as a primary O2 source, they did not test the autoxidizability of NADH dehydrogenase I, which had been deliberately inactivated by the neutral pH used during vesicle preparation. NADH dehydrogenase I interferes with standard O2 detection because it directly reduces exogenous cytochrome c. In order to determine whether NADH dehydrogenase I generates O2, we chemically acetylated cytochrome c and provided it in lower concentrations in the O2 assays (see “Materials and Methods”). Vesicles were then prepared from cells that lacked either NADH dehydrogenase I (nusA) or NADH dehydrogenase II (ndh). The NADH dehydrogenase I-deficient membranes generated O2 during respiration as abundantly as did wild-type membranes, but the NADH dehydrogenase II-deficient membranes generated very little (Table III). The ndh and nus mutations were also transduced into the quinoneless mutants, and once again substantial amounts of O2 were made only when NADH dehydrogenase II was present (not shown). Thus NADH dehydrogenase II, but not NADH dehydrogenase I, appeared to be a significant source of O2.

H2O2 is the other toxic product of enzyme autodestruction, and its production was measured in respiring membranes that contained either NADH dehydrogenase I or II. An artifact that affects H2O2 detection restricted us to observe H2O2 production between 25 and 200 μM NADH (see “Materials and Methods”),

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**Table II**

| Strain | No addition | + KCN |
|--------|-------------|-------|
| 1. Wild-type + NADH | 1.0 | 2.2 |
| 2. Men + NADH | 1.2 | 1.7 |
| 3. Frd + NADH | 1.0 | 1.5 |
| 4. Wild-type + α-glyc-P | 1.0 | 3.9 |
| 5. Men + α-glyc-P | 0.2 | 0.6 |
| 6. Frd + α-glyc-P | 0.1 | 0.7 |
| 7. Ndh II, NdhI-NDHII-α | <0.2 | <0.2 |
| 8. Men, Ubi-α | 2.4 | ND |
| 9. Wild-type + pdh α-α | 6.4 | ND |
| 10. Men, Ubi + pdh α-α | 25.5 | ND |

* Strains were cultured in anaerobic LB medium. O2 production rates are compared to wild-type normalizer to NADH dehydrogenase content.
* NdhII was overproduced 6.0-fold.
* O2 formation accounted for 19% of the NADH oxidase activity of these membranes.
but this is the range of internal NADH concentration inside exponentially growing, glucose-fed cells (19). The H2O2 yield was much higher when electrons passed through NADH dehydrogenase II than through NADH dehydrogenase I (Table III). Since the remainder of the respiratory chain is shared between these two dehydrogenases, the implication was that NADH dehydrogenase II is the preponderant respiratory source of H2O2 as well as O2.

In fact, substantial H2O2 was formed when quinoneless vesicles containing only NADH dehydrogenase II were incubated with NADH (Fig. 1B).

\[ \text{Fig. 1. } O_2^- (A) \text{ and } H_2O_2 (B) \text{ production during incubation of quinoneless (Ubi}^- \text{Men}^-) \text{ vesicles with NADH.} \]  

A, total cytochrome c (cyc) reduction and SOD-resistant cytochrome c reduction during incubation of quinoneless vesicles (0.07 units NADH dehydrogenase II) with 100 \( \mu \)M NADH. By subtraction, \( O_2^- \) production was calculated. B, \( H_2O_2 \) formation determined by dianisidine assay. 30 \( \mu \)M NADH were incubated with quinoneless vesicles (0.23 units of NADH dehydrogenase II). The addition of catalase eliminated the signal (not shown).

Oxidation of Its Flavin—The autoxidation of NADH dehydrogenase II was more closely examined after purification. The enzyme contains an FAD cofactor, which receives electrons from NADH, and a tightly bound ubiquinone cofactor that co-purifies with the enzyme and apparently mediates electron transfer from the FAD to the diffusible quinone pool (33). No metal centers exist in the enzyme.

NADH dehydrogenase II was overexpressed from a plasmid in both wild-type and quinone-deficient backgrounds. Membrane vesicles derived from overexpressing cells generated proportionately more O2 than did wild-type cells (Table II, lines 9 and 10). The enzyme was purified to homogeneity from both the wild-type and quinone-deficient membranes using an established protocol (33). The turnover numbers for O2 and H2O2 production were similar for both forms of the enzyme (Table IV). Thus it is the flavin moiety that directly transfers electrons to molecular oxygen.

Electron transfer to oxygen was much slower than electron transfer to good univalent oxidants such as ferricyanide or plumbagin, indicating that the rate-limiting step in O2 formation is the transfer of electrons from the reduced enzyme to dissolved molecular oxygen. This transfer was not mediated by adventitious metals, since metal chelators such as EDTA and DETAPAC had a slight (~40% maximum) accelerating effect on autoxidation. Similar stimulations have been described for xanthine oxidase and fumarate reductase, which also autoxidize at reduced flavins (21), although the basis of the effect is not known.

The reactivity of NADH dehydrogenase II with oxygen is presumably an accidental consequence of the exposure of its reduced flavin to dissolved oxygen. Accordingly, the reaction followed chemical kinetics and slowed proportionately at low...
TABLE IV

| Enzyme     | NADH oxidation | O$_2$ production | H$_2$O$_2$ production | Univalent flux | % |
|------------|----------------|------------------|-----------------------|---------------|---|
| Native     | 145            | 16.7             | 133                   | 6.3           |   |
| Quinoneless| 131            | 12.7             | 120                   | 5.3           |   |

*Fraction of H$_2$O$_2$ arising from O$_2$.*

Fig. 3. Dependence of NADH dehydrogenase II autoxidation upon oxygen (A) and temperature (B). NADH oxidase activity was measured with the purified enzyme. The two right-most points (determined at 10 and 15 °C) were omitted in the calculation of activation energy.

Concentrations of oxygen (Fig. 3A). The second-order rate constant for enzyme oxidation was $1.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at 37 °C (with saturating NADH). An apparent energy of activation of 26 kJ was calculated from data above 15 °C. At lower temperatures autoxidation was slower than expected from Arrhenius behavior, possibly because of changes in the enzyme structure (Fig. 3B).

Sulfite Reductase Is an Autoxidizable Soluble Protein—The conclusion that isolated respiratory proteins autoxidize primarily or exclusively from flavin moieties raised the question of whether there is any abundant protein in E. coli that spuriously reacts with oxygen at a non-flavin site. This question cannot be answered comprehensively without examination of every redox protein. However, most of the known non-respiratory redox enzymes contain only flavins as redox cofactors. Among the exceptions, sulfite reductase may be the most abundant. It contains FAD, FMN, [4Fe-4S] clusters, and siroheme, and electrons flow in that order from the NAD(P)H donor to sulfite. Sulfite reductase was known to be capable of transferring electrons to a broad range of chemical oxidants, including oxygen (23). We therefore sought to determine which of its moieties were involved in autoxidation.

When aerobic cells were grown on the poor sulfur source djenkolate, which induced sulfite reductase synthesis about 100-fold above that of cystine-supplemented cells, no reliable difference in the total NADPH oxidase activity of the soluble extract could be determined. However, the overproduction of sulfite reductase from a plasmid caused a substantial increase in the NADPH oxidase activity of the whole-cell extract (not shown). Superoxide formation was difficult to measure by established assays, since sulfite reductase directly reduces both native and acetylated cytochrome $c$ (see below).

Holoenzyme was purified by a standard protocol, from SOD-deficient cells in which sulfite reductase had been overproduced. The enzyme spectrum matched that of previous reports. Turnover numbers were 1450 min$^{-1}$ (2160 min$^{-1}$), 4420 min$^{-1}$ (6800 min$^{-1}$), and 18,700 min$^{-1}$ (31100 min$^{-1}$) for sulfite, hydroxylamine, and cytochrome $c$ reduction, respectively. The parenthetical rates were obtained with added FMN and ranged from 93 to 118% of published values (23). The purified enzyme was somewhat FMN-deficient, containing 0.51 FMN/FAD.

Oxidase activity was indeed present; the turnover number was 63 NADPH min$^{-1}$ per holoenzyme, similar to the value 75 min$^{-1}$ measured by Siegel et al. (23). The turnover number was increased somewhat by iron chelators, as seen for other enzymes. Because FMN slowly dissociates from sulfite reductase (44), it was important to verify that the turnover to oxygen was not mediated by the enzymic reduction of free flavins. The sulfite reductase transfers electrons from its FAD moiety to free flavins (31), and the subsequent oxidation of those flavins by molecular oxygen could provide a spurious oxidase activity. To test whether the apparent oxidase activity arose from such a reaction, the NADPH oxidase activity was measured as a function of FMN concentration when free FMN was provided. In fact, the provision of FMN in high concentrations stimulated electron transfer both to oxygen and to other acceptors. However, the stimulation of the oxidase activity was biphasic. Extrapolation of the second phase to low FMN concentrations indicated that the initial phase approximately doubled the activity (Fig. 4). This was presumably achieved by filling the empty FMN sites. The residual effect of higher FMN was less pronounced, exceeded the capacity of the enzyme to stably bind additional FMN at unoccupied sites, and was not saturated at 1 μM. We concluded that the excess FMN stimulated the oxidase activity by acting as an artificial electron acceptor rather than by occupying authentic FMN binding sites in the slightly de-flavinated enzyme. However, substantial basal oxidase activity existed even at very dilute enzymes concentrations (2 nM). In that circumstance the possible amount of free FMN could not have accounted for the oxidase activity, if one assumes a linear relationship between free FMN and spurious oxidase turnover.

Therefore the oxidase activity of the isolated, unsupplemented enzyme reflected bona fide electron transfer directly from the holoenzyme to oxygen, without a free-FMN intermediate. In subsequent oxidase experiments free flavin was not
provided.

**Electron Transfer Occurs from a Flavin**—As reported in earlier studies (23), cyanide blocked electron transfer to sulfite but did not inhibit the oxidase activity. That result suggested that the oxidase reaction did not occur at the siroheme site, although electron transfer from an alternative face of the heme was not formally ruled out. The latter possibility was refuted by the observation that oxidase activity persisted in enzyme that was synthesized by a *cysG* mutant, which generates a siroheme-deficient enzyme (data not shown).

Both the siroheme and the iron-sulfur cluster are contained in the β subunit, whereas FAD and FMN are bound by the α subunit of the holoenzyme. The α subunit (flavoprotein) can be purified in a stable form in the absence of the β subunit, and this was achieved using an established protocol with cells that overexpressed only *cysH*, the flavoprotein structural gene. The purified flavoprotein exhibited a turnover number per FAD cofactor in excellent agreement with that of the holoenzyme (Table V). Siegel and Davis (45) had observed that flavoprotein that was dissociated from the iron protein by denaturants retained 10–18% of the oxidase activity; their low yield was matched by poor recoveries of other reductase activities and presumably reflected enzyme damage during subunit dissociation. To identify more precisely the autoxidizing moiety, we removed FMN by treatment with p-chloromercuri phenylsulfonate. The treated enzyme retained FAD but was effectively devoid of FMN (Table V). Although the FMN-free flavoprotein retained transhydrogenase activity, the oxidase activity was reduced by 75%. This activity loss is quantitatively similar to the residual ferricyanide and menadione reductase activities that others (30) have reported. These experiments indicated either that FMN is the preponderant site of autoxidation or that it electronically interacts with FAD to facilitate the reaction of FAD with oxygen. FAD alone has slight reactivity with oxygen.

**Sulfite Reductase Generates H₂O₂ Not O₂ as Its Major Oxidation Product**—Greater than 97% of the electrons that were transferred to oxygen by sulfite reductase were recovered as H₂O₂ (data not shown); thus the tetravalent reduction of oxygen to water did not occur. This yield represented both the H₂O₂ and the O₂ that were generated as initial products, since any O₂ spontaneously dismutates to form H₂O₂. To determine the initial yield of O₂ an iron-reduction assay was developed (see “Materials and Methods”). We found that only about 10% of the oxidation of the holoenzyme and 20% of the oxidation of the flavoprotein produced O₂ (Table V). In contrast, about 60% of the lesser oxidation of the FMN-depleted flavoprotein generated O₂. It is not clear whether FAD oxidation occurs in the FMN-containing flavoprotein; if so, virtually all of the O₂ from that protein could arise from autoxidation of the FAD. Thus FMN appeared to be the predominant site of sulfite reductase oxidation, with H₂O₂ as the predominant direct product, whereas a small minority of autoxidation occurred at the FAD site, with approximately equal production of O₂ and H₂O₂.

The kinetics of sulfite reductase oxidation reflected its binding interactions with NADPH and chemical interactions with oxygen. The oxidase activity exhibited an apparent *Kₘ* for NADPH of 5 μM in air-saturated buffer, and high concentrations of either NADPH or NADP⁺ did not suppress oxidation (data not shown). The rate of autooxidation was proportionate to oxygen concentration (Fig. 5A). The rate constant for reaction with dissolved oxygen was 2.4 × 10⁵ M⁻¹ s⁻¹ per FMN at 37 °C. The temperature dependence indicated an activation energy of 49.9 kJ/mol (Fig. 5B).

**NADH-reduced Enzyme Reacts with Oxygen but Poorly with an NADP⁺ Analogue**—Eschenbrenner et al. (31) reported the interesting observation that NADH can reduce the FMN moiety of sulfite reductase but that the reduced enzyme then reacts poorly with cytochrome c. This is in stark contrast to NADPH-reduced enzyme. We repeated the observation, finding that NADH bleached the FMN absorbance peak. Interestingly, the NADH-reduced enzyme reacted well with oxygen, showing a turnover number only slightly lower than when NADPH was the reductant. The turnover number to cytochrome c was as low as that to oxygen, suggesting that the slow step might be the reduction of the enzyme. Surprisingly, acetylpyridine dinucleotide phosphate (AcPyNAD⁺), the NADP⁺ analogue that is reduced by reduced FAD, could only be reduced by the NADH-treated enzyme at a very slow rate, whereas acetylpyridine dinucleotide (AcPyNAD⁺), an NAD⁺ homologue, was as reducible as cytochrome c or oxygen (Table VI). This appears to confirm that the NADH- and NADPH-reduced enzymes are electronically dissimilar and that the electrons perhaps localize exclusively on the FMN in the NADH-reduced enzyme. Although the reason for this remains uncertain (perhaps a residual interaction with NAD⁺ affects the electron localization), it supports the conclusion that the preponderant site of electron transfer to oxygen is the FMN moiety rather than the FAD.

![Fig. 4. Acceleration of the NADPH oxidase activity of sulfite reductase holoenzyme by exogenous FMN.](image)

**Table V**

| Enzyme                | FMN/FAD | Turnover numbers to electron acceptors (per FAD) | % Univalent flux to O₂ |
|-----------------------|---------|--------------------------------------------------|------------------------|
|                       |         | SO₂⁻ | cytochrome c | AcPyNAD⁺ | O₂ |       |
| Holoenzyme            | 0.61    | 360  | 4700        | 1390     | 7.5 | 13    |
| Flavoprotein          | 0.57    | 9    | 4300        | 1290     | 6.8 | 20    |
| De-FMN flavoprotein   | <0.03   | ND   | 110         | 1170     | 1.9 | 65    |

Electron transfer from sulfite reductase to oxygen is mediated by flavins

Sulfite reductase, cytochrome c reductase, transhydrogenase, and oxidase turnover numbers (min⁻¹) are normalized per FAD. Cyc, cytochrome c; ND, not determined.
Why Are Flavins the Predominant Sources of $O_2^*$ and $H_2O_2$?

Oxygen is a triplet species, and electron transfers to it from singlet donors must occur in univalent steps. Consequently, $O_2^*$ is the immediate product. The univalent reduction potential of oxygen is low enough that it cannot pull electrons from unwilling donors; the donors must be proficient at univalent redox reactions. For this reason reduced dinucleotides and thiols are relatively stable in air. One anticipates that oxygen will react only with those electron carriers whose univalent oxidation states are stable: those carriers containing transition metals or with sufficient conjugation to stabilize the univalent oxidation products through resonance structures. In biological systems the obvious candidates are iron-sulfur clusters, hemes, quinones, and flavins. In this study we have identified flavins as the primary sites of chemical oxidation of the respiratory chain and of sulfite reductase. In both cases the hemes, iron-sulfur clusters, and quinone pools were not substantial sources of either $O_2^*$ or $H_2O_2$.

Why don’t these other moieties react with oxygen? A number of answers are possible, but a likely one is that, unlike flavins, these moieties are sequestered in environments that are either sterically inaccessible to oxygen or are hydrophobic. The importance of burying clusters is underscored by those exceptional clusters whose catalytic function requires that they be solvent-exposed. These clusters (on nitrogenase, Fnr protein, and dehydratases) react rapidly with oxidants. In contrast, the clusters of sulfite reductase and NADH dehydrogenase I are buried in the protein, where they conduct internal electron transfers, and are stable in air.

The clusters of succinate dehydrogenase and fumarate reductase are presumably near the enzyme surface, since their biochemical function is to reduce directly diffusible quinones, but electron transfer to oxygen may be blocked by the hydrophobicity of the membrane interior. That environment would not tolerate the generation of a hard anion like $O_2^-$. The same issue may prevent the autoxidation of reduced respiratory quinones. A contrasting example is the bc1 complex of other bacteria and eukaryotes, which generates $O_2^*$ when its Q0 site semiquinone reacts with oxygen (46). It would be interesting to see whether this site has a local tolerance for charge that has the side effect of permitting $O_2^*$ formation.

**DISCUSSION**

**Differential Rates of Flavin Autoxidation**—Free reduced flavins react rapidly with oxygen (and may themselves be sources of intracellular $O_2^*$ (47)). The rates at which flavoproteins react with oxygen are generally lower but range over orders of magnitude. Massey (51) has noted that members of the dehydrogenase class typically react slowly, in contrast with electron transferases, oxidases, and monoxygenases. We have seen substantial variation within nominal members of the dehydrogenase class, from undetectably low (NADH dehydrogenase I), to moderate (succinate dehydrogenase, sulfite reductase), to high (NADH dehydrogenase II, fumarate reductase). The different energies of activation for the oxidation of sulfite reductase (50 kJ/mol) and NADH dehydrogenase II (26 kJ/mol) suggest that electronic effects may control these rates; certainly local polypeptide context influences the relative stability of dihydroflavins and flavosemiquinones. However, several other factors are likely to be important, including the degree of flavin exposure and the local electron density. The failure of succinate dehydrogenase to autoxidize at the same efficiency as its homologue, fumarate reductase, may reflect the tendency of these enzymes to distribute their electrons differently; on reduced fumarate reductase electron density may be high on the flavin, in preparation for fumarate reduction; on succinate dehydrogenase the electron density is probably highest on its higher potential iron-sulfur clusters, in anticipation of ubiquinone reduction. It may be that NADH dehydrogenase I does not autoxidize because its electrons are primarily sequestered away from the flavin, on its iron-sulfur clusters.

The rates at which flavoenzymes react with oxygen in vivo will also depend upon the overall enzyme redox status, which in turn depends upon the amount of oxidative substrate present. Thus the elimination by mutation of oxidized ubiquinone as a competitor for their electrons accelerated $O_2^*$ formation.
both by NADH dehydrogenase II and fumarate reductase. We presume that the presence of sulfite would slow the rate at which sulfite reductase generates H$_2$O$_2$, although interference by sulfide prevented us from doing the H$_2$O$_2$ assays that would have tested this idea directly.

**O$_2^\ast$ Versus H$_2$O$_2$ as the Direct Oxidation Product**—The overall turnover number for flavin oxidation reflects the rate-limiting step, which is the transfer of the first electron from the reduced flavin to molecular oxygen. In contrast, the valency of the electron transfer, that is whether superoxide or hydrogen peroxide is the product that leaves the enzyme, is determined by the relative rates at which the nascent O$_2^\ast$ either dissociates from the enzyme or, alternatively, reacts (after a spin flip) with the flavosemiquinone radical to form a peroxy complex (48). Whether flavoenzyme autoxidation generates O$_2^\ast$ or H$_2$O$_2$ is critical to cell physiology, because these two oxidants have qualitatively and quantitatively different impacts upon fitness. O$_2^\ast$ damages dehydratases with high reactivity and specificity, yielding from this enzyme and H$_2$O$_2$ Formation by Enzyme Autoxidation formation, 10$^{-5}$ m is toxic (49). H$_2$O$_2$ is less inhibitory to growth, and levels up to 10$^{-5}$ m can be tolerated. On the other hand, H$_2$O$_2$ has a potentially more lasting effect, since it can attack DNA; 10$^{-4}$ m causes measurable mutagenesis and lethality (50). Overall it seems that cells would be more tolerant of H$_2$O$_2$ than of H$_2$O$_2$ than equivalent O$_2^\ast$ production, and most committed oxidases generate H$_2$O$_2$ as an exclusive product.

NADH dehydrogenase II and sulfite reductase both followed the behavior of typical flavin dehydrogenases (51), forming only a small amount of O$_2^\ast$. Steric factors may affect these yields as follows: if the O$_2^\ast$ is formed within an enzyme cleft, the frequency of collision between it and the flavin semiquinone rises before the O$_2^\ast$ can escape, and that may lessen the yield of diffusible O$_2^\ast$. However, at least in the case of sulfite oxidase, the valency of FMN oxidation is additionally determined by its interaction with the adjacent FAD. By preparing recombinant protein fragments that contained only the FMN-binding site, Fontecave and colleagues (24) were able to study the behavior of this flavin in isolation. The flavin redox potentials of the protein fragment agreed with those determined by Oetstrow et al. (30) for the entire flavoprotein as follows: $-0.322$ and $-0.382$ V for the FADH$_2$/FADH$^\ast$ and FADH/FAD couples, and $-0.327$ and $-0.152$ for the FMNH$_2$/FMNH$^\ast$ and FMNH/FMNH couples. These potentials are low enough to favor oxygen reduction ($E^\circ'_o$(O$_2$/O$_2^\ast$) = $-0.18$ V). The striking feature is that the univalent oxidation of reduced FMNH$_2$ will generate a semiquinone product that, with respect to oxygen, could be fairly stable. In fact this was observed as follows: while the reduced recombinant FMN fragment oxidized rapidly upon exposure to air, the product was a stable neutral flavosemiquinone of its own oxidation required hours. Since the enzyme lost only one electron, O$_2^\ast$ must have been the exclusive product. That observation contrasts with our finding that H$_2$O$_2$ rather than H$_2$O$_2$ is formed by the respira-
O$_2$ and H$_2$O$_2$ Formation by Enzyme Autoxidation

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