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ANAEROBIC INDUCTIONS OF ACTIVE FORMS OF SUPEROXIDE DISMUTASES IN *ESCHERICHIA COLI*

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Escherichia coli growing anaerobically respond to NO_3^- with a ~3-fold induction of active FeSOD and a ~5.5-fold induction of an inactive, but activatable form of MnSOD (pro-MnSOD). Paraquat, which mediates anaerobic electron flow to NO_3^-, increased the induction of pro-MnSOD to ~25-fold. Strains with defects in the SOD genes or which lacked nitrate reductase activity failed to accumulate active or pro-forms of SODs in response to $NO_3^- \pm PQ^{++}$. Diamide caused anaerobic induction of *active* MnSOD and this effect was also observed in a glutathione-negative strain. These inductions required *de novo* synthesis of protein, even when cell content of pro-MnSOD had been elevated by exposure to $NO_3^- + PQ^{++}$ prior to addition of diamide.

These results indicate that oxidation of a cell component increases biosynthesis of the SOD gene product and this postulated oxidation can be caused by terminal electron acceptors, such as dioxygen or NO_3^-. In addition, it appears that insertion of the correct metal can be rate-limiting, leading to competition by other metals and to the accumulation of inactive, incorrectly substituted pro-forms. Metal insertion may be dependent upon the valence of the metal, which may be influenced, in turn, by the redox status of the cells. Diamide and redox active agents such as ferricyanide may thus allow anaerobic production of *active* MnSOD by favoring the production of a complexed form of Mn(III) which can compete favorably with other metal cations for the active site of nascent MnSOD.

KEY WORDS: Pro-MnSOD, anaerobic respiration, paraquat, reversible resolution, diamide.

Abbreviations: pro-MnSOD – enzymatically inactive form of MnSOD which is activated by treatment with Mn(II) under defined conditions; PQ^{++} – paraquat; GSH – reduced glutathione.

INTRODUCTION

Escherichia coli synthesizes two distinct homodimeric superoxide dismutases (SODs). One of these contains iron (FeSOD)[1] while the other contains manganese.[2] Anaerobically-grown cells contain only active FeSOD but oxygen causes induction of MnSOD and appearance of a hybrid of FeSOD and MnSOD (HySOD).[3] Anaerobically-grown *E. coli* also contain an inactive form of MnSOD which can be converted into the active enzyme by treatment with manganous salts under mildly denaturing conditions.[4] This inactive, but activatable form of MnSOD has been termed pro-MnSOD.[5] Pro-MnSOD is induced anaerobically by terminal electron sinks such as NO_3^- and this effect is dramatically increased by the simultaneous presence of the electron carrier paraquat.[4,5] Under anaerobic conditions, flow of electrons to sinks such as NO_3^-, facilitated by paraquat, is evidently sufficient to elicit biosynthesis of the MnSOD polypeptide.

The availability of strains of *E. coli* with defects in the sodA gene (MnSOD)[6] and of other strains carrying a multicopy plasmid bearing this gene[7] allowed exploration

of the effects of gene dosage on the production of pro-MnSOD. In addition, a strain of *E. coli* lacking glutathione (GSH)[8] allowed examination of the potential role of this thiol in regulation of MnSOD biosynthesis. The results reported here, together with other studies,[9-11] suggest that MnSOD biosynthesis is under multifactorial redox control.

MATERIALS AND METHODS

Bacterial Strains

E. coli B B_{12}^- was obtained from the American Type Culture Collection (ATCC 29682). *E. coli* K12 strain AB2463 (pDT1-5)[7] was a gift from Dr. Fred Archibald, Pulp and Paper Research Institute of Canada, Pointe Claire, Quebec, Canada. *E. coli* superoxide dismutase mutants QC781 (sodA), QC773 (sodB) and QC779 (sodAsodB) were generously provided by Dr. Daniele Touati, Institut Jacques Monod, Centre National de la Recherche Scientifique, Université Paris. The double mutant carries the mutations (sodA::MudPR13)25 and ø(sodB-kan)1-Δ2. The parental wild type strain was GC4468.[6] *E. coli* K12 strains AB1157 and JTG10 were supplied by Dr. Bruce Demple, Harvard University, Cambridge, Massachusetts.[8]

Growth Conditions

E. coli was grown under anaerobic conditions as previously described.[5]

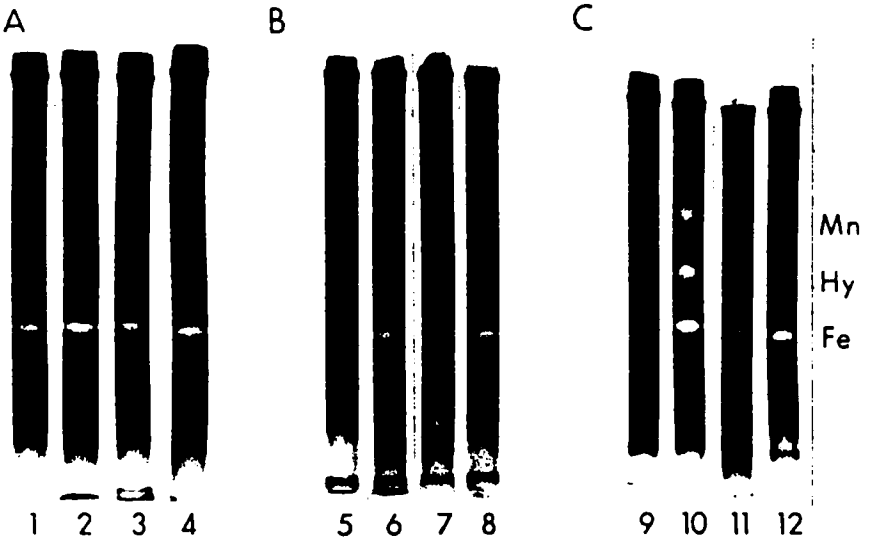

FIGURE 1 Effect of nitrate on anaerobic induction of MnSOD/HySOD in a strain of *E. coli* bearing a multicopy plasmid carrying the sodA gene. *E. coli* K12 cells were grown under anaerobic conditions at 37°C for 7 h in TSY medium (AB2463) or in TSY medium supplemented with 30 μg/ml ampicillin (AB2463 + pDT1-5). Soluble extracts were applied to polyacrylamide gels at 70 μg of protein/gel. After electrophoresis, the electropherograms were stained for SOD activity. A, AB2463; B, AB2463 + pDT1-5; C, AB2463 + pDT1-5, medium supplemented with 0.1 mM $MnCl_2$. Lanes 1, 5 and 9, control; lanes 2, 6 and 10, 50 mM NO_3^-; lanes 3, 7 and 11, 1.0 mM PQ^{++}; lanes 4, 8 and 12, 50 mM NO_3^- + 1.0 mM PQ^{++}.

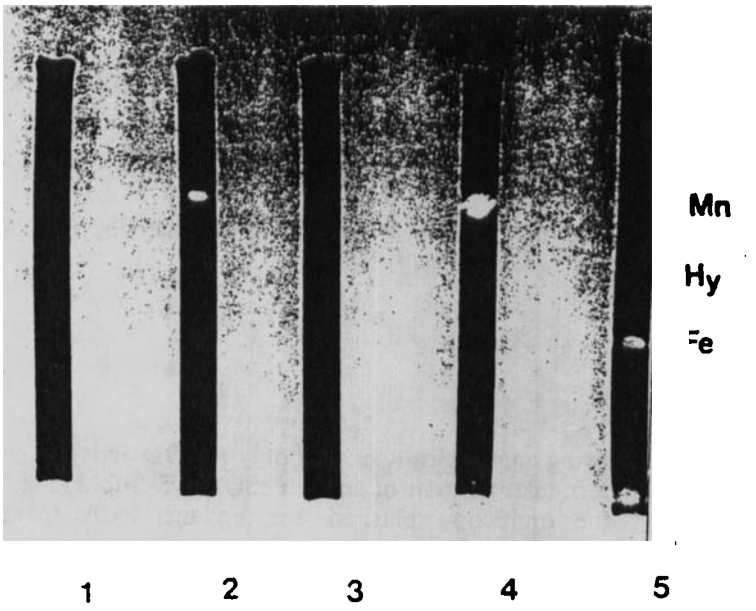

FIGURE 2 Reversible resolution and Mn(II) reconstitution. *E. coli* K12 cells were grown under anaerobic conditions in TSY medium for 5 h at 37°C. Soluble extracts were subjected to reversible resolution and Mn(II) reconstitution.[5] Reconstituted extracts were applied to polyacrylamide gels at 150 μg 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^{++}, lane 4, 50 mM NO_3^- + 1.0 mM PQ^{++}, lane 5, 50 mM NO_3^- + 1.0 mM PQ^{++}, native activity (extract not subjected to reversible resolution and reconstitution).

Enzyme Assays

Cells from 25 ml experimental cultures were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, washed once with 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.8, and resuspended in 1.0 ml of this buffer. Cells were lysed by two passages through a French pressure cell at 20,000 pounds/square inch. Lysates were 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 500 volumes of KP_i/EDTA buffer with one change of buffer. SOD was assayed by the xanthine oxidase/cytochrome *c* method.[12] Protein content was estimated colorimetrically with bovine serum albumin serving as the standard.[13] SOD electromorphs were separated by electrophoresis on 7% polyacrylamide gels[14] and bands of SOD activity were visualized by activity staining.[15] The relative amount of SOD isozymes was quantitated on gels by determination of peak areas of activity bands by linear scanning densitometry on a Zeineh model SL-504-XL laser densitometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli.[16] Soluble extracts were applied to 10% polyacrylamide slab gels (200 × 200 × 1.5 mm) and electrophoresis was carried out at 25 mA/slab. Western blot analysis was carried out by a modification of the method of Towbin *et al.*[17] Electrophoretic transfer of proteins from gels to nitrocellulose membranes (0.45 μm, Bio-Rad) was performed on a Multiphor II Nova Blot Transfer unit (LKB)

for 60 min at 25°C. Unbound sites were blocked by incubation with 3% gelatin in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 30 min at 25°C. Membranes were incubated overnight at 25°C with protein A-purified MnSOD antibody (~ 20 μg/ml) in 1% gelatin-TBS. Immunoreactive sites were visualized by incubation with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) (1:2000 dilution) in 1% gelatin-TBS for 2 h at 42°C, followed by color development with hydrogen peroxide and 4-chloro-1-naphthol.

Reversible resolution and Mn(II) reconstitution was carried out as previously described.[5]

RESULTS

Gene Dosage Effects

E. coli (AB2463) growing anaerobically exhibit only FeSOD activity and NO_3^- ± p-araquat increases the cellular content of active FeSOD 2.5–3-fold (Figure 1A). The strain containing the multicopy plasmid bearing the sodA (MnSOD) gene

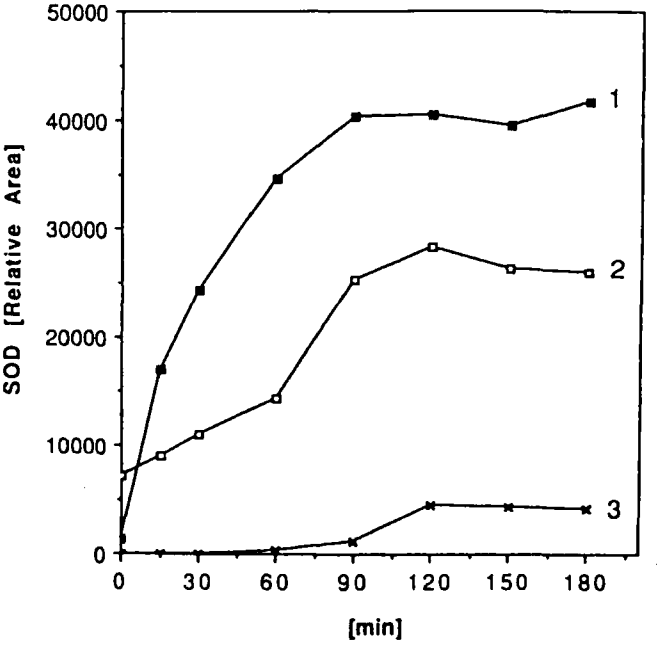

FIGURE 3 Kinetics of anaerobic induction of *E. coli* SODs by NO_3^- + PQ^{++}. *E. coli* B cells were grown under anaerobic conditions in 200 ml of TSY medium at 37°C. After 2 h (A_{600nm} = 0.26) an aliquot was removed (zero time) and the remaining medium was supplemented with anaerobically-equilibrated NO_3^- and PQ^{++}, to final concentrations of 50 mM and 1.0 mM, respectively. At indicated times, 20 ml aliquots were removed, placed on ice, and immediately harvested at 4°C. ProMnSOD samples were subjected to reversible resolution and Mn(II) reconstitution. Soluble extracts were applied to polyacrylamide gels at 80 μg of protein/gel. After electrophoresis, gels were stained for SOD activity. The relative amount of SOD isozymes was estimated by linear scanning densitometry. Line 1, proMnSOD; line 2, FeSOD; line 3, native MnSOD.

(AB2463 + pDT1-5) also shows induction of FeSOD in addition to appearance of the HySOD (Figure 1B, lane 6). The appearance of HySOD indicates that an increase in the MnSOD gene copy number is accompanied by increased production of the MnSOD polypeptide, while the absence of an active band of MnSOD indicates that the protein is inactive under these conditions. However, enrichment of the medium with Mn(II) resulted in accumulation of active MnSOD (Figure 1C), as previously reported.[4]

Cell extracts were subjected to reversible resolution which revealed an approximate 5.5-fold induction of pro-MnSOD by NO_3^- alone, and in the additional presence of paraquat, a 25-fold induction (Figure 2). A significant gene dosage effect on pro-MnSOD levels was also observed, as evidenced by the NO_3^--mediated 6-fold increase in pro-MnSOD levels in the plasmid-bearing strain compared to the parental strain (data not shown).

Kinetics of Induction of FeSOD and Pro-MnSOD

Pro-MnSOD accumulates rapidly after addition of an electron sink, as evidenced by a 13-fold increase in reconstitutable MnSOD activity following 15 min of anaerobic growth in the presence of NO_3^- plus paraquat (Figure 3). Active MnSOD was detectable only after a lag period of 60 min. FeSOD, like pro-MnSOD, was induced rapidly following anaerobic addition of NO_3^- plus paraquat. Western blot analysis demonstrated that the induction of pro-MnSOD was due to *de novo* synthesis of the

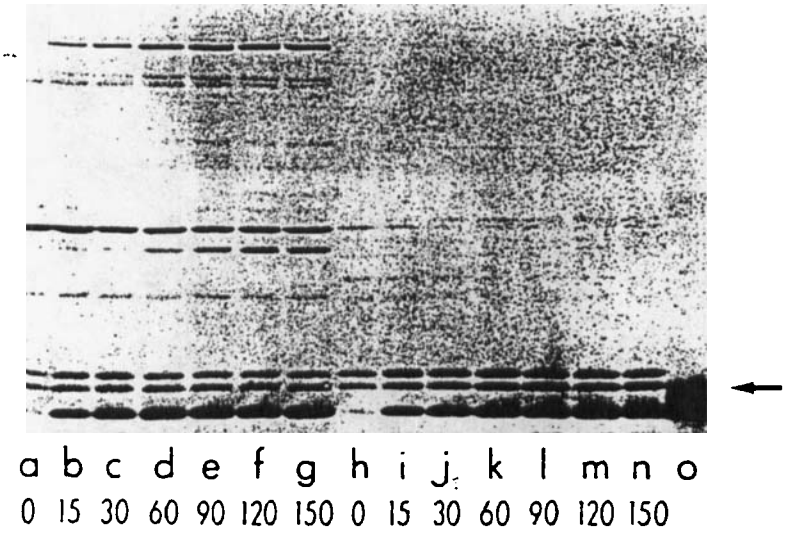

FIGURE 4 Western blot analysis illustrating the effect of NO_3^- + PQ^{++} on proMnSOD biosynthesis in *E. coli*. *E. coli* B cells were grown as described in Figure 3. Soluble extracts were applied to a 10% SDS-polyacrylamide slab gel and electrophoresis was carried out at 25 mA/slab. Western blot analysis was as described in "Materials and Methods." Lanes a–g, native extracts, 50 μg of protein/lane; lanes h–n, Mn(II)-reconstituted extracts, 25 μg of protein/lane; lane o, at the arrow, contains 0.2 μg of purified *E. coli* MnSOD. Values represent min following anaerobic exposure to 50 mM NO_3^- + 1.0 mM PQ^{++}, as described in Figure 3.

MnSOD polypeptide. The rapid appearance and subsequent accumulation of the MnSOD polypeptide is evident prior to and following reversible resolution (Figure 4). The 13-fold increase in reconstitutable MnSOD activity was in good agreement with the 15-fold increase in immunologically-detectable MnSOD polypeptide after 15 min of anaerobic induction with NO_3^- plus paraquat.

Effect of Defects in the sodA Gene on Pro-MnSOD

Pro-MnSOD appears to be a product of the sodA gene, based on cross-reactivity with rabbit anti-*E. coli* MnSOD and reconstitution and activation with Mn(II). This possibility was further examined through the use of mutants with defects in the SOD genes. As shown in Figure 5, anaerobic growth of *E. coli* in the presence of NO_3^- plus paraquat induced pro-MnSOD in the parental strains and in sodB strains (lanes 2 and 6); whereas strains with defects in the MnSOD gene (sodA and sodAsodB) failed to produce pro-MnSOD (lanes 4 and 8).

Effect of Diamide on Anerobic Induction of MnSOD

We have previously reported that glutathione (GSH) suppressed *in vitro* synthesis of *E. coli* MnSOD and that diamide, a glutathione oxidant, resulted in anaerobic induction of active MnSOD.[9] This raised the possibility that mutants lacking GSH[8]

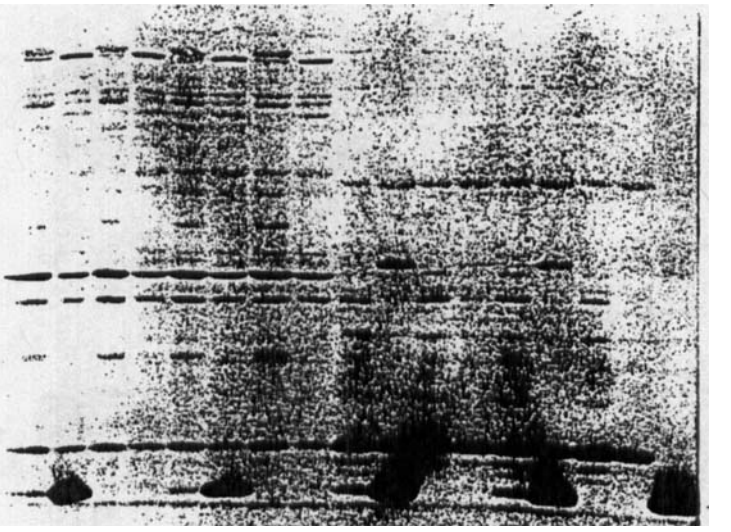

FIGURE 5 Western blot analysis of the effects of NO_3^- + PQ^{++} on proMnSOD biosynthesis in SOD mutants of *E. coli*. *E. coli* K12 cells were grown anaerobically in TSY medium for 5.25 h at 37°C. Soluble extracts, at 150 μg of protein/lane, were applied to a 10% SDS-polyacrylamide slab gel and electrophoresis was carried out at 25 mA/slab. Western blot analysis was as described in "Materials and Methods." Lanes 1, 2, 9 and 10, GC4468 (parent strain); lanes 3, 4, 11 and 12, QC781 (sodA); lanes 5, 6, 13 and 14, QC773 (sodB); lanes 7, 8, 15 and 16, QC779 (sodAsodB). Lanes 1–8, native extracts; lanes 9–16, Mn(II)-reconstituted extracts; lane 17, 0.2 μg of purified *E. coli* MnSOD at the arrow. Odd-numbered lanes, controls; even-numbered lanes, growth in the presence of 50 mM NO_3^- + 1.0 mM PQ^{++}.

might be anaerobically derepressed for MnSOD. As shown in Figure 6, diamide resulted in the induction of active MnSOD in both the parental strain (AB1157) and a strain lacking GSH (JTG10) (lanes 2 and 4). Cells lacking GSH were not constitutive for MnSOD biosynthesis (Figure 6, lane 3, and Western blot analysis, data not shown).

Anaerobic induction of *active* MnSOD by diamide suggested that in addition to increasing the biosynthesis of the MnSOD polypeptide this compound might also influence the valence state of the manganese, facilitating metal insertion under anaerobic conditions. As shown in Figure 7 (lane 6), diamide failed to cause the conversion of pro-MnSOD to active MnSOD when *de novo* protein synthesis was inhibited. This does not eliminate the possibility that diamide influences metal availability or valence state in a manner dependent upon protein synthesis, or that the anaerobically-synthesized MnSOD polypeptide rapidly acquires a metal other than manganese at the active site which cannot then be displaced *in vivo*.

DISCUSSION

Under anaerobic conditions *E. coli* contain active FeSOD and an enzymatically inactive MnSOD (pro-MnSOD), characterized by its conversion to active MnSOD under conditions devised for resolution and reconstitution of this enzyme and by its

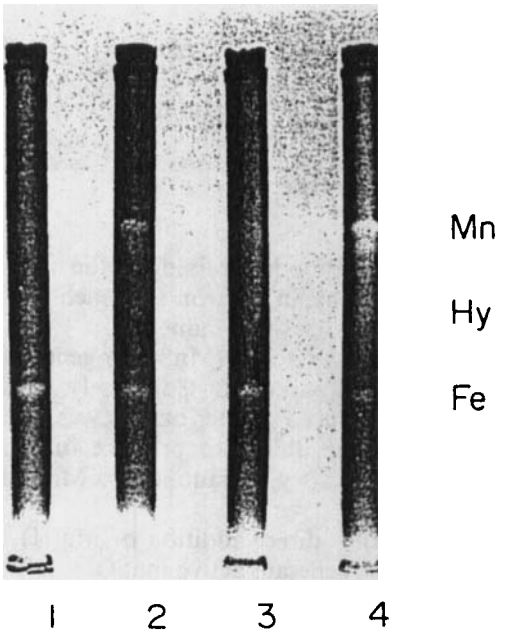

FIGURE 6 Effect of diamide on anaerobic induction of MnSOD on *E. coli*. *E. coli* K12 cells were grown under anaerobic conditions at 37°C for 1.75 h in TSY medium (AB1157) or in TSY medium supplemented with 25 μg/ml kanamycin (JTG10). Diamide (500 μm) was then added and cells were grown an additional 3.25 h. Soluble extracts were applied to polyacrylamide gels at 60 μg of protein/gel, and after electrophoresis, stained for SOD activity. Lanes 1 and 2, AB1157, without (1) and with (2) diamide; lanes 3 and 4, JTG10, without (3) and with (4) diamide.

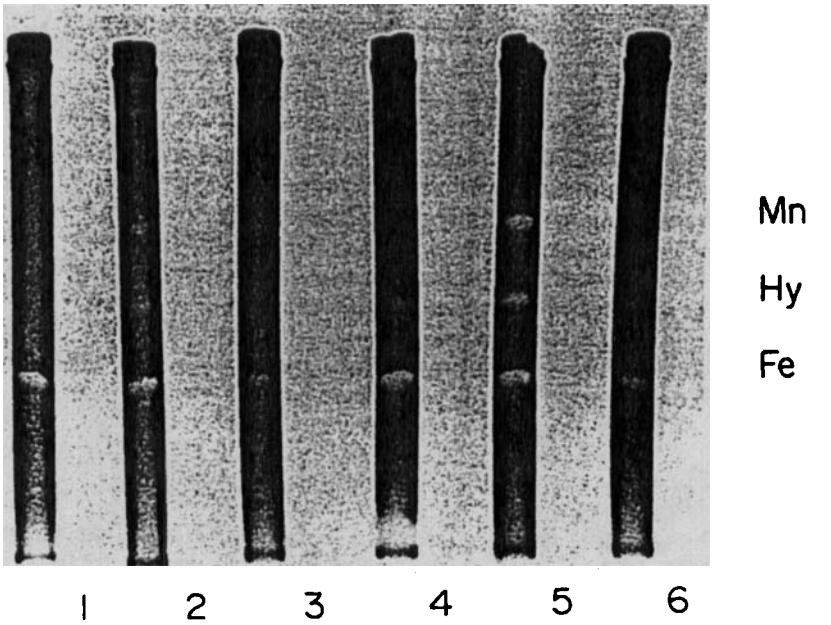

FIGURE 7 The effect of diamide on pro-MnSOD activation. *E. coli* B cells were grown anaerobically at 37°C for 2 h in TSY medium. Chloramphenicol (150 μg/ml) was added as indicated and cultures were incubated an additional 15 min at 37°C. Where indicated, cultures then received diamide (500 μM) and cells were incubated an additional 30 min. Soluble extracts (50 μg of protein/gel) were applied to polyacrylamide gels and stained for SOD activity. Lanes 1 and 4, no addition; lanes 2 and 5, 500 μM diamide; lanes 3 and 6, 150 μg/ml chloramphenicol + 500 μM diamide. Lanes 1–3, TSY medium, lanes 4–6, TSY medium supplemented with 50 mM KNO_3 + 1.0 mM PQ^{++}.

immunological reactivity with antibodies raised to the native polypeptide. Pro-MnSOD is anaerobically induced by an electron sink such as NO_3^- and the electron carrier paraquat dramatically increases this induction.

E. coli strains with defects in the sodA (MnSOD) gene failed to produce pro-MnSOD in response to NO_3^- plus paraquat. Conversely, strains bearing the sodA gene on a multicopy plasmid produced greater basal levels of pro-MnSOD than the parental strain while retaining the ability to produce further enhanced levels of pro-MnSOD in the presence of NO_3^- ± paraquat. Pro-MnSOD thus appears to be a product of the sodA gene.

We have previously shown that direct addition of Mn(II) to extracts of *E. coli* containing pro-MnSOD did not generate active mnSOD unless acidic guanidinium chloride and a chelating agent were first used to remove endogenous metals.[5] Similarly, apo-MnSOD prepared by dialysis against acidic guanidinium chloride, followed by dialysis against neutral Tris buffer, could not be reactivated by simple addition of Mn(II) unless the apo-MnSOD was prepared with buffers whose trace metal content had been reduced by treatment with Chelex 100.[5] This indicates that the apoenzyme readily binds trace metals from the buffer, rendering it unresponsive to the subsequent additon of Mn(II). These findings, coupled with the demonstration that enrichment of the growth medium with Mn(II) allowed anaerobic induction of *active* MnSOD by

NO_3^- plus paraquat,[4,5] support the contention that pro-MnSOD is MnSOD with a metal other than manganese at the active site.

The anaerobic induction of pro-MnSOD by NO_3^- and enhancement thereof by paraquat, in conjunction with other studies,[9-11] suggests a role for cellular redox status in the regulation of MnSOD biosynthesis in *E. coli*. Relevant to this supposition is our observation that potassium ferricyanide results in the induction of active MnSOD under anaerobic conditions.[4] We have previously reported that GSH, at concentrations similar to those found within *E. coli*,[18] suppressed transcription of the MnSOD gene *in vitro*.[9] In addition, diamide, a GSH oxidizing agent, caused anaerobic induction of active MnSOD in *E. coli*, suggesting a role for this thiol in MnSOD regulation. However, mutants which lack GSH fail to synthesize MnSOD under anaerobic conditions but MnSOD is induced by diamide in these mutants, suggesting that intracellular GSH levels *per se* do not operate as a regulatory component(s). Diamide, however, has been shown to interact with cellular components other than GSH and therefore lacks specificity.[19,20] It is possible that diamide effectively lowers the NADPH/$NADP^+$ ratio by oxidizing GSH and other thiols, such as thioredoxin and glutaredoxin, and thereby affects the redox status of the cell. This redox state change may then allow biosynthesis of the MnSOD polypeptide. Factors which influence the activation of the nascent polypeptide, such as metal availability and valence state, also appear to be responsive to redox status of the cell.

Acknowledgements

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