**Inductions of Superoxide Dismutes in *Escherichia coli* under Anaerobic Conditions**

ACCUMULATION OF AN INACTIVE FORM OF THE MANGANESE ENZYME*

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*Escherichia coli* growing anaerobically respond to NO; with a 3-fold induction of the iron-containing superoxide dismutase. Mutants lacking nitrate reductase do not show this response. Anaerobically grown cells also contain an inactive form of the manganese-containing superoxide dismutase (MnSOD) which can be activated by addition of Mn(I1) salts in the presence of acidic guanidinium chloride, followed by dialysis against neutral buffer. Direct addition of Mn(I1) to a neutral solution of the inactive MnSOD does not impart activity. This inactive MnSOD thus behaves as would the apoenzyme or the enzyme bearing a metal other than Mn(I1) at its active sites.

Terminal electron acceptors, such as NO; or trimethylamine N-oxide, increase the amount of inactive MnSOD produced by anaerobic *E. coli*. Paraquat, which is itself ineffective in this regard, markedly augments the effect of these terminal electron acceptors. It appears that flow of electrons to sinks such as NO; or trimethylamine N-oxide, facilitated by paraquat, is sufficient to elicit biosynthesis of the MnSOD protein and that O; is not needed for this process. Yet, oxygenation and concomitant O; production do appear important for the insertion of manganese into the growing MnSOD polypeptide, possibly because O; oxidizes Mn(I1) to Mn(III), and the latter is the valence state most effective in combining with the apoenzyme.

Oxygenation of the growth medium increases the biosynthesis of SOD1 in *Streptococcus faecalis* (1), *Escherichia coli* (1-5), *Photobacterium leiognathi* (6, 7), *Saccharomyces cerevisiae* (8), *Bacteroides fragilis* (9), *Propionibacterium shermanii* (10), and other microorganisms (11-18). Increasing pO; should increase intracellular O; production. Other manipulations also expected to elevate intracellular O; such as exposure to viologens or to a variety of quinones, were similarly found to induce the biosynthesis of SOD (19-24). This led to the view that O; or something engendered from O; was crucial for the induction of SOD biosynthesis in *E. coli*.

The need for a more complex model was made clear by the observations that the MnSOD of *E. coli*, although strikingly induced by O; or NO; or trimethylamine N-oxide, facilitated by paraquat, is sufficient to elicit biosynthesis of the MnSOD protein and that O; is not needed for this process. Yet, oxygenation and concomitant O; production do appear important for the insertion of manganese into the growing MnSOD polypeptide, possibly because O; oxidizes Mn(I1) to Mn(III), and the latter is the valence state most effective in combining with the apoenzyme.

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*Bacterial Strains—* *E. coli* BB11 was obtained from the American Type Culture Collection (ATCC 29662). *E. coli* K12 strains (M1-7) were kindly provided by Dr. K. V. Rajagopalan, Duke University Medical Center, Dept. of Biochemistry.

**Growth Conditions—** *E. coli* was grown at 37 °C on trypticase soy yeast extract (TSY medium), pH 7.0, supplemented with 1.0 mg/liter vitamin B12 (for ATCC 29682), or on MOPS minimal medium (30), modified by increasing the concentration of MOPS to 80 mM (31).

Glucose (0.5%) was added as the carbon and energy source in the MOPS medium. Cultures were grown in Ryan or Erlenmeyer flasks at a volume/volume medium ratio of 1:1. Aerobic growth was achieved at 200 rpm on a rotary shaker in air, whereas anaerobic growth was accomplished in a Coy anaerobic chamber. Anaerobic conditions were maintained by growing the overnight inoculating cultures and the experimental cultures in medium equilibrated in the Coy chamber. Cultures were initiated with either 2% (TSY) or 4% (MOPS) inoculum from overnight cultures.

**Materials and Methods**

**Enzyme Assays—** Cells from experimental cultures were harvested by centrifugation at 10,000 × g for 10 min at 4 °C; washed once with 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8; and resuspended in 1.0 ml of this buffer. The washed cells were lysed by two passages through a French pressure cell. Lysozyme was clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and the cell-free extracts were dialyzed overnight at 4 °C against 250-300 volumes KP/EDTA buffer with one change of buffer. SOD was assayed by the xanthine oxidase/cytochrome c method (32). Catalase was assayed in terms of the disappearance of H2O2 followed at 240 nm (33).
amidine in 25% HCl was substituted for sulfanilic acid. Protein content was estimated colorimetrically with bovine serum albumin serving as the standard (35). SOD electrophorograms were separated by electrophoresis on 7% polyacrylamide gels (36), and bands of SOD activity were visualized by activity staining (37). Catalase was localized on gels as described by Clare et al. (38). Reversible resolution of the inactive MnSOD was performed as previously described (39).

Chemicals—Trypticase soy broth was from Baltimore Biological Laboratories. Yeast extract was from Difco. Acrylamide and methylenebisacrylamide were purchased from Bio-Rad. Radamid, diamide, paraquat, trimethylamine N-oxide, cytochrome described by Clare et al., xanthine, nitro blue tetrazolium, 8-hydroxyquinoline, and MOPS buffer were from Sigma. Potassium nitrate and Me2SO were obtained from Mallinkrodt Chemical Works. Potassium ferricyanide was from T. Raker Chemical Co., and guanidinium chloride was from Schwarz/Mann.

RESULTS

Induction of SOD by Nitrate—E. coli grown anaerobically in MOPS medium contained 8.0 units of SOD activity/mg of protein (Table I). Electrophoretic separation and activity staining of the extracts revealed the presence of only FeSOD, as expected (3) (Fig. 1, lane 2). Aerobically grown cells contained 22 units of SOD activity/mg of protein, containing approximately equal amounts of MnSOD and FeSOD and a smaller amount of the hybrid SOD (lane 1). NO3- (50 mM) did not significantly change the total amount or the isozymic distribution of SOD present in aerobically grown cells, but did result in a 3-fold increase in SOD content in anaerobically grown cells (26 units/mg of protein). Activity gels revealed this induction to be entirely due to the FeSOD isozyme (lane 3).

Surprisingly, catalase was more abundant in anaerobically grown cells than in aerobically grown cells. This has also been observed in Salmonella following growth in minimal medium (40). NO3- resulted in an induction of catalase under both aerobic and anaerobic conditions (Table I). Aerobically grown cells contained both hydroperoxidases I and II (41-43) as shown by Fig. 1 (lane 4), and aerobic nitrate induced primarily hydroperoxidase I (lane 5). In contrast, cells grown in the absence of O2 contained only hydroperoxidase I (lane 6), and nitrate resulted in the appearance of hydroperoxidase II (lane 7) (43). Sulfate (50 mM), unlike nitrate, had no effect on cell content of SODs or of hydroperoxidases under aerobic or anaerobic conditions (Table I).

Effects of Paraquat on Anaerobic Induction of SOD—We have previously noted that the induction of MnSOD in E. coli by paraquat is dioxygen-dependent (22), and the results in Table II again demonstrate the inability of paraquat to induce SOD under anaerobic conditions. These data also show that in the presence of paraquat, NO3- resulted in an induction of SOD, although to a smaller extent than with NO2- alone. Activity-stained electrophorograms demonstrated a complete lack of MnSOD and showed that this induction was due entirely to an increase in cell content of FeSOD (data not shown). In contrast to these results, Smith and Neidhardt (5) have reported anaerobic induction of the MnSOD polypeptide by NO2-, and Hassan2 has observed the anaerobic induction of MnSOD activity by paraquat plus KNO3.

We have previously shown that growth of E. coli B in simple medium enriched with Mn(II) resulted in an elevation of MnSOD and, furthermore, that manganese in TSY medium appears to be in a more accessible form than manganese in a simple medium (44). Therefore, we examined the effects of NO2- ± paraquat on E. coli grown anaerobically in TSY medium or in MOPS medium supplemented with Mn(II). Addition of NO2- (50 mM) to E. coli B growing anaerobically in TSY medium resulted in a 2.5-fold increase in total SOD similar to results observed in MOPS medium. Paraquat (1.0 mM) alone had no effect on SOD content and did not enhance the induction of activity caused by NO2-. However, as shown in Fig. 2, cells grown in TSY in the combined presence of paraquat plus nitrate demonstrated a weak band of the hybrid SOD (lane 8, caret). Mn(II)-enriched MOPS medium did not affect SOD isozyme content of the cells, and a hybrid band was not observed in response to NO2- + PQ2+.

NO2- can serve as a terminal electron acceptor for E. coli

\[ \text{H. M. Hassan, personal communication.} \]

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**Table I**

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>( \rho_{\text{SOD}} )</th>
<th>Specific activity (units/mg)</th>
</tr>
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<tbody>
<tr>
<td>Aerobic</td>
<td>0.94</td>
<td>22.2</td>
</tr>
<tr>
<td>+50 mM KNO3</td>
<td>0.98</td>
<td>18.0</td>
</tr>
<tr>
<td>+50 mM Na2SO4</td>
<td>0.90</td>
<td>20.1</td>
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<tr>
<td>Anaerobic</td>
<td>0.40</td>
<td>8.0</td>
</tr>
<tr>
<td>+50 mM KNO3</td>
<td>0.43</td>
<td>25.9</td>
</tr>
<tr>
<td>+200 mM KNO3</td>
<td>0.33</td>
<td>24.0</td>
</tr>
<tr>
<td>+50 mM Na2SO4</td>
<td>0.39</td>
<td>10.6</td>
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</table>

**Table II**

<table>
<thead>
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<th>Growth conditions</th>
<th>( \rho_{\text{SOD}} )</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.39</td>
<td>9.8</td>
</tr>
<tr>
<td>50 mM KNO3</td>
<td>0.42</td>
<td>28.5</td>
</tr>
<tr>
<td>1.0 mM PQ2+</td>
<td>0.36</td>
<td>10.1</td>
</tr>
<tr>
<td>50 mM KNO3 + 1.0 mM PQ2+</td>
<td>0.34</td>
<td>21.4</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Effect of nitrate on SOD and catalase electrophorograms. E. coli B cells were grown in MOPS medium at 37°C. Anaerobic growth was carried out for 8 h. Aerobic growth was in air at 200 rpm for 5 h. Soluble extracts were applied to polyacrylamide gels at 60 µg of protein/gel. After electrophoresis, the electrophorograms were stained for enzymatic activity. Lanes 1–3, SOD activity; lanes 4–7, catalase activity. Lanes 1 and 4, aerobic; lanes 2 and 6, anaerobic; lanes 3 and 7, anaerobic + 50 mM NO3-; lane 5, aerobic + 50 mM NO3-. Hy, hybrid; HP, hydroperoxidase.
Anaerobic Inductions of SODs in E. coli

An inactive FeSOD dimer and of an active MnSOD dimer. Application of this procedure to extracts from the NO\textsubscript{3} plus paraquat-induced nitrate reductase-positive strain of E. coli resulted in the disappearance of the hybrid band and failure of an MnSOD band to appear (Fig. 3). This suggested the presence of an inactive MnSOD subunit in the hybrid, which, in turn, suggested the possibility that NO\textsubscript{3} was causing an aerobic induction and accumulation of a pool of inactive MnSOD.

If the inactive MnSOD was devoid of activity due to a lack of the prosthetic Mn(III) or, alternatively, due to occupation of the manganese site by another metal, it might be possible to activate MnSOD by application of a procedure for reversible removal and replacement of the active site metal. We have previously reported that SODs in crude extracts can be resolved by exposure to low pH in the presence of guanidinium chloride. The apoenzymes produced are inactive and can be activated only by the metal found in the native enzyme (39). As shown in Fig. 4, this procedure revealed that NO\textsubscript{3} and, to a greater extent, NO\textsubscript{3} plus paraquat resulted in anaerobic induction of MnSOD. Similar results were observed with E. coli B. This MnSOD was inactive until subjected to metal removal by exposure to low pH in the presence of guanidinium chloride followed by reconstitution with manganese. A small amount of inactive MnSOD could be detected in extracts of cells grown anaerobically in the absence of NO\textsubscript{3} or paraquat. Paraquat in the absence of NO\textsubscript{3} did not induce this inactive MnSOD.

It has previously been noted that apo-MnSOD could be reconstituted when Mn(II) was added at low pH and the pH was subsequently raised during dialysis against neutral buffer (48). Conversely, reconstitution was not observed when Mn(II) was added to a neutral solution of apo-MnSOD. Addition of MnCl\textsubscript{2} to extracts of E. coli grown anaerobically under conditions which elicited induction of the inactive MnSOD (paraquat + NO\textsubscript{3}) similarly failed to elicit the appearance of active MnSOD. These results are consistent with the contention that the inactive MnSOD induced by NO\textsubscript{3} is either an apo-MnSOD or a MnSOD in which the active site is occupied by a metal other than manganese.

(45). If the anaerobic induction of SOD were due to electron flow to NO\textsubscript{3}, then a mutant defective in the dissimilatory nitrate reductase should fail to show this induction. The nitrate reductase mutant MJ-421, which lacks the molybdopterin cofactor (46), and the parental strain (MJ-7) were compared for their ability to induce SOD in response to NO\textsubscript{3} in anaerobic TSY medium. The results, shown in Table III, demonstrate that the parental strain induced nitrate reductase, SOD, and catalase in response to NO\textsubscript{3}; whereas the mutant exhibited no detectable nitrate reductase and did not increase cellular content of SOD or catalase in response to nitrate. Polyacrylamide gel electrophorograms of extracts from these cells, stained for SOD activity, showed anaerobic induction of FeSOD in the parental strain in response to NO\textsubscript{3} alone and induction of both FeSOD and hybrid SOD by NO\textsubscript{3} plus paraquat (data not shown). When grown aerobically, both strains exhibited MnSOD, FeSOD, and hybrid SOD bands. In addition, both strains responded to aerobic exposure to paraquat by induction of MnSOD.

Anaerobic Induction of Inactive MnSOD—We have previously demonstrated (47) that treatment of the E. coli hybrid SOD with H\textsubscript{2}O\textsubscript{2} inactivates the FeSOD subunit and results in a resegregation of subunits, with the resultant appearance of

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**TABLE III**

Effect of paraquat and nitrate on SOD, catalase, and nitrate reductase activities in E. coli K\textsubscript{12}.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>A\textsubscript{max}</th>
<th>Specific activity</th>
<th>NaRC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOD</td>
<td>Catalase</td>
</tr>
<tr>
<td>A. Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM KNO\textsubscript{3}</td>
<td>0.54</td>
<td>11.7</td>
<td>2.0</td>
</tr>
<tr>
<td>1.0 mM PQ\textsuperscript{2+}</td>
<td>0.54</td>
<td>11.0</td>
<td>3.8</td>
</tr>
<tr>
<td>50 mM KNO\textsubscript{3} + 1.0 mM PQ\textsuperscript{2+}</td>
<td>0.54</td>
<td>18.0</td>
<td>10.3</td>
</tr>
<tr>
<td>B. Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM KNO\textsubscript{3}</td>
<td>0.47</td>
<td>8.4</td>
<td>3.0</td>
</tr>
<tr>
<td>1.0 mM PQ\textsuperscript{2+}</td>
<td>0.45</td>
<td>7.6</td>
<td>2.0</td>
</tr>
<tr>
<td>50 mM KNO\textsubscript{3} + 1.0 mM PQ\textsuperscript{2+}</td>
<td>0.41</td>
<td>8.9</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* NaR, nitrate reductase (nmol NO\textsubscript{3}·min\textsuperscript{-1}·mg\textsuperscript{-1}); ND, not detectable.

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(39). As shown in Fig. 4, this procedure revealed that NO\textsubscript{3} and, to a greater extent, NO\textsubscript{3} plus paraquat resulted in anaerobic induction of MnSOD. Similar results were observed with E. coli B. This MnSOD was inactive until subjected to metal removal by exposure to low pH in the presence of guanidinium chloride followed by reconstitution with manganese. A small amount of inactive MnSOD could be detected in extracts of cells grown anaerobically in the absence of NO\textsubscript{3} or paraquat. Paraquat in the absence of NO\textsubscript{3} did not induce this inactive MnSOD.

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**Fig. 3. Effect of hydrogen peroxide on E. coli SODs induced by NO\textsubscript{3} + PQ\textsuperscript{2+}.** E. coli K\textsubscript{12} (strain MJ-7) cells were grown anaerobically in TSY medium containing 50 mM KNO\textsubscript{3} and 1.0 mM PQ\textsuperscript{2+} at 37°C. Lane 1, extract incubated in 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8, for 30 min at 25°C; lane 2, extract incubated in KP/EDTA buffer containing 5.0 mM H\textsubscript{2}O\textsubscript{2}, 1.0 mM KCN for 30 min at 25°C. Extracts, at 120 μg of protein/gel, were then subjected to electrophoresis and stained for SOD activity.
Anaerobic Inductions of SODs in E. coli

We have suggested (28) that Mn(III) is more effective than Mn(II) in combining with apo-MnSOD and that one manner in which O_2 facilitates biosynthesis of active MnSOD is by oxidizing Mn(II) to Mn(III). If insertion of manganese is inefficient under anaerobic conditions, then enrichment of the growth medium with Mn(II) might allow the anaerobic induction of active MnSOD by paraquat plus NO_3. E. coli did indeed accumulate active MnSOD when grown anaerobically in TSY medium containing 50 mM NO_3, 1.0 mM paraquat, and 0.1 mM MnCl_2 (Fig. 5A, lane 5). In contrast, aerobic exposure of E. coli in the presence of chloramphenicol following anaerobic growth in the presence of PQ^2+ and NO_3 did not result in an increase in active MnSOD (Fig. 5B, lanes 6–10). Chloramphenicol was added immediately prior to oxygenation to prevent de novo synthesis of MnSOD. Thus, the inactive MnSOD present within anaerobically grown cells (PQ^2+ and NO_3) could not be activated by aeration under these conditions.

In addition to NO_3, E. coli can utilize TMAO or Me_2SO as terminal electron acceptors (45, 49). The compounds were therefore examined for their ability to induce SOD and catalase under anaerobic conditions. As shown in Table IV, both TMAO and Me_2SO induced catalase, with TMAO-mediated induction approximately equal to that of NO_3-mediated induction. Anaerobic growth in TSY medium supplemented with Me_2SO resulted in a 2-fold increase in SOD compared to a 1.6-fold increase in the presence of TMAO. Polyacrylamide gel electrophoresis and activity staining of both native extracts and extracts following metal removal and treatment with Mn(II) from cells grown anaerobically in the presence of a variety of electron acceptors revealed SOD isozyme inductions similar to those observed following growth in NO_3 Me_2SO and, to a smaller extent, TMAO resulted in the induction of only FeSOD; whereas TMAO and PQ^2+ resulted in the additional appearance of hybrid SOD (Fig. 6A). Reversible resolution and Mn(II) reconstitution followed by linear scanning densitometry of activity-stained bands re-

![Figure 4](image1.png)

**FIG. 4. Reversible resolution and Mn(II) reconstitution.** E. coli K12 (strain MJ-7) cells were grown under anaerobic conditions in TSY medium for 5 h at 37 °C. Soluble extracts were subjected to reversible resolution and Mn(II) reconstitution as described under "Materials and Methods." Reconstituted extracts were applied to polyacrylamide gels at 150 µg of protein/gel and, after electrophoresis, stained for SOD activity. Lane 1, control; lane 2, 50 mM NO_3; lane 3, 1.0 mM PQ^2+; lane 4, 50 mM NO_3 + 1.0 mM PQ^2+; lane 5, 50 mM NO_3 + 1.0 mM PQ^2+, native activity (extract not subjected to reversible resolution and reconstitution).

![Figure 5](image2.png)

**FIG. 5. Effect of oxygenation and Mn(II) on active MnSOD synthesis.** E. coli B cells were grown under anaerobic conditions in TSY medium for 5 h at 37 °C. Chloramphenicol (150 µg/ml) was added, and cultures were incubated an additional 15 min at 37 °C. Indicated cultures were then aerated at 200 rpm in air for 60 min while anaerobic controls were maintained in a Coy chamber. Cell extracts were subjected to electrophoresis at 70 µg of protein/gel and electropherograms were stained for SOD activity. Lanes 1 and 6, control; lanes 2 and 7, 50 mM NO_3; lanes 3 and 8, 1.0 mM PQ^2+; lanes 4 and 9, 50 mM NO_3 + 1.0 mM PQ^2+; lanes 5 and 10, 50 mM NO_3 + 1.0 mM PQ^2+ + 0.1 mM MnCl_2. A, lanes 1–5, anaerobic cultures; B, lanes 6–10, cultures air-exposed for 60 min.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>( A_{600} )</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOD</td>
</tr>
<tr>
<td>Control</td>
<td>0.60</td>
<td>7.6</td>
</tr>
<tr>
<td>50 mM KNO_3 + 1.0 mM PQ^2+</td>
<td>0.56</td>
<td>18.3</td>
</tr>
<tr>
<td>50 mM TMAO</td>
<td>1.02</td>
<td>12.3</td>
</tr>
<tr>
<td>50 mM TMAO + 1.0 mM PQ^2+</td>
<td>0.81</td>
<td>13.1</td>
</tr>
<tr>
<td>50 mM Me_2SO</td>
<td>0.67</td>
<td>15.4</td>
</tr>
<tr>
<td>50 mM Me_2SO + 1.0 mM PQ^2+</td>
<td>0.58</td>
<td>11.7</td>
</tr>
</tbody>
</table>
under anaerobic conditions devised for resolution and reconstitution of this enzyme, but not by addition of Mn(II) salts at neutral pH. This is consistent with the behavior expected of apo-MnSOD or of MnSOD bearing iron or another metal cation at the active site (48). We cannot now distinguish between these possibilities. We have undertaken the isolation of the inactive MnSOD in order to determine its metal content and to allow direct comparison with the holoenzyme. The anaerobic induction of inactive MnSOD by NO_3 provides an explanation for the apparent discrepancy between our current findings and those reported by others (5). Thus, NO_3 induced both active FeSOD, which is detected by measuring SOD activity, and inactive MnSOD, detected by us only after reversible resolution and Mn(II) reconstitution. The quantitation of polypeptide spots following two-dimensional gel electrophoresis would fail to differentiate between active and inactive forms of the enzyme.

Anaerobic induction of the inactive MnSOD is augmented by NO_3, TMAO, and, to a lesser extent, Me_2SO. PQ^+ sharply increases this effect of these terminal electron acceptors. However, PQ^{2+} by itself is unable to cause anaerobic induction of the inactive MnSOD, even when present at 50 mM. This may be due to the different redox potentials of paraquat and the terminal electron acceptors. Thus, Me_2SO (E'\_0 = +0.13 V), TMAO (E'\_0 = +0.82 V) all have redox potentials which are positive with respect to NAD(P)H (E'\_0 = -0.32 V). Hence, electron flow from NAD(P)H to these acceptors is energetically favorable. In contrast, the redox potential of PQ^{2+} (E'\_0 = -0.44 V) is negative with respect to NAD(P)H, and net reduction of PQ^{2+} is an endergonic process. Thus, although PQ^{2+} may mediate electron flow from NAD(P)H to electron sinks such as NO_3, TMAO, or O_2, it is not itself capable of serving as an efficient electron sink.

We have shown that a variety of electron sinks, under anaerobic conditions, can derepress the biosynthesis of the MnSOD polypeptide. However, it appears that insertion of manganese at the active site occurs most readily when biosynthesis occurs in the presence of dioxygen. Furthermore, in the presence of dioxygen, manganese insertion and the appearance of MnSOD activity are facilitated by increasing the production of O_2 through the actions of a variety of redox active viologens and quinones (19-24). Since O_2 oxidizes Mn(II) to Mn(III) (51) and the manganese in resting state MnSOD is Mn(III), we have previously suggested that induction of MnSOD was facilitated by O_2 because Mn(III) would be more abundant under conditions which increased O_2 production (28). This hypothesis remains reasonable and is supported by the increased accumulation of active MnSOD seen when anaerobic TSY medium containing PQ^{2+} plus NO_3 was supplemented with 100 μM Mn(II). Spectroscopic techniques have shown that aeration of Staphylococcus aureus was accompanied by net oxidation of Mn(II) to Mn(III) (52). Fe(CN)_6^{3-}, under anaerobic conditions may also be able to cause oxidation of Mn(II) to Mn(III) within E. coli and could thus account for the appearance of active MnSOD. Further support for this possibility comes from the observation that the redox potential for the oxidation of Mn(II) to Mn(III) is dramatically shifted for manganese bound to enzyme (approximately 0.511 V) compared to manganese free in solution (approximately 1.56 V) (53, 54).

**FIG. 6. Effect of terminal electron acceptors on native and Mn(II)-reconstituted SOD electromorphs.** E. coli B cells were grown under anaerobic conditions in TSY medium for 5 h at 37 °C. Soluble extracts were applied to polyacrylamide gels, subjected to electrophoresis, and stained for SOD activity. A, native activity, 70 μg of protein/gel; B, Mn(II)-reconstituted activity, 150 (lanes 6-12) and 70 (lanes 13 and 14) μg of protein/gel. Lanes 1 and 8, control; lanes 2 and 9, 50 mM NO_3; lanes 3 and 10, 50 mM TMAO; lanes 4 and 11, 50 mM TMAO + 1.0 mM PQ^{2+}; lanes 5 and 12, 50 mM Me_2SO + 1.0 mM PQ^{2+}; lanes 6 and 13, 25 mM Fe(CN)_6^{3-}; lanes 7 and 14, 25 mM Fe(CN)_6^{3-} + 1.0 mM PQ^{2+}.

vealed that Me_2SO (1.9-fold), TMAO (5.9-fold), and, to a greater extent, TMAO + PQ^{2+} (11.9-fold) resulted in induction of inactive MnSOD (Fig. 6B). Fe(CN)_6^{3-}, an artificial electron acceptor, was unique among the compounds tested in causing anaerobic production of active MnSOD, and this was more dramatic in the presence of PQ^{2+} (Fig. 6A).

Since PQ^{2+} at 1.0 mM augmented the anaerobic induction of the inactive MnSOD by 50 mM NO_3, we examined the effect of 50 mM PQ^{2+} alone on induction of inactive MnSOD. Anaerobic cultures of E. coli in TSY medium were able to accumulate PQ^{2+}, the reduced form of paraquat, as evidenced by development of a blue color during anaerobic growth. This color was not observed when NO_3 was also present, indicating that electron flow was from NADPH → PQ^{2+} → NO_3. We have previously described a soluble NADPH:PQ^{2+} oxidoreductase in extracts of E. coli (23). Nevertheless, addition of 50 mM PQ^{2+} to anaerobic cultures of E. coli did not lead to induction of the inactive form of MnSOD (data not shown).

The ability of Fe(CN)_6^{3-} to cause anaerobic induction of active MnSOD suggested that depletion of intracellular GSH might be a factor in this induction. Diamide, which also depletes GSH (50), was tested and was also found to result in anaerobic induction of active MnSOD (data not shown).
The anaerobic induction of the inactive MnSOD polypeptide by NO\textsubscript{2} or TMAO and enhancement thereof by PQ
eliminate the possibility that O\textsubscript{2} per se plays a direct role in this aspect of MnSOD biosynthesis. We have recently reported that depletion of GSH derepresses the biosynthesis of the MnSOD in E. coli, both in vivo and in an in vitro transcription/translation system (55). Addition of an electron sink to anaerobic cultures of E. coli may have the effect of decreasing the steady-state concentration of NAD(P)H within the cell. A decrease in this reductant, utilized by glutathione reductase, would ultimately lead to a lowering of the intracellular glutathione concentration. The observation that agents such as ferricyanide and diamide, which have been shown to lower intracellular GSH levels, also result in anaerobic induction of active MnSOD is in accord with this view.

REFERENCES