Detection and Quantification of Superoxide Formed within the Periplasm of Escherichia coli

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Many gram-negative bacteria harbor a copper/zinc-containing superoxide dismutase (CuZnSOD) in their periplasms. In pathogenic bacteria, one role of this enzyme may be to protect periplasmic biomolecules from superoxide that is released by host phagocytic cells. However, the enzyme is also present in many nonpathogens and/or free-living bacteria, including Escherichia coli. In this study we were able to detect superoxide being released into the medium from growing cultures of E. coli. Exponential-phase cells do not normally synthesize CuZnSOD, which is specifically induced in stationary phase. However, the engineered expression of CuZnSOD in growing cells eliminated superoxide release, confirming that this superoxide was formed within the periplasm. The rate of periplasmic superoxide production was surprisingly high and approximated the estimated rate of cytoplasmic superoxide formation when both were normalized to the volume of the compartment. The rate increased in proportion to oxygen concentration, suggesting that the superoxide is generated by the adventitious oxidation of an electron carrier. Mutations that eliminated menaquinone synthesis eradicated the superoxide formation, while mutations in genes encoding respiratory complexes affected it only insofar as they are likely to affect the redox state of menaquinone. We infer that the adventitious autoxidation of dihydromenaquinone in the cytoplasmic membrane releases a steady flux of superoxide into the periplasm of E. coli. This endogenous superoxide may create oxidative stress in that compartment and be a primary substrate of CuZnSOD.

In 1969, McCord and Fridovich reported the isolation from bovine erythrocytes of a protein that scavenged superoxide (29) by the following chemical reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. They subsequently demonstrated that this activity, which they designated superoxide dismutase (SOD), is ubiquitous among aerobic organisms. Eukaryotes contain a copper- and zinc-cofactored isozyme (CuZnSOD) in their cytosol and a manganese-cofactored isozyme in their mitochondria. Bacteria employ in their cytoplasm either manganese- or iron-cofactored enzymes that are structurally homologous to one another and to the eukaryotic mitochondrial manganesezyme.

The discovery of SOD implied both that superoxide is formed as a by-product of aerobic metabolism and that, if it is not scavenged, it can harm cells. Subsequent studies have revealed that molecular oxygen adventitiously steals electrons from the reduced cofactors of redox enzymes, thereby forming superoxide in all aerobic organisms (19, 28). Further, superoxide can directly oxidize the iron-sulfur clusters of a family of dehydratases, inactivating the enzymes and blocking the pathways to which they belong (10, 12, 13). By rapidly scavenging superoxide, SOD protects these enzymes and permits metabolism to occur in environments that contain oxygen (19, 28).

In 1974 a CuZnSOD was isolated for the first time from a bacterium, Photobacterium leiognathi, a symbiont of the ponyfish (35). The structure of that enzyme closely resembled that of the eukaryotic CuZnSOD, and initially it was suspected that the gene had been laterally transferred from host to symbiont. However, CuZnSODs have since been discovered in all clades of the Proteobacteria and in sequenced members of several other bacterial phyla. It now seems clear that eukaryotes inherited their CuZnSODs from the bacterial ancestor of the mitochondrion.

In gram-negative bacteria, CuZnSODs are invariably located in the periplasm. Thus, there must be conditions under which this compartment is exposed to superoxide. The enzymes that are known to release superoxide are all cytosolic, and $O_2^-$ cannot cross membranes at physiological pH (22, 27); therefore, bacterial CuZnSOD must exist to scavenge $O_2^-$ that is either generated inside the periplasm or that diffuses into it from outside the cell. Some studies have implicated periplasmic SODs in bacterial virulence, raising the prospect that these enzymes scavenge superoxide that is released by the NADPH oxidase of phagocytes (7, 9, 14, 23, 36, 43, 46). However, several observations suggest that this cannot be the sole role of the enzyme. First, periplasmic SODs are found in Caulobacter crescentus, a free-living organism (41), and in nonpathogenic strains of Escherichia coli (2). Second, Salmonella species synthesize both chromosomal and phage-encoded isozymes, and only the latter are required for pathogenesis (23, 46). Implicitly, the chromosomally encoded CuZnSOD, which is a close homologue of the E. coli enzyme, has a role unrelated to pathogenesis. Finally, Salmonella and E. coli mutants that lack periplasmic SODs exhibit a mild sensitivity to $H_2O_2$ in vitro (15). Although the basis of that sensitivity is not understood, its existence implies that superoxide must stress the periplasm even when the bacteria are grown in pure culture. This observation raises the prospect that periplasmic superoxide might be formed by the bacterium itself.

This process has been observed in one specialized situation. Huycke and colleagues found that the gram-positive bacterium...
Enterococcus faecalis, a natural heme auxotroph, releases superoxide into the medium when heme is not provided (17, 18). In the absence of this cofactor, cytochrome d oxidase cannot be activated, and respiration cannot proceed. The superoxide is apparently generated on the outer aspect of the cytoplasmic membrane as a vent for electrons that have entered the respiratory chain. Supplementation of hematin restores oxidative function and eliminates superoxide excretion.

In contrast, gram-negative bacteria that express periplasmic SOD can synthesize their own heme and do not face this dilemma. Nevertheless, we were interested in determining whether superoxide might be formed within the periplasm during aerobic growth. We report here that substantial superoxide is indeed released into this compartment as an incidental by-product of respiration, apparently due to the adventitious oxidation of menaquinone.

MATERIALS AND METHODS

Chemicals. Cytochrome c (horse heart, type IV), superoxide dismutase (bovine erythrocytes), horse radish peroxidase, thiamine, Casamino Acids, ampicillin, chloramphenicol, kanamycin, potassium ferricyanide, EDTA, isopropylthiogalactoside, 30% hydrogen peroxide, NADH, phosmobutane, lactose, and fumarate were from Sigma. Tryptone, yeast extract, and Bacto agar were purchased from Becton Dickinson. Potassium phosphate salts, ammonium sulfate, sodium citrate, sodium chloride, glucose, potassium cyanide, calcium chloride, magnesium sulfate, and Tris base were from Fisher. Ampicillin red and PicocGreen reagent were obtained from Molecular Probes, and Coomassie reagent and ovalbumin were from Pierce.

Strains. Bacterial strains used in this study are listed in Table 1. Plasmids were transformed by electroporation. Mutations were introduced by P1 transductions (32) and selection on antibiotic-containing plates. The presence of mutant alleles was subsequently confirmed using screens for the appropriate phenotypic properties. Inheritance of the menA allele was verified by the inability of the mutant to grow on anaerobic glycerol-fumarate plates within 4 days. The ubiCA mutants were unable to grow on aerobic succinate plates, and they also exhibited a diminished rate of growth on aerobic LB plates. The menA ubiCA double mutants were unable to grow on aerobic LB plates at all. Enzymatic assay of NADH dehydrogenase II activity was used to confirm the presence of ndh proficiency was tested in minimal A salts containing 30 mM succinate. Ampicillin (2 µg/ml) was added to minimal glucose complex media; therefore, measurements were performed in minimal glucose media that was supplemented with 0.2% glucose and 20 µg/ml of chloramphenicol.

Buffers, media, and growth conditions. LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl, pH 7.0) (32) was routinely supplemented with 0.2% glucose (or 0.2% lactate plus 0.7 mM isopropyl-β-D-thiogalactopyranoside [IPTG] if specified). Most bacterial growth in defined medium employed minimal A salts (7.5 mM ammonium sulfate, 2 mM sodium citrate, 33 mM potassium dihydrogenphosphate, 60 mM potassium hydrophosphate, 1 mM magnesium sulfate, 5 mg/liter thiamine; pH 7.3) (32) that were supplemented with 0.2% Casamino Acids, 0.5 mM thymophtan, and 0.2% glucose. Where indicated below, Casamino Acids were replaced with a 0.5 mM concentration of specified amino acids. Menaquinone mutants were screened in medium containing minimal A salts, 0.6% glycerol, and 30 mM fumarate, and aerobic respiratory proficiency was tested in minimal A salts containing 30 mM succinate. Ampicillin (0.1 mg/ml) was added to cultures of plasmid-bearing strains. Washing buffer (WB) contained 7.5 mM ammonium sulfate, 6 mM sodium chloride, 33 mM potassium dihydrogenphosphate, 60 mM potassium hydrophosphate, and 1 mM magnesium, adjusted to pH 7.3. Phosphate buffer contained 50 mM potassium phosphate at pH 7.8.

Aerobic cultures were grown in flasks with vigorous shaking at 37°C. Anaerobic cultures were grown in an anaerobic chamber (90% nitrogen, 5% hydrogen, 5% carbon dioxide; Coy Laboratory Products). To remove oxygen, anaerobic buffers and media were moved into the chamber immediately after being autoclaved, and they were allowed to equilibrate with the anaerobic atmosphere for at least 24 h prior to use.

Detection and measurement of extracellular superoxide. The production of extracellular superoxide was monitored using the ability of superoxide to reduce ferricytochrome c that was added to the cell suspension. This approach was used previously to detect the superoxide that is abundantly released by Enterococcus faecalis (18). The method was modified here to permit the quantitation of the lesser amount that is generated by E. coli and to distinguish superoxide that is generated directly by the bacteria from that which is formed by the autoxidation of cell metabolites.

To measure total extracellular superoxide production, cells either from exponential-phase (optical density at 600 nm [OD600] of 0.25) or stationary-phase (OD600 of 2.4) cultures were centrifuged, washed with WB, and resuspended to an OD600 of 0.2 in two 50-ml flasks, each containing 10 ml of prewarmed WB supplemented with 0.2% glucose and 20 µM cytochrome c. (Complex medium cannot be used, because medium components directly reduce cytochrome c). Superoxide dismutase (30 U/ml) was added to one of the two flasks, and they were incubated at 37°C in a shaking water bath. At intervals, 1.5 ml of cell suspension was withdrawn from each flask and filtered using syringe filters. The filtrates were kept on ice, and the amount of reduced cytochrome c was determined as soon as possible (within 2 to 3 min) by the method described below.

To quantify superoxide formation by excreted metabolites, cell suspensions were incubated without the addition of cytochrome c. At various time points, cells were removed by filtration, cytochrome c was added to the filtrate, each was counted to one of four each containing a 0.2 mM cytochrome c. Samples were then incubated at 37°C for 15 min, which is sufficient to completely oxidize the superoxide metabolites. To quantify the amount of reduced cytochrome c in each sample, the absorbance spectrum was recorded between 570 and 530 nm. Then 0.2 mM of potassium ferricyanide was added to the filtrates to oxidize cytochrome c, and the spectra were again recorded. The amount of reduced cytochrome c was calculated using the ferricyanide-induced absorbance change at 550 nm (A550 upon reduction of cytochrome c, 0.21 mM 1 cm-1) relative to the 556.5 nm isobestic point. These reduced-minus-oxidized spectra provided the precision that was needed to obtain quantitatively reproducible measurements. The fraction of cytochrome c reduction that had been mediated by superoxide was then determined by comparing the degrees of reduction in the paired samples with and without SOD (Fig. 1).

The procedure produced in this way revealed the total amount of extracellular superoxide that had been produced in cell cultures, the amount that had been generated by the autoxidation of metabolites, and, by subtraction, the amount produced directly by the cells. We anticipate that essentially all of the superoxide that is produced in the periplasm will be detected by this assay. While superoxide can spontaneously disproportionate, react with 20 µM cytochrome c (k = 2.5 x 10⁻⁴ M⁻¹ s⁻¹) (11) is overwhelmingly favored. Similarly, it is unlikely that significant superoxide will disproportionate before it leaves the periplasm. Using the measured rate of superoxide formation and the dismutation rate constant, we calculate that the half-life of superoxide should exceed 0.6 s—which, given the high diffusion coefficient of small molecules (10⁻⁵ cm²/s), implies a mean single-dimension diffusion distance of 35 µm. The distance between inner and outer membranes is only about 0.1 µm, so most superoxide molecules should persist for long enough to encounter a porin and efflux from the cell. Periplasmic superoxide formation would be underestimated only if a periplasmic molecule reacted catalytically with superoxide at a high rate. No such molecule (other than SOD) is known.

Statistical analysis. All experimental results were determined multiple times with discrete independent cultures. Where rates of superoxide formation differed significantly from those of wild-type cells, the rates were measured four to five times, and error bars represent the standard deviations of those data.

Measurement of hydrogen peroxide formation. Total H₂O₂ formation was measured using the fluorescent dye Amplex red (37). This method is subject to interference by medium components and cannot be performed at all in complex media; therefore, measurements were performed in minimal glucose medium that was supplemented with His, Pro, Leu, Arg, Thr, Phe, Tyr, and Trp. These amino acids are sufficient for good growth of strains that cannot scavenge H₂O₂. Cells (strains J1377 and SSK6) were grown for at least five generations in this medium to an OD₆₅₀ of 0.25. They were then pelleted at room temperature and washed once with WB. The cells were resuspended to an OD₆₅₀ of 0.1 in 10 ml of the same prewarmed medium in a 37°C shaking water bath. Every 3 min, 1.5 ml of cell suspension was withdrawn and filtered using a syringe filter. To 0.5 ml of the cell filtrate were added 1 ml of 0.2 mM solution of Amplex red in 50 mM phosphate buffer (pH 7.8) and then 0.5 ml of horseradish peroxidase (0.02 mg/ml). Fluorescence was measured after 30 seconds in a Shimadzu RF-Mini 150 fluorometer. Fluorescence values were converted to H₂O₂ concentration using a
plus 0.2% glucose at an OD 600 of 0.1. Measurements were carried out at 37°C with a Clark oxygen electrode. Cells were centrifuged, washed, and resuspended in WB medium.

NADH oxidation was monitored by the decrease in absorbance of NADH at 340 nm in the presence of 3 mM KCN and 0.1 mM plumbagin. SodCI activity was assayed according to the xanthine oxidase/cytochrome c method (29); in parallel samples, 2 mM KCN, an inhibitor of CuZnSOD, was added to distinguish CuZnSOD activity from that of the cytosolic manganese- and iron-containing SODs. Total protein was determined using Coomassie reagent.

**RESULTS**

Detection of extracellular superoxide. When cytochrome c was added to a culture of growing cells, it was continuously reduced, as determined by an increase in absorbance at 550 nm (Fig. 1A, line 1). Reduction occurred at a rate proportional to cell density, approximately 6 pmol per 0.1 OD600 unit of cells.
The addition of superoxide dismutase to the culture diminished the rate by about 30% (line 2), indicating that approximately one-third of the cytochrome c reduction was mediated by superoxide and that the remainder occurred when either the growing cells or an excreted metabolite directly transferred an electron to the cytochrome c.

We focused upon the extracellular superoxide. None was produced by sterile medium. Therefore, in principle it could arise from two sources: through its direct release by the cells and through the autoxidation of an excreted metabolite. When cells were removed by filtration and cytochrome c was added to the filtrate, a small amount of SOD-inhibitable cytochrome c reduction still occurred (Fig. 1B). Because superoxide itself spontaneously dismutates within seconds of its formation, this persistent reduction indicated that a low-molecular-weight metabolite was excreted by the cells and gradually reacted with oxygen in the medium, producing superoxide. Kinetic analysis (see Materials and Methods) indicated that this metabolite was responsible for only a minor fraction of the extracellular superoxide that was released by the cultures. The identity of the excreted compound was not determined. We inferred that the remaining superoxide (Fig. 1A, line 3) was either released directly by the bacteria or produced by autoxidation of a metabolite with such a short half-life that it did not survive the filtration process.

**Superoxide is formed within the periplasm.** In subsequent analyses, the superoxide that was formed by the filterable metabolite was measured and subtracted from total superoxide formation. If the residual superoxide was released directly by cells, then its release might be blocked by the presence of SOD within the periplasm. Because the periplasmic SOD of *E. coli* is synthesized only in stationary phase, the *Salmonella enterica* serovar Typhimurium sodC1 gene, placed behind a *lac* promoter, was transformed on a plasmid into *E. coli*. The consequent periplasmic SOD activity was measured in a sodA sodB sodC strain that lacks interfering SOD isozymes and was found to be about 10 U/mg. For the sake of comparison, *E. coli* and *Salmonella* laboratory strains typically contain about 3 U/mg and 15 U/mg of periplasmic SOD, respectively, when they are in stationary phase.

The SodC-expressing plasmid eliminated the release of superoxide from wild-type *E. coli* (Fig. 2A), indicating that the superoxide is released into the medium from the periplasm. In contrast, sevenfold overproduction of cytosolic FeSOD did not diminish superoxide release, and mutations that eliminated cytosolic FeSOD and MnSOD activities caused at most a marginal (<10%) increase in superoxide release (data not shown). Thus, the superoxide that is released by the cell must be formed within the periplasm.

The plasmid had no effect upon superoxide formation by the filterable metabolite (data not shown). This result is consistent with our previous observation that periplasmic SOD cannot scavenge superoxide that is formed in the extracellular medium before it reacts with extracellular cytochrome c (22).

**Periplasmic superoxide formation is accelerated by high oxygen concentration.** The previous experiments were conducted with cells suspended in air-saturated growth medium. When pure oxygen was bubbled into the cultures, increasing the dissolved oxygen concentration by four- to fivefold, the rate of periplasmic superoxide production increased proportionately (Fig. 2B). This result suggests that the superoxide is formed by the adventitious oxidation of a reduced molecule.

Prior reports demonstrated that heme-starved *E. faecalis* generates extracellular superoxide at a rate which is far above what we observed with *E. coli* (17, 18). To ensure that the discrepancy in rates did not arise from differences in methodology, we measured superoxide production by *E. faecalis* under the same conditions that we used for *E. coli*. Indeed, the rate for *E. faecalis* was approximately 25-fold faster than that for *E. coli*. In contrast to the case with *E. coli*, superoxide formation by *E. faecalis* cultures was not significantly accelerated by hyperoxygenation. It is assumed that heme-starved *E. faecalis* generates superoxide at some site on its incomplete electron transport chain. The unresponsiveness to oxygen concentration indicates either that electron transfer to oxygen is not the rate-limiting step in superoxide formation or that the autoxidizing component has a saturable oxygen-binding site. Therefore, it seems likely that superoxide production within the periplasm of *E. coli* and on the surface of *E. faecalis* occurs by distinct mechanisms.
Superoxide is released by the respiratory chain. One candidate for the site of superoxide formation in *E. coli* was the pyroquinoline quinone (PQQ) moiety of periplasmic glucose dehydrogenase. *E. coli* does not synthesize PQQ, but *E. coli* scavenges it from laboratory media, including the LB medium in which cells had been precultured prior to the measurement of superoxide production. PQQ autoxidizes rapidly, which led to the misidentification of purified glucose dehydrogenase as a glucose oxidase.

However, superoxide formation by intact cells was not diminished when the cells were precultured in defined, PQQ-free media (data not shown). Further, supplementation with PQQ or the use of lactose instead of glucose media did not alter the rates of superoxide production. Thus, glucose dehydrogenase is evidently not the primary superoxide source.

The respiratory chain lies within the cytoplasmic membrane, which raised the possibility that superoxide might be formed by autoxidation of a component that faces the periplasm. To test this possibility, we examined mutant strains that were severely defective in respiration by virtue of mutations in both quinones (*menA ubiCA* mutant) or both NADH dehydrogenase enzymes (*nuo ndh* mutant). The former strain grows poorly in air, so to permit comparisons with a wild-type strain, all three strains were precultured anaerobically and then shifted to aerobic medium for the period of superoxide measurement. The wild-type strain generated superoxide at a rate 30% higher than if it had been precultured aerobically. The *nuo ndh* mutant produced superoxide at a far lower rate (Fig. 3). Virtually no superoxide was released by a *menA ubiCA* mutant which possesses both NADH dehydrogenases but lacks ubiquinone and menaquinone, the electron carriers that deliver electrons from respiratory dehydrogenases to the cytochrome oxidases.

In aerobic *E. coli*, ubiquinone is the predominant quinone, while menaquinone (and its derivative demethylmenaquinone) comprises about 20% of the quinone pool (45, 47, 50). A *ubiCA* single mutant exhibited a 10-fold decrease in respiration and a diminished growth rate relative to those of wild-type cells, but the formation of periplasmic superoxide was elevated significantly (Fig. 4A). This result suggested that when ubiquinone is absent, the electron flow is diverted to a more autoxidizable component of the respiratory chain. Indeed, mutants lacking menaquinone (*menA* mutants) exhibited a four- to fivefold diminution in superoxide formation, despite robust growth and a wild-type respiratory rate (Fig. 4A). These results indicated that reduced menaquinone either transferred electrons to periplasmic oxygen directly or delivered them to an enzyme complex that did.

Localizing the site of superoxide formation. Menaquinone has a lower reduction potential (*E. coli*) of −0.074 V than ubiquinone (+0.113 V) and thus is a stronger electron donor. It is the obligatory carrier in anaerobic respiratory processes that employ low-potential terminal acceptors, such as fumarate. The value of its synthesis in aerobic cells is unclear. During growth in aerobic glucose media, both α-glycerolphosphate dehydrogenase and NADH dehydrogenase activities are relatively abundant and are capable of reducing menaquinone. The *nuo ndh* mutants exhibited a low level of residual H₂O₂ formation. In this medium NADH dehydrogenase II is much more active than NADH dehydrogenase I; accordingly, we found that *ndh*...
mutants were twofold diminished in periplasmic superoxide formation but that nuo mutants were unaffected (Fig. 4B). These results may simply reflect the role of these dehydrogenases in delivering electrons to menaquinone, or they may reveal that menaquinone autoxidizes while complexed to the dehydrogenase active sites. The latter possibility parallels the behavior of ubiquinone while bound in the semiquinone form to the Qo and Qi sites of the mammalian bc1 complex (16, 44). However, 10-fold overproduction of NdhII, which might be expected to increase the concentration of NdhII-complexed menaquinone, did not increase superoxide formation (Fig. 4C).

Elimination of fumarate reductase had no impact upon superoxide production (data not shown). The same was true of cytochrome o oxidase, indicating that neither of these two enzymes was the site of formation (Fig. 4D). Mutations in cyd, encoding cytochrome o oxidase, caused lower rates of respiration and growth and a twofold acceleration of superoxide formation. The effect was amplified when both cyo and cyd were eliminated. A plasmid that overproduced cytochrome o oxidase restored wild-type respiration and growth rates and suppressed superoxide formation.

Collectively, these data are consistent with the notion that mutations that alter the redox state of menaquinone—either by affecting electron delivery to it or transfer of electrons away from it—have congruent impacts upon superoxide formation. Further, we have been unable to identify any respiratory complex that is essential for superoxide production. The simplest explanation is that uncomplexed dihydromenaquinone itself is the predominant superoxide source.

Menaquinone is not the primary source of endogenous hydrogen peroxide. The rate at which E. coli generates hydrogen peroxide can be measured using an ahp katG katE strain which lacks the three primary enzymes that scavenge H2O2 (38). Under the conditions of these experiments, this strain generated H2O2 at a rate of 25 pmol per min per 0.1 OD unit of cell suspension. At the same time, extracellular superoxide was formed at a rate of 5 pmol per min per 0.1 OD unit. Upon dismutation, the latter superoxide would account for only 2.5 pmol of H2O2, or about 10% of the total. Further, while the addition of a menA mutation eliminated superoxide formation, H2O2 production was essentially unaffected (a <20% change, the limit of the experimental precision [data not shown]). Thus, while the primary process of endogenous H2O2 formation re-
growing cells. The mechanism of formation differed from that of exponentially so therefore not synthesized in LB medium until E. coli condition, sodC superoxide production was detected. This was true even when resuspended in medium lacking a fresh carbon source, no stationary phase (15). When these stationary-phase cells were used. Note that no superoxide was detected in the medium of SSK9, SSK18, SSK19, SSK20, SSK21, SSK22, SSK23, and SSK24 were used. Periplasmic O2 mains unidentified, it is clearly distinct from the process of periplasmic O2− formation.

Periplasmic superoxide formation by stationary-phase cells. Periplasmic superoxide dismutase is regulated by RpoS and is therefore not synthesized in LB medium until E. coli enters stationary phase (15). When these stationary-phase cells were resuspended in medium lacking a fresh carbon source, no superoxide production was detected. This was true even when sodC was eliminated. Thus, at least under this stationary-phase condition, E. coli does not produce significant periplasmic superoxide.

When these cells were resuspended in medium containing glucose, however, respiration immediately resumed, and superoxide was exuded from the sodC mutants (Fig. 5). In this situation, wild-type (SodC+) cells did not release superoxide, apparently because it was scavenged by the periplasmic SOD. Surprisingly, the refed stationary-phase cells initially generated superoxide even when menA was deleted, suggesting that the mechanism of formation differed from that of exponentially growing cells. The nnu ndh and cyo ccd mutants did not release superoxide, however, indicating that even under this circumstance, the superoxide depended upon flux through the respiratory chain.

**FIG. 5.** Superoxide release by stationary-phase cells depends upon respiration but does not require menaquinone. Black bars, superoxide release by exponential-phase cells; gray bars, superoxide release by stationary-phase cells immediately upon resuspension in glucose medium. The sodC mutant AS454 and the respiratory mutants SSK3, SSK9, SSK18, SSK19, SSK20, SSK21, SSK22, SSK23, and SSK24 were used. Note that no superoxide was detected in the medium of sodC derivatives of any of these stationary-phase cells.

**DISCUSSION**

A role for periplasmic superoxide dismutase. Early investigations into bacterial periplasmic superoxide dismutases focused on the possibility that they might defend pathogens against superoxide that they encounter as part of the host immune response. However, that idea was inadequate to rationalize the presence of the enzyme in nonpathogenic strains of E. coli and in free-living bacteria, such as Caulobacter (42). The discovery that aerobic E. coli releases superoxide into its periplasm provides an explanation for its synthesis of periplasmic superoxide dismutase.

The rate of periplasmic superoxide formation is quite high: about 3 μM/s, when normalized to the estimated periplasmic volume. That value is comparable to the 5 μM/s that has been estimated for superoxide formation in the cytosol (20). The latter compartment requires the protection of MnSOD and/or FeSOD, as mutants that lack these SODs are defective in aerobic metabolism and biosynthesis, due to the inactivation of key enzymes (6). In contrast, we have not observed any growth defect in sodC mutants (15). In part, this may be because the efflux of periplasmic superoxide through the outer membrane pores is rapid and may keep periplasmic superoxide levels moderate even in sodC mutants. However, the larger puzzle is the identity of the periplasmic or cell surface biomolecules that periplasmic SOD serves to protect.

Most of the enzymes that are damaged by cytosolic superoxide belong to the family of dehydratases that have exposed [4Fe-4S] clusters. Superoxide directly oxidizes these exposed clusters, thereby destabilizing them and leading to inactivation of the enzymes and the pathways to which they belong (10, 12, 13, 24, 25). However, the periplasm is not known to contain enzymes that belong to this family. There are respiratory complexes embedded in the cytoplasmic membrane that utilize Fe-S clusters, but these clusters are typically buried within polypeptides and are not affected by superoxide. Evidently, a second class of superoxide-sensitive enzymes or structural molecules is present in the periplasm. The identification of this molecule(s) is a priority, both to complete our understanding of endogenous superoxide stress in the periplasm and to gain insight into the mechanism by which phagocytic superoxide might incapacitate pathogenic bacteria.

**Mechanism of superoxide formation.** Superoxide is a charged species at neutral pH; therefore, it should not efficiently cross membranes (22, 27). This possibility has nevertheless been raised as a possible explanation for the role that Saccharomyces cerevisiae cytosolic SOD plays in protecting mitochondrial enzymes (48). Our own calculations suggest that the release of superoxide from the E. coli cytosol into the periplasm should be vanishingly rare (<1 in 106). This prediction was supported by the fact that genetic alternations that either raised or lowered the amount of cytosolic SOD had no detectable impact upon the rate of superoxide release into the external medium. Therefore, most or all periplasmic superoxide must be formed within the periplasm.

Molecular oxygen is a relatively poor univalent electron acceptor (midpoint potential [E0] = −0.16 V), so only good univalent electron donors can transfer an electron to it. Several cytosolic enzymes that can generate superoxide have been identified, and in all cases a reduced flavin (univalent E0 ≈ −0.22 V) is the direct electron donor (31). There are no flavoenzymes in the periplasm of E. coli. However, the chemical principle was sustained by our finding that in exponentially growing cells, the donor is menaquinone, another good univalent reductant (E0 = −0.074 V). In contrast, ubiquinone (E0 = +0.113 V) seemed uninvolved, despite its greater abundance than menaquinone.

There are three mechanisms by which menaquinone might deliver electrons to oxygen, and at this point we cannot exclude any of them. The first is that dihydromenaquinone might trans-
fer an electron directly, generating superoxide and a mena-
semiquinone radical that would subsequently transfer an elec-
tron to a second molecule of oxygen. Electron spin restric-
tions may impede the transfer of both electrons to the same mole-
cule of oxygen (with H2O2 as a product): the orbital structure of
oxygen requires that it accept electrons only in univalent
steps, and the superoxide-menasequione collision complex
may not survive long enough to permit the second transfer
reaction. Thus, dihydromenaquinone oxidation may predomi-
nantly generate two distinct molecules of superoxide.

A second mechanism would be driven by reaction between
reduced and oxidized menaquinones, generating two mole-
cules of menaquinone (i.e., comproportionation), as fol-

\[
\text{MQH}_2 + \text{MQ} \rightarrow 2 \text{MOH}
\]

\[
2 \text{MOH} + 2 \text{O}_2 \rightarrow 2 \text{MQ} + 2 \text{O}_2^- + 2 \text{H}^+
\]

Oxygen, a radical species itself, reacts much more rapidly with
semiquinones than with dihydroquinones (34). This fact may
argue against this mechanism, as this model might predict that
comproportionation comprises the rate-limiting step. In con-
trast, we observed that oxygen was a limiting reactant.

The third mechanism also emphasizes the autoxidizability of
semiquinone species. Semiquinones are formed as momentary
intermediate species when quinones are divalent reduced by
dehydrogenases, and they are also transiently transformed when
dihydroquinones pass their electrons to metal centers in ter-
ninal oxidases. Much of the superoxide that is formed by
mammalian mitochondria has been ascribed to the autoxidation
of the ubisemiquinone radicals that are formed during the
catalytic cycle of the bc1 complex (16, 44). The dependence of
superoxide formation upon oxygen concentration would not be
an objection to this model, since the radical intermediates are
so fleeting that their ability to be oxidized depends upon the
concentration of the oxidant. However, we found that each of
the major respiratory complexes could individually be re-
moved without diminishing periplasmic superoxide produc-
tion. We tentatively conclude that unbound dihydromaqui-
none is likely to be the autoxidizing species.

Interestingly, Huycke and colleagues observed that E. fae-
calis generates superoxide when its respiratory chain lacks a
functional cytochrome d oxidase and that this activity requires
demethylmenaquinone (18). The parallel to our observations is
striking, but some differences suggest that the mechanisms may
be distinct. First, E. faecalis forms superoxide 25 times more
rapidly than does E. coli. E. coli strains did not approach that
rate even when cytochrome oxidases were deleted to force
electron accumulation on menaquinone. Second, superoxide
formation in E. coli occurred in proportion to oxygen concen-
tration, which is what one would expect if the rate-limiting step
were the adventitious oxidation of a reduced electron carrier.
However, in E. faecalis, superoxide formation was not in-
creased when oxygen levels were elevated. These observations
suggest the possibility that E. faecalis, a natural heme aux-
troph that must frequently find itself in heme-deficient envi-
nments, might have a respiratory enzyme that catalyzes qui-
none oxidation as a way to discharge an incomplete electron
transport chain. Siegele et al. suggested that in E. coli cyto-CD
mutants, the heme-less cytochrome d oxidase might do this
directly (39). However, we did not observe any increase in
periplasmic superoxide formation in these mutants (data not
shown).

Finally, we observed that when stationary-phase cells were
refed glucose, superoxide was initially formed at substantial
rates even in strains lacking menaquinone. Thus, menaquinone
oxidation is not the sole source of periplasmic superoxide in E.
coli. This superoxide formation in refed cells was eliminated by
combinations of mutations that either blocked electron entry
into the respiratory chain (nuo ndh mutant) or that blocked
electron flow out of it (cyo cyd mutant). This observation sug-
gests that the autoxidizing species may be a partially reduced
enzyme, as it fits the expectation that enzymatic semiquinones
are most abundant when enzymes are actively turning over.

The structure of the respiratory chain in stationary-phase cells
is not well studied, and we have not analyzed this situation
further. However, it is notable that even in exponentially grow-
ing cells, the absence of menaquinone did not completely elim-
inate superoxide formation. Therefore, the superoxide source
in the refed cells could be the same residual source that is
detected in the menaquinoneless mutants.

**Why is periplasmic SOD induced only in stationary phase?**

Like many oxidative defenses, periplasmic SOD is positively
regulated by the RpoS system and is strongly induced in sta-
tionary phase. However, in our experiments, the highest yield
of superoxide was obtained when cells were growing exponen-
tially; stationary-phase cells evolved periplasmic superoxide
only after they were diluted into fresh medium. Thus, the
timing of SOD synthesis appeared not to correlate with super-
oxide formation. This apparent contradiction may turn on the
reason that cells enter stationary phase. While in our experi-
ments growth stopped because carbon sources were exhausted,
in natural habitats cells may also enter stationary phase for lack
of a phosphorus, nitrogen, or sulfur source. If a carbon source
is present, superoxide formation might still occur. Indeed,
some recent work by Moreau indicates that oxidative stress
might be especially severe under these conditions (33).

We tested this idea by culturing sodC mutants in a defined
glucose medium containing limiting phosphate. When these
cells stopped growing, they were diluted into phosphate-free
medium, and superoxide release was measured. Periplasmic
superoxide formation occurred at a rate that was 50 to 60% of
that of exponentially growing cells (data not shown).

Still, it is not obvious why periplasmic SOD would not be
made in exponentially growing cells. We speculate that cells
may be particularly vulnerable to oxidative damage in station-
ary phase; injuries may be most consequential when cells lack
the energy and material to repair or replace the damaged
molecules. In fact, several other antioxidant enzymes are
strongly induced in stationary phase as part of the RpoS regu-
lon, including catalase, exonuclease III, and the iron-seques-
tering protein Dps (8).

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