ANAEROBIC INDUCTION OF SUPEROXIDE DISMUTASE IN *ESCHERICHIA COLI* BY NITRATE RESPIRATION

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Previously, we demonstrated that nitrate respiration increases the synthesis of iron-containing superoxide dismutase (Fe SOD) in anaerobically grown *Escherichia coli* cells. In this study, we developed a system with which the anaerobic induction of Fe SOD by nitrate respiration in *E. coli* was separated from the induction of the nitrate respiration system. The anaerobic induction of Fe SOD required the presence of both nitrate and an electron donor, and the induction of Fe SOD by nitrate respiration was inhibited by chloramphenicol or rifampicin. When glucose was used as the electron donor, carbonyl cyanide *p*-trifluoro-methoxyphenyl-hydrazone (FCCP) but not *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibited the induction of Fe SOD by nitrate respiration, but when formate was used as the donor, both FCCP and DCCD inhibited the induction of Fe SOD by nitrate respiration.

Superoxide dismutase (SOD), which catalyzes dismutation of the superoxide anion radical (O_2^-) into O_2 and H_2O_2 , is generally regarded as protecting the cells from oxygen toxicity.

Escherichia coli has three types of SOD: iron-containing SOD (Fe SOD) (1), manganese-containing SOD (Mn SOD) (2), and a SOD with a protein moiety, this is a hybrid of Fe SOD and Mn SOD (hybrid SOD) (3).

We have shown that *E. coli* cells, grown anaerobically by fermentation, had a low level of SOD and contained Fe SOD only (4). We also showed that *E. coli* cells, grown anaerobically by anaerobic respiration with an electron acceptor such as nitrate, fumarate, dimethyl sulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO), contained increased levels of Fe SOD (5). In addition, it was reported that when *E.*

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coli cells were grown anaerobically by nitrate respiration, Mn SOD and hybrid SOD were also synthesized (4-6). Recently SCHIAVONE and HASSAN reported that Mn SOD was induced under anaerobic condition when *E. coli* cells were grown in the presence of potassium ferricyanide, copper-cyanide complex, ammonium persulfate or hydrogen peroxide (7). On the other hand, PRIVALLE and FRIDOVICH showed that anaerobically grown *E. coli* cells had an inactive form of Mn SOD and that the addition of a terminal electron acceptor, such as nitrate or TMAO, increased the amount of inactive Mn SOD in anaerobically grown *E. coli* (8).

Generally speaking, growing cells are not useful for the study of the control mechanism in enzyme synthesis, since selection of the cells with high enzyme may occur during the growth. Therefore, we intended to develop a system with which anaerobic induction of SOD by nitrate respiration is possible with little accompanying cell growth. We also attempted to separate the induction of SOD from the induction of nitrate respiration in *E. coli* cells. After establishment of the system, we examined the effects of several factors on the anaerobic induction of SOD by nitrate respiration.

MATERIALS AND METHODS

Bacteria and growth conditions. *Escherichia coli* K12 W1895 (Hfr, *met*) was used in this study.

The composition of the peptone-glucose medium (PSG medium) and the procedure of anaerobic culture were as described previously (4). Cell concentrations were measured in a cuvette with a 10-mm light path using a Hitachi EPO B photometer at 660 nm, and expressed by absorbance as A_{660} . An A_{660} value of 1.0 corresponds to 1.6 mg of cell protein per ml.

Short term incubation of cell suspensions. The cells grown anaerobically in PSG medium with 40 mM KNO_3 for 2 h were harvested by centrifugation and suspended in 40 ml of an incubation mixture containing 0.25% casamino acids, 10 μM FeCl_3 , 10 μM MnSO_4 , 0.1 M potassium phosphate buffer (pH 7.2) and other additions such as nitrate. The suspension was added to a 100-ml Sakaguchi-flask, then the gas phase of the flask was changed to pure N_2 (O_2 less than 0.01%). The suspension was incubated for 4 h at 30°C with shaking on a reciprocating shaker at 120 strokes per min with an amplitude of 5 cm.

Assays. Cell-free extracts were prepared by sonic disruption as described previously (4).

SOD activity was estimated by the method of MCCORD and FRIDOVICH (9) with a slight modification (4). One unit of SOD activity is defined as the amount of enzyme which produces 50% inhibition of cytochrome *c* reduction. SOD activity was calculated from the results of more than three independent experiments. SOD isozymes were separated by electrophoresis on 7.5% polyacrylamide gels, then the gels were stained by the method of BEAUCHAMP and FRIDOVICH (10).

Nitrate reductase (NaR) activity, with methyl viologen (MV) as an electron

acceptor, was assayed by the method of GAUTHIER et al.(11) with a slight modification. The assay was carried out in an open tube containing 20 μmol of KNO_3 , 0.1 μmol of methyl viologen, 19 μmol of NaHCO_3 , 9 μmol of sodium dithionite and 100 μmol of potassium phosphate buffer (pH 7.2) in a final volume of 1 ml. The reaction was started by adding a freshly prepared solution of sodium dithionite in NaHCO_3 , and the reaction mixture was incubated for 10 min at 30°C. One unit of NaR activity was defined as the amount of enzyme that produces 1 μmol of nitrite in 10 min. Nitrite was measured by the diazo-coupling method as described previously (4).

Protein was measured by the biuret reaction according to the method of ITZHAKI and GILL (12).

RESULTS

Effect of nitrate on anaerobic SOD synthesis in growing cells

We reported that when *E. coli* cells were cultured anaerobically for 18 h, cellular SOD activity increased about 4-fold by the operation of nitrate respiration(4). In that study, we did not examine the kinetics of cell growth or enzyme synthesis. In the present study, the cells, grown anaerobically for 18 h by fermentation, were harvested, inoculated in the fresh medium and cultured anaerobically for 4 h by nitrate respiration. The initial cell density of the fresh suspension was about 25% of the cell density level of the preculture. Figure 1 shows the kinetics of cell growth and changes in SOD or MV-NaR activities during the anaerobic culture by nitrate respiration. MV-NaR activity increased with little lag and after 2 h the activity reached almost the same level as the cells grown

Fig. 1. Kinetics of anaerobic synthesis of enzymes in *E. coli*.

Cells cultured anaerobically for 18 h without KNO_3 were harvested, suspended in fresh medium and cultured anaerobically for 4 h with 40 mM KNO_3 . Growth ($\square$), MV-NaR activity ($\bullet$) and SOD activity ($\circ$).

anaerobically for 18 h by nitrate respiration. During the culture, SOD activity started to increase after a 2 h lag and after growth for 4 h the SOD activity reached only 35% of the cells grown anaerobically for 18 h by nitrate respiration. In the cells grown anaerobically for 4 h by nitrate respiration, Fe SOD was detected but Mn SOD and hybrid SOD were not detected by gel electrophoresis (data not shown).

Induction of SOD separated from the induction of nitrate respiration system

We attempted to develop a system with which SOD is induced independently of the induction of the nitrate respiration system. The results in Fig. 1 show that when cells were cultured anaerobically for 2 h with 40 mM KNO_3 , MV-NaR is almost fully induced but SOD remained at the original level. Therefore, cells, cultured anaerobically for 2 h with 40 mM KNO_3 and already contained a high level of MV-NaR, were harvested, washed and suspended in a mixture containing 0.25% casamino acids, 10 μM FeCl_3 , 10 μM MnSO_4 and 0.1 M potassium phosphate buffer (pH 7.2), then incubated anaerobically for 4 h with or without additions such as nitrate or electron donors. The results in Fig. 2 show that when the cells were incubated anaerobically with 40 mM KNO_3 and 40 mM formate, only SOD was induced without alteration of the level of MV-NaR activity. Moreover, the level of the nitrate respiration system, from formate to nitrate, was not affected by chloramphenicol during the incubation,

Fig. 2. Nitrite accumulation and SOD activity in resting cells of *E. coli*.

Cells, cultured anaerobically for 18 h without KNO_3 , were inoculated in the fresh medium containing 40 mM KNO_3 and cultured anaerobically for 2 h. Then cells were harvested, washed once with 20 mM potassium phosphate buffer (pH 7.2), then suspended in the incubation mixture and incubated anaerobically for 4 h. The composition of the incubation mixture was 40 mM KNO_3 , 40 mM formate, 10 μM FeCl_3 , 10 μM MnSO_4 , 0.25% casamino acids and 0.1 M potassium phosphate buffer (pH 7.2).

A: Nitrite accumulation in the mixture during the incubation of cells without chloramphenicol ($\circ$) or with 200 $\mu\text{g}/\text{ml}$ chloramphenicol ($\bullet$). MV-NaR activity of the cells incubated without chloramphenicol ($\square$). B: SOD activity of cells incubated without chloramphenicol ($\circ$) or with 200 $\mu\text{g}/\text{ml}$ chloramphenicol ($\bullet$).

Table 1. Effect of chloramphenicol or rifampicin on anaerobic induction of SOD in *E. coli* by nitrate respiration.

Additions to incubation mixture ^a		Cell density (<i>A</i> ₆₆₀)	SOD activity (unit/mg protein)
Electron donor	Inhibitor		
Formate (40 mM)	None	0.321	11.8 (2.2) ^b
Formate (40 mM)	Chloramphenicol (150 µg/ml)	0.216	6.9 (1.0)
Formate (40 mM)	Rifampicin (50 µg/ml)	0.177	7.3 (0.9)
Glucose (10 mM)	None	0.452	12.2 (1.9)
Glucose (10 mM)	Chloramphenicol (150 µg/ml)	0.211	5.9 (0.6)
Glucose (10 mM)	Rifampicin (50 µg/ml)	0.149	7.0 (0.6)
Before incubation		0.257	6.3 (0.6)

Cells were incubated as described in the legend to Fig. 2.

^a The composition of the incubation mixture was 40 mM KNO₃, 10 µM FeCl₃, 10 µM MnSO₄, 0.25% casamino acids and 0.1 M potassium phosphate buffer (pH 7.2).

^b Standard deviation.

Table 2. Anaerobic induction of SOD in *E. coli* by nitrate respiration.

Additions to incubation mixture ^a		Cell density (<i>A</i> ₆₆₀)	SOD activity (unit/mg protein)
Electron donor	Electron acceptor		
Formate (40 mM)	None	0.210	4.5 (1.0) ^b
DL-Lactate (40 mM)	None	0.215	4.3 (1.2)
Glycerol (40 mM)	None	0.197	5.7 (1.3)
Glucose (10 mM)	None	0.420	4.7 (1.0)
None	KNO ₃ (40 mM)	0.215	4.8 (1.0)
Formate (40 mM)	KNO ₃ (40 mM)	0.317	7.7 (1.8)
DL-Lactate (40 mM)	KNO ₃ (40 mM)	0.239	5.5 (1.3)
Glycerol (40 mM)	KNO ₃ (40 mM)	0.338	8.8 (1.0)
Glucose (10 mM)	KNO ₃ (40 mM)	0.524	8.0 (1.3)
Before incubation		0.266	4.4 (0.9)

Cells were incubated as described in the legend to Fig. 2.

^a The composition of the incubation mixture was 0.25% casamino acids, 10 µM FeCl₃, 10 µM MnSO₄ and 0.1 M potassium phosphate buffer (pH 7.2).

^b Standard deviation.

as shown by the fact that the inhibitor did not affect the rate of nitrite accumulation from nitrate and formate.

By using the method of incubation as shown in Fig. 2, it was possible to examine the effect of several factors on the synthesis of SOD by nitrate respiration. The results in Fig. 2B and Table 1 show that adding chloramphenicol or adding rifampicin inhibited SOD induction by nitrate respiration. The results in Table 2 show that both nitrate and some electron donor were required to induce SOD: among the donors tested, glucose, glycerol and formate were equally effective. In the

Table 3. Effect of paraquat on anaerobic induction of SOD in *E. coli* by nitrate respiration.

Additions to incubation mixture ^a			Cell density (<i>A</i> ₆₆₀)	SOD activity (unit/mg protein)
Electron donor	Electron acceptor	Paraquat (mM)		
None	None	0.5	0.188	3.9 (0.8) ^b
Formate (40 mM)	None	0.5	0.180	4.0 (0.8)
Glucose (10 mM)	None	0.5	0.431	4.4 (0.4)
None	KNO ₃ (40 mM)	0.5	0.212	4.1 (1.1)
Formate (40 mM)	KNO ₃ (40 mM)	0.0	0.308	7.5 (1.9)
Formate (40 mM)	KNO ₃ (40 mM)	0.5	0.271	6.3 (1.5)
Glucose (10 mM)	KNO ₃ (40 mM)	0.0	0.507	8.9 (2.0)
Glucose (10 mM)	KNO ₃ (40 mM)	0.5	0.482	8.0 (1.7)
Before incubation			0.268	4.1 (1.0)

Cells were incubated as described in the legend to Fig. 2.

^a The composition of the incubation mixture was 0.25% casamino acids, 10 μ M FeCl₃, 10 μ M MnSO₄ and 0.1 M potassium phosphate buffer (pH 7.2).

^b Standard deviation.

Table 4. Effect of DCCD or FCCP on anaerobic induction of SOD in *E. coli* by nitrate respiration.

Additions to incubation mixture ^a		Cell density (<i>A</i> ₆₆₀)	SOD activity (unit/mg protein)
Electron donor	Inhibitor		
Formate (40 mM)	None	0.335	9.6 (0.8) ^b
Formate (40 mM)	DCCD (100 μ M)	0.181	5.4 (0.8)
Formate (40 mM)	FCCP (5 μ M)	0.143	5.1 (0.9)
Glucose (10 mM)	None	0.402	8.7 (0.9)
Glucose (10 mM)	DCCD (100 μ M)	0.238	10.0 (1.2)
Glucose (10 mM)	FCCP (5 μ M)	0.184	5.7 (0.9)
Before incubation		0.270	5.6 (0.7)

Cells were incubated as described in the legend to Fig. 2.

^a The composition of the incubation mixture was 40 mM KNO₃, 10 μ M FeCl₃, 10 μ M MnSO₄, 0.5% ethanol, 0.25% casamino acids and 0.1 M potassium phosphate buffer (pH 7.2). DCCD or FCCP was added as ethanol solutions.

^b Standard deviation.

system, only Fe SOD was synthesized but no Mn SOD or hybrid SOD (Fig. 3B, C).

Effect of paraquat on anaerobic SOD synthesis

Aerobic synthesis of Mn SOD and hybrid SOD was reported to be stimulated by adding paraquat (13). Therefore, the effect of paraquat on the anaerobic Fe SOD induction by nitrate respiration was examined. The results shown in Table 3

Fig. 3. SOD isozyme pattern in resting cells of *E. coli*.

Cells, cultured anaerobically for 2 h with 40 mM KNO_3 , were harvested and washed as shown in the legend to Fig. 2. The cells were suspended in an incubation mixture containing 0.25% casamino acid, $10 \mu\text{M}$ FeCl_3 , $10 \mu\text{M}$ MnSO_4 and 0.1 M potassium phosphate buffer (pH 7.2) and incubated anaerobically for 4 h with the following additions: B, 40 mM KNO_3 and 40 mM formate; C, 40 mM KNO_3 and 10 mM glucose; D, 10 mM glucose and 0.5 mM 8-HQ; E, 40 mM KNO_3 , 40 mM formate and 0.5 mM 8-HQ; F, 40 mM KNO_3 , 10 mM glucose and 0.5 mM 8-HQ. Gel A was before incubation.

Cells were disrupted and the SOD isozyme pattern was detected by gel electrophoresis.

indicate that adding paraquat had no effect on Fe SOD induction by nitrate respiration.

Effect of DCCD or FCCP on anaerobic SOD induction by nitrate respiration

Nitrate respiration in *E. coli* generates a transmembrane proton motive force, and ATP is synthesized by proton-translocating adenosine triphosphatase (H^+ -ATPase) (14). Carbonyl cyanide *p*-trifluoro-methoxyphenyl-hydrazone (FCCP) is an uncoupler of oxidative phosphorylation and *N,N'*-dicyclohexylcarbodiimide (DCCD) binds to a specific protein moiety of H^+ -ATPase. In the present study, the effects of these inhibitors on anaerobic induction of Fe SOD by nitrate respiration were studied. When glucose was used as the electron donor, FCCP but not DCCD inhibited the anaerobic Fe SOD induction by nitrate respiration. When formate was used as the donor, not only FCCP but also DCCD inhibited the anaerobic Fe SOD induction by nitrate respiration (Table 4).

Effect of iron chelators on anaerobic SOD synthesis

MOODY and HASSAN (15), PUGH and FRIDOVICH (16) reported that removal of

Table 5. Effect of 8-HQ on anaerobic induction of SOD in *E. coli* by nitrate respiration.

Additions to incubation mixture ^a			Cell density (A_{660})	SOD activity (unit/mg protein)
Electron donor	Electron acceptor	8-HQ (mM)		
Glucose (10 mM)	None	0.0	0.366	5.1 (0.5) ^b
Glucose (10 mM)	None	0.5	0.260	2.7 (0.8)
Formate (40 mM)	KNO ₃ (40 mM)	0.0	0.325	9.3 (1.1)
Formate (40 mM)	KNO ₃ (40 mM)	0.5	0.244	6.4 (0.8)
Glucose (10 mM)	KNO ₃ (40 mM)	0.0	0.393	8.5 (1.1)
Glucose (10 mM)	KNO ₃ (40 mM)	0.5	0.306	28.5 (4.8)
Before incubation			0.271	5.2 (0.7)

Cells were incubated as described in the legend to Fig. 2.

^a The composition of the incubation mixture was 0.5% ethanol, 0.25% casamino acids, 10 μ M FeCl₃, 10 μ M MnSO₄ and 0.1 M potassium phosphate buffer (pH 7.2). 8-HQ was added as ethanol solutions.

^b Standard deviation.

iron from culture media induced the synthesis of Mn SOD under anaerobic conditions. Therefore, the effect of iron chelators on anaerobic induction of SOD by nitrate respiration was examined. Adding 8-hydroxyquinoline (8-HQ) induced anaerobic Mn SOD synthesis even when cells were incubated in the absence of nitrate (Fig. 3D). Nitrate respiration further stimulated anaerobic Mn SOD synthesis by 8-HQ, while the amount of Fe SOD decreased by 8-HQ even with nitrate respiration (Fig. 3E, F). Only when cells were incubated with glucose and nitrate, the total level of SOD increased by 8-HQ (Table 5).

DISCUSSION

The results shown in Figs. 2 and 3 show that Fe SOD was increased by nitrate respiration without alteration of the nitrate respiration system. During the incubation of cell suspensions by using formate as the electron donor for nitrate respiration, the increase of cell density was only 25% of the original density, while the specific activity of SOD increased to about twice the original activity. A twofold increase of cell density occurred when glucose is used as the electron donor. We are developing a system, in which glucose is used as the electron donor but the increase of cell density is less than 15% of the original density; with this system, SOD activity was increased about twofold by nitrate respiration (data not shown).

The anaerobic induction of Fe SOD required both nitrate and an electron donor (Table 2). Chloramphenicol or riphampicin inhibited the Fe SOD induction by nitrate respiration (Table 1). These results show that nitrate respiration induce de novo synthesis of Fe SOD.

Anaerobic Mn SOD synthesis by adding an iron chelator occurred even when

cells were incubated with glucose alone (Fig. 3D), but the anaerobic Mn SOD synthesis by adding iron chelator was further stimulated by nitrate respiration (Fig. 3E, F). Therefore, the anaerobic Mn SOD synthesis by iron chelator is partially dependent on nitrate respiration.

The results in Table 4 show that FCCP inhibited Fe SOD induction by nitrate respiration whether the electron donor was formate or glucose. When formate was used as the electron donor in nitrate respiration, that is, when ATP was generated only through the electron transport system, DCCD inhibited anaerobic Fe SOD induction, but when glucose was used as the donor, that is, when ATP was generated by both fermentative and electron transport systems, DCCD did not inhibit the anaerobic Fe SOD induction. These results indicate that both ATP and proton motive force are required to induce Fe SOD by nitrate respiration.

PUGH et al. reported that the enrichment of the medium with ferrous ion elevated the Fe SOD in aerobically grown *E. coli* (17). LODGE and EMERY reported that anaerobic ferrienterobactin transport in *E. coli* cells, grown anaerobically with nitrate, was inhibited by KCN or by dinitrophenol (18).

These results indicate that the uptake of iron is stimulated by the operation of nitrate respiration. If so, the relationship between intracellular Fe ion and the induction of Fe SOD by nitrate respiration is not yet clear. Some models to explain the regulation of anaerobic Mn SOD synthesis were proposed (7, 15, 16, 19), but a model to explain the regulation of anaerobic Fe SOD synthesis has not been proposed. More studies are necessary to elucidate the mechanism of Fe SOD synthesis by nitrate respiration.

REFERENCES

- 1) F. J. YOST, Jr. and I. FRIDOVICH, *J. Biol. Chem.*, **248**, 4905 (1973).
- 2) B. B. KEELE, Jr., J. M. MCCORD, and I. FRIDOVICH, *J. Biol. Chem.*, **245**, 6176 (1970).
- 3) H. W. DOWGHERTY, S. J. SADOWSKI, and E. BAKER, *J. Biol. Chem.*, **253**, 5220 (1978).
- 4) K. MIYAKE, *J. Gen. Appl. Microbiol.*, **32**, 527 (1986).
- 5) K. MIYAKE and S. HINO, *J. Gen. Appl. Microbiol.*, **34**, 105 (1988).
- 6) H. M. HASSAN and C. S. MOODY, *J. Biol. Chem.*, **262**, 17173 (1987).
- 7) J. R. SCHIAVONE and H. M. HASSAN, *J. Biol. Chem.*, **263**, 4269 (1988).
- 8) C. T. PRIVALLE and I. FRIDOVICH, *J. Biol. Chem.*, **263**, 4274 (1988).
- 9) J. M. MCCORD and I. FRIDOVICH, *J. Biol. Chem.*, **244**, 6049 (1969).
- 10) C. BEAUCHAMP and I. FRIDOVICH, *Anal. Biochem.*, **44**, 276 (1971).
- 11) D. K. GAUTHIER, G. D. CLARK-WAKER, W. T. GARRARD, Jr., and J. LASCELLES, *J. Bacteriol.*, **102**, 797 (1970).
- 12) R. F. ITZHAKI and D. M. GILL, *Anal. Biochem.*, **9**, 401 (1964).
- 13) H. M. HASSAN and I. FRIDOVICH, *J. Biol. Chem.*, **252**, 7667 (1977).
- 14) W. J. INGLEDEW and R. K. POOLE, *Microbiol. Rev.*, **48**, 222 (1984).
- 15) C. S. MOODY and H. M. HASSAN, *J. Biol. Chem.*, **259**, 12821 (1984).
- 16) S. Y. R. PUGH and I. FRIDOVICH, *J. Bacteriol.*, **162**, 196 (1985).
- 17) S. Y. R. PUGH, J. L. DIGUISEPPI, and I. FRIDOVICH, *J. Bacteriol.*, **160**, 137 (1984).
- 18) J. S. LODGE and T. EMERY, *J. Bacteriol.*, **160**, 801 (1984).
- 19) P. R. GARDNER and I. FRIDOVICH, *J. Biol. Chem.*, **262**, 17591 (1987).