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Induction of Superoxide Dismutases in *Escherichia coli* B by Metal Chelators

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The effects of metal salts, chelating agents, and paraquat on the superoxide dismutases (SODs) of *Escherichia coli* B were explored. Mn(II) increased manganese-containing SOD (MnSOD), whereas Fe(II) increased iron-containing SOD (FeSOD). Chelating agents induced MnSOD but decreased FeSOD and markedly increased the degree of induction seen with Mn(II). Paraquat also exerted a synergistic effect with Mn(II). High levels of MnSOD were achieved in the combined presence of Mn(II), chelating agent, and paraquat. All of these effects were dependent on the presence of oxygen. MnSOD, not ordinarily present in anaerobically grown *E. coli* cells, was present when the cells were grown anaerobically in the presence of chelating agents. These results are accommodated by a scheme which incorporates autogenous repression by the apoSODs and competition between Fe(II) and Mn(II) for the metal-binding sites of the apoSODs. It is further supposed that oxygenation and intracellular O_2^- production favor MnSOD production because O_2^- oxidizes Mn(II) to Mn(III), which competes favorably with Fe(II) for the apoSODs.

Escherichia coli produces two distinct homodimeric SODs. One of these contains iron (FeSOD) (25, 25a, 30), and the other contains manganese (MnSOD) (14, 26). When grown anaerobically, *E. coli* cells contain only FeSOD, but when grown aerobically they contain FeSOD, MnSOD, and a hybrid of these (HySOD) (9). Redox-active compounds, which can increase intracellular production of O_2^-, enhance the biosynthesis of MnSOD (8–11). These results led to the view that FeSOD is constitutive in *E. coli*, whereas MnSOD is under repression control. Subsequent observations indicated that this was an inadequately detailed view of the controls on the biosynthesis of these enzymes. Thus, chelating agents were seen to increase the content of MnSOD in cells (H. M. Hassan and I. Fridovich, unpublished data), whereas enriching a glucose-plus-salts medium with Mn(II) increased MnSOD and enrichment with Fe(II) increased FeSOD (23). The latter observations were accommodated by supposing that metal insertion was rate limiting in the biosynthesis of holoSODs and that the corresponding apoenzymes acted as autogenous suppressors (23).

It seemed likely that further exploration of the effects of dioxygen, chelating agents, and metals on the biosynthesis of SODs in *E. coli* would lead to a more complete understanding of the factors governing the production of these enzymes. We now report the results of such studies and propose a mechanism that accommodates the available data. A parallel and independent investigation has duplicated some of our observations, but has led to a different proposed mechanism (19; C. S. Moody and H. M. Hassan, Fed. Proc. 43:1718, 1984).

(A preliminary report of these results has been published [Fed. Proc. 43:2057, 1984].)

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are: SOD, superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; FeSOD, iron-containing superoxide dismutase; HySOD, hybrid superoxide dismutase; TSY, tryptic soy-yeast extract; VB, Vogel Bonner; PQ, paraquat (2+ ionic charge); 8-HQ, 8-hydroxyquinoline; DTPA, diethylenetriamine pentaacetic acid; EDTA, ethylenediamine tetraacetic acid disodium salt; A_{600}, absorbance measured at 600 nm.

Cells. *E. coli* B B_{12}- (ATCC 29682) was grown at 37°C, aerobically or anaerobically, in VB (28) or TSY medium as previously described (23). The initial pH of all media was 7.0, and vitamin B_{12} was present at 1.0 mg/liter. Culture flasks were acid washed and thoroughly rinsed in deionized water. After 16 h of growth in 10 ml of medium, cells were sedimented at 20,000 × *g* for 15 min at 4°C, washed three times in 5 mM potassium phosphate–1.0 mM EDTA at pH 7.8 and 4°C, and then suspended in 0.5 to 1.5 ml of this buffer. The washed cells were lysed during two passages through a French press at 20,000 lb/in^2. Cell lysates were clarified at 13,000 × *g* and 4°C for 10 min in a Fisher model 235A microfuge. SOD was assayed by the xanthine oxidase-cytochrome *c* procedure (18), and protein was measured as described by Lowry et al. (17), with bovine serum albumin as the calibrating standard. When it was necessary to measure the protein content of whole cells, the suspensions were clarified by incubation for 90 min at 90°C in 0.33 M NaOH before the Lowry method was used. When this was done, the calibrating standard was treated identically.

SOD isozymes. SOD isozymes were separated by electrophoresis on 7% polyacrylamide gels (7). The electropherograms were stained for SOD activity as previously described (4), and the amount of each isozyme was then estimated by linear scanning densitometry at 560 nm. Cells were prepared for metal analysis by being washed five times with citrate buffer, two times with citrate-EDTA buffer, and two times with water and were then suspended in water, as described by Archibald (1). The metal content was measured by atomic absorption with a Perkin Elmer model 107 fitted with a graphite furnace.

Materials. TSY medium was purchased from Difco Laboratories, salts were from Fisher Scientific Co., and other organics were from Sigma Chemical Co. Manganese sulfate was from Sigma. Iron as Fe(II) was added as reagent-grade $FeSO_4 \cdot 7H_2O$ from the Mallinkrodt Chemical Works.

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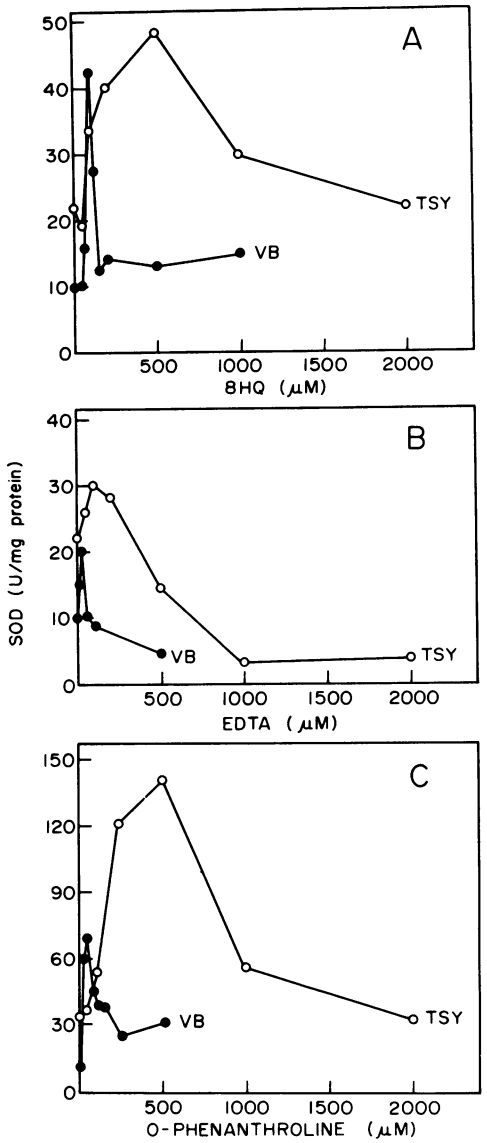

FIG. 1. Induction of SOD in aerobic TSY and VB media. 8-HQ (A), EDTA (B), or *o*-phenanthroline (C) was added to VB (●) or TSY (○) medium at the time of inoculation with *E. coli* B. Cells were harvested in the stationary phase of growth (after 16 h at 37°C), lysed, extracted, and assayed for total SOD and protein content. The specific activity of SOD in the extracts is here graphed as a function of the concentration of the chelating agent in the medium.

RESULTS

Induction of SOD by chelating agents. Addition of progressively greater amounts of 8-HQ, EDTA, or *o*-phenanthroline caused first an increase and then a decrease in the SOD content of cells grown in their presence (Fig. 1). Similar effects were seen with α,α'-dipyridyl and DTPA (data not shown). These effects were due to the chelating properties of these compounds, since *m*-phenanthroline, which lacks chelating ability, had no effect (data not shown). With each of these chelating agents, the biphasic response was seen over a much narrower range of concentrations with VB medium than with TSY medium (Fig. 1). This may be due to the presence of a variety of competing chelating agents in the complex TSY medium. 8—HQ, and to an even greater extent *o*-phenanthroline, caused larger maximal increases in SOD content than did EDTA; moreover, higher than optimal levels of 8-HQ or *o*-phenanthroline eliminated the increase seen at the optimal levels, but did not suppress the SOD content below that seen in the absence of a chelating agent (Fig. 1A and C). In contrast, higher than optimal levels of EDTA markedly diminished net SOD content (Fig. 1B).

Induction by metals, chelating agents, and PQ. The effects of adding Fe(II), Mn(II), PQ, 8-HQ, and EDTA to the aerobic VB medium on the growth and SOD content of *E. coli* cells were explored. Some growth was seen in all cases (Fig. 2).

It is clear that Fe(II) and Mn(II) each modestly increased the SOD content and that their effects were additive when both were present. 8-HQ increased SOD content by itself and also increased the effect of Mn(II). The effects of 8-HQ and Fe(II) were additive. The combination of 8-HQ plus Mn(II) plus Fe(II) had a synergistic effect. PQ by itself caused only a very small induction, but it markedly increased the effect of Mn(II) and had a much smaller influence on the effect of Fe(II). Moreover, Fe(II) reduced induction by PQ plus Mn(II). PQ and 8-HQ showed no cooperation, and 8-HQ eliminated the cooperative response of Mn(II) plus PQ. PQ plus 8-HQ also acted to inhibit growth, as indicated by the low A_{600} attained when they were present. Impressive levels of SOD were achieved in cells grown in the combined presence of Fe(II), PQ, and 8-HQ and of Fe(II), Mn(II), PQ, and 8-HQ in spite of the growth-inhibiting effects of PQ and 8-HQ. EDTA, like 8-HQ, was

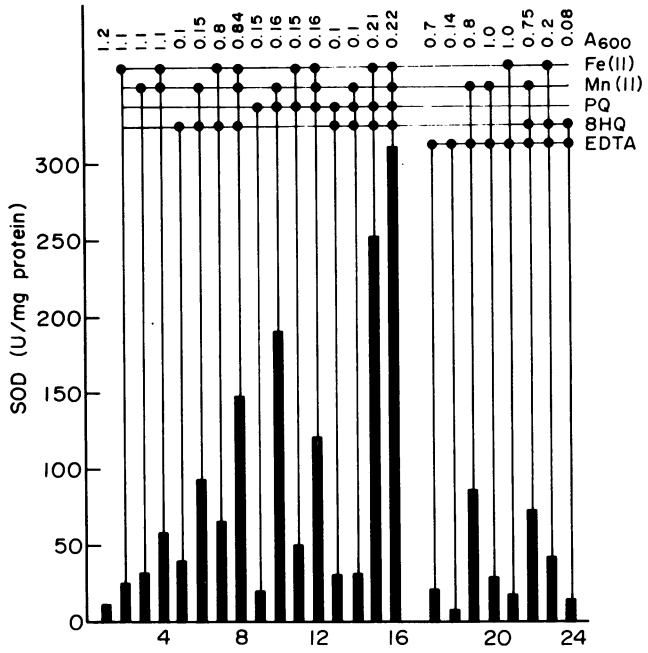

FIG. 2. Effect of Mn(II), Fe(II), PQ, 8-HQ, EDTA, and combinations thereof on SOD activity of *E. coli* B. Bacteria were grown in aerobic VB medium with the indicated additions, harvested, and assayed for SOD activity and protein content. The specific activity of SOD in the extracts is here given as a function of the substances added to the growth medium. The following concentrations were used: Fe(II), 100 μM; Mn(II), 100 μM (except bar 19, 1 μM); PQ, 10 μM; 8-HQ, 100 μM; and EDTA, 100 μM (except bars 17 and 19, 25 μM). The initial A_{600} was 0.05; the A_{600} values shown at the top were obtained after 16 h of incubation.

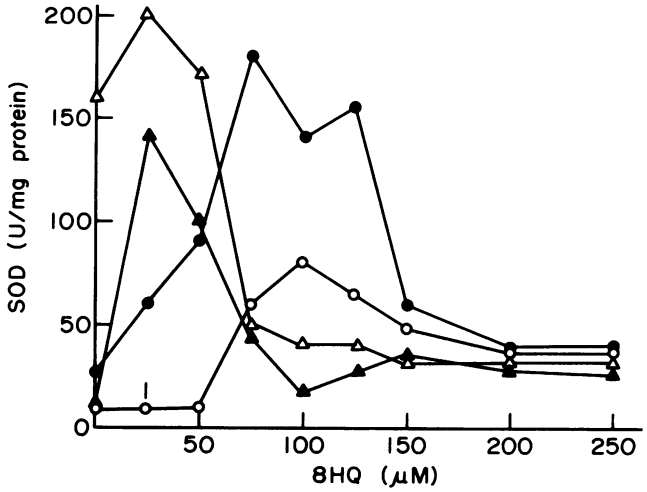

FIG. 3. Effect of PQ and Mn(II) on response of *E. coli* B SOD to 8-HQ. *E. coli* B cells were cultured in aerobic VB medium with various concentrations of 8-HQ and no other additions (○), 100 μM Mn(II) (●), 100 μM Mn(II) plus 10 μM PQ (△), or 10 μM PQ (▲). The specific SOD activities of cell extracts are here plotted as a function of the 8-HQ concentration.

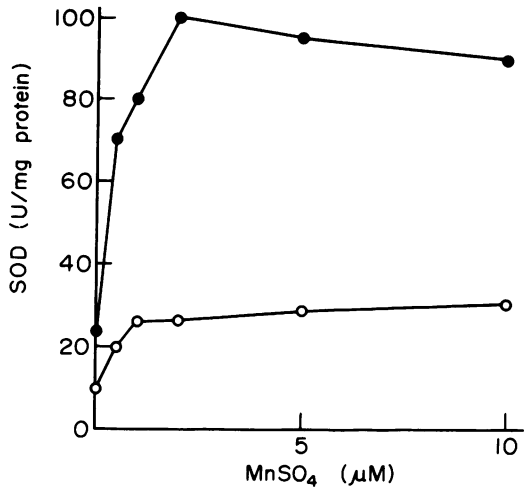

FIG. 4. Effect of PQ on SOD induction by Mn(II). *E. coli* B cells were grown in aerobic VB medium containing various concentrations of Mn(II) with (●) and without (○) 10 μM PQ. Specific SOD activity is here plotted as a function of the Mn(II) concentration in the medium.

able to enhance the effect of Mn(II), but was less effective than 8-HQ.

Effects of Mn(II) and PQ on induction by 8-HQ. Chelating agents added to the VB medium increased the SOD content of cells grown in that medium only within a narrow range of chelator concentrations. It appeared possible that the amount of chelator needed for optimal induction of SOD might be shifted by the presence of Mn(II), PQ, or both. A concentration of 8-HQ that provided optimal induction in the absence of Mn(II) and PQ might thus provide little induction in their presence. Such a shift of the optimum might provide an explanation for the actual suppression of induction seen when 100 μM 8-HQ was added in the presence of Mn(II) plus PQ (Fig. 2, bars 10 and 14).

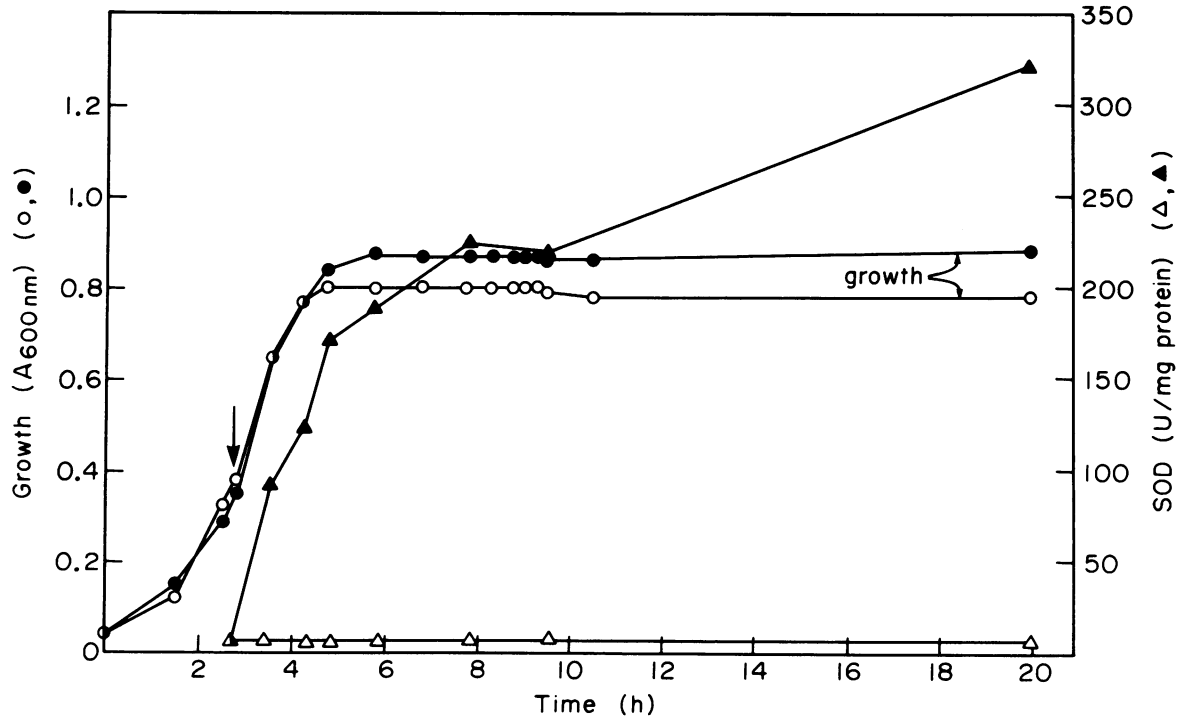

FIG. 5. Effect of Mn(II), Fe(II), 8-HQ, and PQ on growth and SOD content of *E. coli* B. Cells were grown aerobically in duplicate in VB medium. After 165 min of growth, 100 μM Mn(II), 100 μM Fe(II), 100 μM 8-HQ, and 10 μM PQ were added to one culture. Growth was monitored by absorbance, and samples were taken at intervals for extraction and assay of specific SOD activity. Symbols: ○, △, no additions to medium; ●, ▲, with additions (components were added at the time point indicated by the arrow.

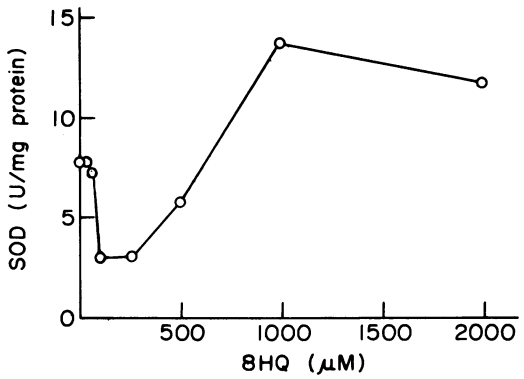

FIG. 6. Anaerobic effect of 8-HQ on SOD in *E. coli* B. Cells were harvested after 16 h of growth in anaerobic TSY medium containing the indicated concentrations of 8-HQ. The cells were then lysed, and the extracts were assayed for SOD activity and protein content.

The SOD content of *E. coli* cells grown in VB medium as a function of 8-HQ concentration was therefore explored several ways: in the absence of other components, with 100 μM Mn(II), with 100 μM Mn(II) plus 10 μM PQ, and with 10 μM PQ (Fig. 3). Optimal induction of SOD was seen at 100 μM 8-HQ in the absence of other components or when only Mn(II) was also added. In contrast, optimal induction was seen at 25 μM 8-HQ when PQ or PQ plus Mn(II) were present. In the latter cases, no induction was seen at 100 μM 8-HQ, which was the optimal concentration in the absence of PQ. Since 8-HQ was routinely used at 100 μM in the experiment shown in Fig. 2, we can now understand why it induced strongly when Mn(II) was present but not when PQ with or without Mn(II) was present in the medium.

Effect of PQ on induction of SOD by Mn(II). The interaction of PQ and 8-HQ, which resulted in a marked shift in the amount of 8-HQ needed for optimal induction of SOD (Fig. 3), suggested that a similar exploration be performed for PQ and Mn(II). Figure 4 shows the effect of Mn(II), added to VB medium, on the SOD content of *E. coli* cells with and without 10 μM PQ. We note that PQ was synergistic with Mn(II) in raising the SOD content, but did not noticeably shift the range of Mn(II) concentrations within which a response was seen.

Growth inhibition and SOD induction. The combination of Mn(II), Fe(II), 8-HQ, and PQ caused a large increase in the content of SOD in the cells while suppressing their growth (Fig. 2). The growth-inhibiting effect of the combination of these four additives could be avoided without decreasing the induction of SOD by adding them at mid-log phase rather than at the outset of growth (Fig. 5). Thus, cultures growing in VB showed the usual increase in A_{600} and no increase in SOD content. When Fe(II), Mn(II), PQ, and 8-HQ were all added at the same time to a mid-log-phase culture, it completed its growth cycle while showing a great increase in the amount of SOD. It is not yet clear why mid-log-phase cultures are immune to the growth-inhibiting effects of these additives. Changes in the composition of the medium and in the cells both need to be considered and will be explored in future work. For the present, the strategy of making additions at mid-log-phase will be very useful for isolating SODs in that it allows both very high specific SOD activity and high cell yield to be achieved simultaneously.

Anaerobic induction of MnSOD. Induction of total SOD in *E. coli* has always been thought to be dioxygen dependent. However, progressively higher concentrations of 8-HQ in anaerobic TSY medium caused first a decrease and then an increase in SOD content (Fig. 6). Furthermore, when these cell extracts were analyzed for their SOD isozyme content by polyacrylamide gel electrophoresis and activity staining, it became apparent that 8-HQ caused the anaerobic biosynthesis of active MnSOD (Fig. 7). This effect of 8-HQ was seen in both TSY and VB media. *o*-Phenanthroline was also able to induce anaerobic increases in the SOD content of *E. coli* (Fig. 8). In contrast, *m*-phenanthroline, which is not a chelating agent, was ineffective. EDTA and DTPA lowered the SOD content of anaerobically grown *E. coli* cells at all concentrations examined.

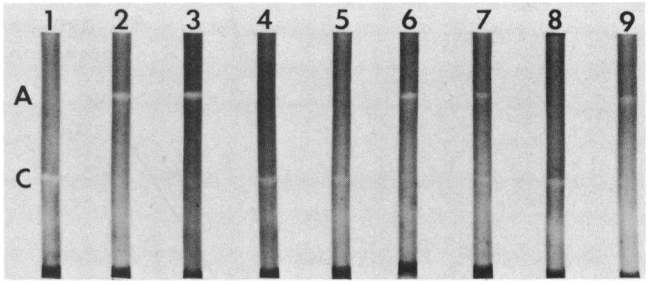

FIG. 7. Effect of Mn(II) and 8-HQ on SOD isozymes in anaerobically grown *E. coli* B cells. The bacteria were anaerobically grown in either TSY (gels 1 through 7) or VB (gels 8 and 9) medium modified as indicated below. Soluble extracts were prepared and analyzed for protein. Samples containing 50 μg of protein were applied to each gel, and after electrophoresis, the gels were stained for SOD activity. A, MnSOD band; C, FeSOD band. Gels: 1, no additions; 2, 500 μM 8-HQ; 3, 1,000 μM 8-HQ; 4, 100 μM Mn(II); 5, 100 μM Mn(II) plus 100 μM 8-HQ; 6, 100 μM Mn(II) plus 500 μM 8-HQ; 7, 100 μM Mn(II) plus 2,000 μM 8-HQ; 8, 100 μM 8-HQ; 9, 100 μM Mn(II) plus 100 μM 8-HQ.

Effects of dioxygen on enzyme induction and metal uptake. The effects of Fe(II), Mn(II), and 8-HQ on the SOD and metal content of *E. coli* cells grown in VB medium were examined aerobically and anaerobically (Table 1). It is clear that the induction of SOD was in all cases dioxygen dependent, whereas metal uptake was not. This was true for both the VB and TSY media.

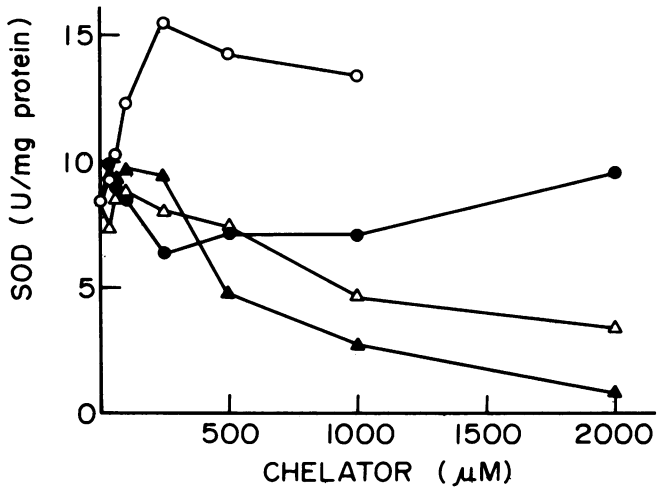

FIG. 8. Anaerobic effects of *o*-phenanthroline (○), *m*-phenanthroline (●), EDTA (△), and DPTA (▲). *E. coli* B cells were harvested after 16 h of growth in anaerobic TSY medium containing the indicated chelator. The bacteria were lysed, extracted, and assayed for SOD activity and protein content.

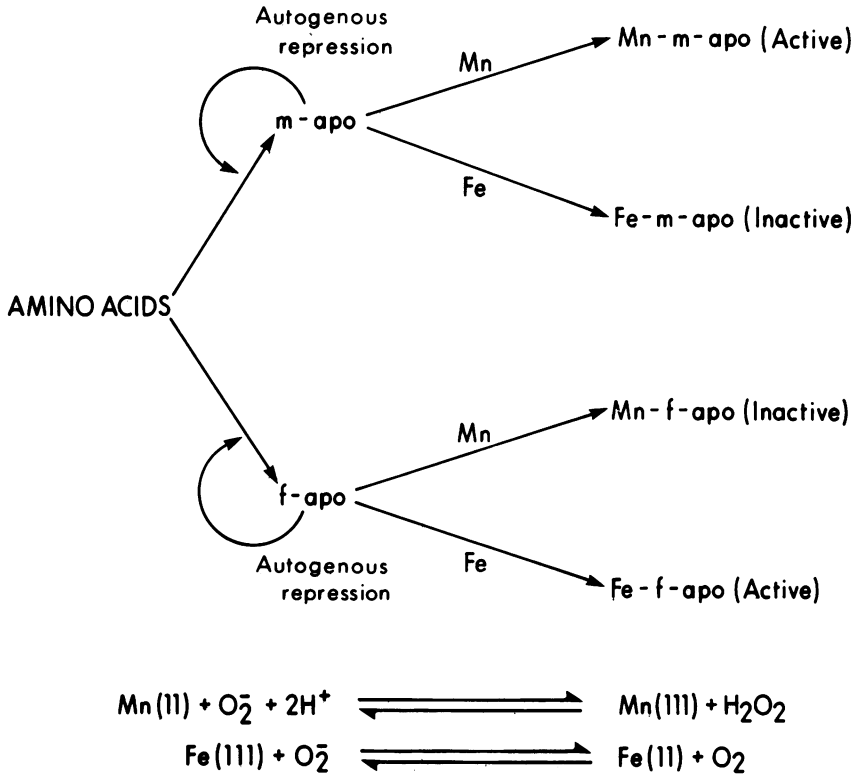

FIG. 9. Effects of metals on the biosynthesis of SODs—a proposal. Abbreviations: m-apo, apoprotein of MnSOD; f-apo, apoprotein of FeSOD. The valences of the metals are not shown. It is assumed that Fe(II) binds to both m-apo and f-apo more avidly than Mn(II), but not more avidly than Mn(III). The reactions illustrated at the bottom indicate why O_2^- increases the availability of Mn(III).

DISCUSSION

In a previous report (23), we sought to explain the induction of MnSOD by Mn(II) and of FeSOD by Fe(II) by supposing that apoSODs act as autogenous repressors, whereas holoSODs do not. Elevating the concentration of the appropriate metal speeds the conversion of apo- to holoenzymes, thus lowering the steady-state level of the apoSODs and reducing the degree of repression. We may now refine this hypothesis to accommodate the observed biphasic effect of chelating agents, the shift in the optimum concentration of chelating agent seen in the presence of PQ, the synergism seen between Mn(II) and PQ, and the anaerobic induction of MnSOD by chelating agents.

In vitro studies (5, 20–22) have shown that the *E. coli* apoMnSOD can bind either Mn(II) or Fe(II), but that only the former imparts catalytic activity. Moreover, O_2^- oxidizes Mn(II) to Mn(III) (2, 12, 15, 16, 29) but reduces Fe(III) to Fe(II) whether that iron is in cytochrome *c* (3), hemoglobin (27), or the EDTA complex (13). In accord with the proposal we will develop below, it has been shown that in *Staphylococcus aureus* manganese becomes oxidized to Mn(III) in aerobic cells and reduced to Mn(II) in anaerobic cells (7a). Competition between iron and manganese for the apoSODs and the effect of the valence state of the metals on that competition are key features of our proposal (Fig. 9).

Suppose that both apoMnSOD and apoFeSOD can bind either Mn(II) or Fe(II) and, as is the case in vitro, that Mn(II) specifically imparts activity to apoMnSOD and that Fe(II) does the same for apoFeSOD. Further suppose that Fe(II) shows greater affinity for both apoSODs than does Mn(II), but not than does Mn(III). Under anaerobic conditions, the cell would contain Fe(II) and Mn(II), and the former competes favorably for both apoSODs. It follows that only active FeSOD will be made anaerobically. When dioxygen is present, some O_2^- will be made, and O_2^- oxidizes Mn(II) to Mn(III) but reduces Fe(III) to Fe(II). Mn(III) will then compete favorably with Fe(II) for apoMnSOD, and active MnSOD will appear. If we increase the rate of O_2^- production through the action of PQ, more Mn(III) will be available and more MnSOD will be produced, as was observed.

Chelating agents which bind Fe(II) more tightly than Mn(II) or Mn(III) will diminish the competitive effect of Fe(II) and will thus increase the production of active MnSOD. When added at higher concentrations, the chelating agents will also bind Mn(II) and Mn(III) and make them unavailable to the apoMnSOD. The result will be augmented SOD production up to some optimal level of chelator and suppression of SOD production at still higher levels (Fig. 1). For a variety of ligands which contain N as the donor atom, iron is bound more tightly than is manganese (6). Thus, for 8-HQ the first-affinity constant for Fe(II) is 8.0, whereas for Mn(II) it is 6.8 (24). When PQ is present, O_2^- production is elevated and more of the manganese is present as Mn(III), which has a higher affinity for the apoMnSOD. Competition by Fe(II) is then less severe, and less of the chelating agent will be required to offset it. As a consequence, the optimal concentration of chelating agent is lower in the presence of PQ than in its absence (Fig. 3). By converting the low-affinity Mn(II) to the higher-affinity Mn(III), PQ should increase the ability of manganese to induce SOD and thus exert a synergistic effect with it (Fig. 2 and 4).

Our scheme suggests that MnSOD is not made anaerobically merely because Mn(II) cannot compete with Fe(II) for

TABLE 1. Synergism between Mn(II), Fe(II), and 8-HQ in VB medium[a]

Addition to medium	Amt (μM)	SOD (U/mg of protein)		Mn content (nmol/mg of protein)		Fe content (nmol/mg of protein)	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Mn(II)	0	10	9	0.06	0.06	1.76	6.5
	100	32	8	1.0	2.6	3.5	7.8
Fe(II)	500	39	8	0.19	0.05	10.0	9.3
8-HQ	100	43	4	0.3	0.19	6.8	7.1
	1,000	15	—[b]	0.18	—	1.4	—
Mn(II) + 8-HQ	100 + 100	96	3	1.0	1.6	1.6	2.9
Fe(II) + 8-HQ	500 + 1,000	100	8	0.19	0.17	—	—

[a] *E. coli* B cells were harvested after 16 h of growth in either aerobic or anaerobic VB medium.
[b] —, Not measured.

the apoMnSOD. It follows that chelating agents which may have a selective affinity for Fe(II) over Mn(II) should allow anaerobic production of MnSOD, and that was shown (Fig. 6 and 7). Moody and Hassan (19; Fed. Proc., 1984) have also observed the anaerobic induction of MnSOD by chelating agents and in addition have achieved the same result by growing cells in iron-poor medium. They postulate an iron-containing repressor protein, distinct from FeSOD, which is active as a repressor only when it contains iron. This is an interesting proposal which explains their observations but does not accommodate all of our results, such as induction by Mn(II) and the synergism between Mn(II) and chelating agents.

Chelating agents which make Fe(II) less available for competition with manganese for apoMnSOD will also make Fe(II) less available for combination with apoFeSOD. This effect was seen with EDTA, which induced MnSOD while virtually eliminating FeSOD and HySOD (data not shown). It will now be important to test the consequences, and thus the validity, of the hypothetical scheme shown in Fig. 9.

ACKNOWLEDGMENTS

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