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Induction of superoxide dismutase in *Escherichia coli* by heat shock

(heat shock proteins/oxidative stress)

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ABSTRACT **Exposure of midlogarithmic-phase cultures of *Escherichia coli* B to 48°C for 1 hr elicited an induction of the manganese-containing superoxide dismutase (MnSOD), which became more pronounced during 1 hr of recovery at 37°C. This induction required protein biosynthesis, since it was suppressed by chloramphenicol. Induction of MnSOD appeared to be a response to a heat-mediated increase in O_2^- production because it was dioxygen-dependent and because heating to 48°C doubled the cyanide-resistant fraction of the total respiration.**

Exposure of a wide variety of living cells to moderately elevated temperatures elicits the selective synthesis of a small number of highly conserved proteins (1). These are referred to as heat shock proteins, and they appear to impart resistance towards elevated temperatures and towards oxidative stress (2, 3). Moreover, heat shock proteins can be induced by a number of oxidants such as hydrogen peroxide or quinones and by depletion of intracellular thiols with diamide, iodoacetamide, or Cd^{2+} (4–8). Exposure to oxygen after anaerobic incubation induces heat shock proteins in *Drosophila* (9), CHO cells (10), and liver (11). The relationship between heat shock and oxidative stress has been emphasized by the finding that a variety of phosphorylated dinucleotides, termed "alarmones," accumulate under both conditions (5, 6).

Since superoxide dismutase (SOD) is induced in response to the oxidative stress imposed by dioxygen (12–14) or by redox active compounds, such as viologens or quinones (15–17), it appeared possible that heat stress might cause increased biosynthesis of SOD. The literature contains conflicting data in this regard. Thus, exposure of *Staphylococcus aureus* to 52°C caused a linear decrease in SOD activity (18) and thermally-stressed cells exhibited heightened susceptibility towards oxidative stress (19). In contrast, heat shock induced SOD in mammalian cells (20, 21). We have examined the effects of heat shock on *Escherichia coli* and now report a dioxygen-dependent induction of the manganese-containing SOD (MnSOD) in these cells.

MATERIALS AND METHODS

Culture Methods. *E. coli* B vitamin B_{12}^- (ATCC 29682) was grown at 37°C on trypticase soy yeast extract (TSY) medium (3% trypticase soy broth/0.5% yeast extract) or on trypticase peptone (TPY) medium (2% trypticase peptone broth/0.5% bactopeptone/0.5% NaCl/0.25% K_2HPO_4). These media were initially at pH 7.0 and contained 1.0 mg/liter of vitamin B_{12}. Cultures were grown in Ryan or Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. Aerobic growth was maintained at 200 rpm in air, while anaerobic growth was accomplished in a Coy chamber under an atmosphere of 85% N_2/10% H_2/5% CO_2. Cultures were started with 2% inocula from overnight cultures and were grown to midlogarithmic phase ($A_{600} \simeq 0.6$). These cultures were heat-shocked by transfer to a water bath shaker at 48°C. Subsequent recovery involved return to 37°C after heat shock.

Enzyme Assays. Cells from 25 ml of cultures were collected at 10,000 × *g* for 10 min at 4°C and were washed once with cold 50 mM potassium phosphate/0.1 mM EDTA at pH 7.8 before being resuspended in 1.0 ml of this buffer. The washed cells were lysed by two passages through a French pressure cell at 20,000 pounds/in^2 (1 pound/in^2 = 6.9 × 10^3 Pa). Lysates were clarified at 14,000 × *g* for 10 min at 4°C in a Fisher model 235A microcentrifuge, and the resultant clarified extracts were dialyzed overnight at 4°C. SOD was assayed by the xanthine oxidase/cytochrome *c* method (22). Catalase was assayed in terms of the disappearance of H_2O_2 followed at 240 nm (23), and protein concentration was estimated colorimetrically with bovine serum albumin serving as the standard (24). SOD electromorphs were separated by electrophoresis on 7% polyacrylamide gels (25), and bands of SOD activity were visualized by activity staining (26). The gel support used for gel illumination obscured the bottom of the gels, and the achromatic region at the bottom of the stained portion of the gel is due to the tracking dye. The relative amount of SOD electromorphs was estimated by linear scanning densitometry. Bands due to iron-containing SOD (FeSOD) were distinguished from those due to MnSOD by soaking the electropherograms for 60 min at room temperature in 20 mM H_2O_2/1.0 mM KCN/50 mM potassium phosphate/0.1 mM EDTA, pH 7.8, prior to staining for SOD activity. This treatment selectively inactivates FeSOD (27).

Respiration of *E. coli* suspended in TPY medium was measured polarographically at 37°C or 48°C under a Clarke electrode in the absence or presence of 1.0 mM NaCN. Cells from a midlogarithmic-phase culture were washed, resuspended in fresh TPY medium, and kept at 0°C until used (never longer than 3 hr). The reaction mixture contained 2.0 ml of TPY medium, air-equilibrated at the indicated temperature.

Materials. Trypticase soy broth and trypticase peptone were from Baltimore Biological Laboratory Microbiology Systems. Yeast extract and Bacto peptone were from Difco. Acrylamide and methylenebisacrylamide were purchased from Bio-Rad; chloramphenicol, cytochrome *c* (type III), xanthine, vitamin B_{12}, and nitroblue tetrazolium, from Sigma; and riboflavin, from Eastman Organic Chemicals.

RESULTS

Induction of MnSOD by Heat Shock. When midlogarithmic cultures of *E. coli*, growing aerobically in TSY medium at 37°C, were exposed for 1 hr to 48°C, there was a modest induction of MnSOD, which was increased in Mn(II)-enriched medium and which became impressive during recov-

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Abbreviations: SOD, superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; FeSOD, iron-containing superoxide dismutase.

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ery at 37°C (Fig. 1). Induction of MnSOD was accompanied by a slight decline in FeSOD which, although not entirely obvious in Fig. 1, could be shown by linear scanning densitometry of these gels (data not shown). Exposure to 48°C also caused a 60% loss of catalase activity (Table 1). There was also an apparently irreversible cessation of growth. Polyacrylamide gel electrophoresis followed by staining for catalase activity (28) demonstrated that both HP-I and HP-II (29, 30), the electrophoretically distinct hydroperoxidases of *E. coli*, participated in this decline in catalase activity (data not shown). When extracts from cells, grown at 37°C, were incubated at 48°C, the rate of inactivation of catalase was similar to that seen when the cells were exposed to 48°C, prior to preparation of extracts. Thus, it appears that the decline in catalase activity at 48°C reflected thermal inactivation. The increase in MnSOD that occurred after incubation at 48°C for 1 hr followed by recovery at 37°C for 1 hr was largely suppressed by chloramphenicol at 150 μg/ml (Fig. 2) and, therefore, may be presumed to represent *de novo* synthesis of this enzyme. In contrast, chloramphenicol did not influence the loss of catalase activity at 48°C.

TSY medium contains 0.25% glucose, derived from the commercial trypticase soy powder. We have noted (31) that glucose decreases the biosynthesis of SOD in *E. coli*. During the course of our current studies we noted that glucose suppresses the biosynthesis of MnSOD. This glucose effect could cloud the results of heat shock experiments because exhaustion of glucose by the cell suspension could result in induction of MnSOD, and such depletion of glucose might occur just prior to or during heat shock or recovery from heat shock. Therefore, the effects of heat shock and of recovery were examined in TPY medium, which is devoid of glucose (31). Induction of MnSOD during recovery from heat shock was evident when the manipulations were carried out in TPY medium (Fig. 3). However, in TPY medium, enrichment with 100 μM $MnSO_4$ did not increase the extent of this induction. The induction of MnSOD was accompanied by a small decrease in FeSOD. As was seen in TSY medium, exposure

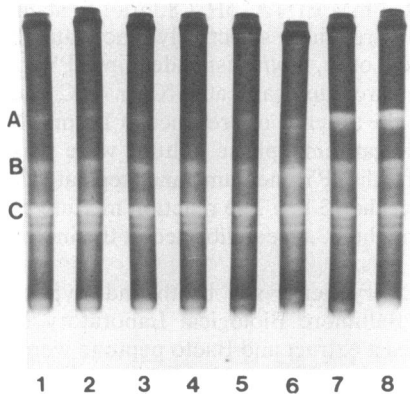

FIG. 1. Effects of heat shock on SOD electromorphs (TSY medium). *E. coli* B were grown on TSY medium at 37°C to midlogarithmic phase. At that time 100 μM $MnSO_4$ was added (where specified), and the temperature was raised to 48°C (where specified). After 1 hr at 48°C (where specified), the cultures were allowed to recover for 1 hr at 37°C. Soluble extracts were prepared and applied to polyacrylamide gels at 70 μg of protein per gel. After electrophoresis, the electropherograms were stained for SOD activity. Bands: A, the MnSOD electromorphs; B, the hybrid SOD electromorphs; C, the FeSOD electromorphs. Gels: 1 and 2, control 1 hr past midlogarithmic phase without (gel 1) or with (gel 2) addition of 100 μM Mn(II); 3 and 4, control 2 hr past midlogarithmic phase without (gel 3) or with (gel 4) addition of 100 μM Mn(II); 5 and 6, 48°C 1 hr past midlogarithmic phase without (gel 5) or with (gel 6) addition of 100 μM Mn(II); 7 and 8, 48°C 1 hr past midlogarithmic phase, with recovery at 37°C for 1 hr, without (gel 7) or with (gel 8) addition of 100 μM Mn(II).

Table 1. Effect of heat shock on growth, SOD, and catalase content of *E. coli* B in TSY medium

Growth conditions past midlogarithmic phase*	A_{600}	Specific activity, units/mg	
		SOD	Catalase
Control at 37°C			
1 hr	0.96	18.5	32.6
1 hr + Mn(II)	0.95	17.0	31.2
2 hr	1.10	24.0	42.0
2 hr + Mn(II)	1.09	24.8	44.1
Heat shock at 48°C			
1 hr	0.83	18.2	12.5
1 hr + Mn(II)	0.81	18.0	11.4
1 hr + 37°C 1 hr	0.84	26.5	12.9
1 hr + Mn(II) + 37°C 1 hr	0.83	34.4	13.3

*Growth conditions were as described in Fig. 1. Mn(II) was at 100 μM.

to 48°C in TPY medium resulted in 60–70% loss of catalase activity and in an irreversible cessation of growth (Table 2).

Anaerobic Heat Shock. A relationship between heat shock and oxidative stress has been suggested (5, 6). Therefore, the dioxygen dependence of induction of MnSOD by heat shock was explored. Heating to 48°C followed by recovery at 37°C, when performed under anaerobic conditions, did not cause induction of MnSOD (Fig. 4). The only SOD evident in extracts of anaerobically-grown *E. coli* is the FeSOD, whose activity can be eliminated by treatment with H_2O_2 (Fig. 4).

Effect of Heat Shock on Respiration. We have previously used cyanide-resistant respiration as an upper-limit measure of intracellular production of O_2^- (15). If heat shock does increase the rate of O_2^- production in *E. coli*, we should anticipate that this would be reflected by an increase in the cyanide-resistant respiration. The respiration of midlogarithmic *E. coli* suspended in fresh TPY medium was measured with and without 1.0 mM CN^- at 37°C, 43°C, and 48°C. The corresponding percentage of cyanide-resistant respirations were 4.3 ± 0.3%, 6.3%, and 8.2 ± 0.4%. Thus, it appears that exposure to elevated temperatures does increase cyanide-resistant respiration.

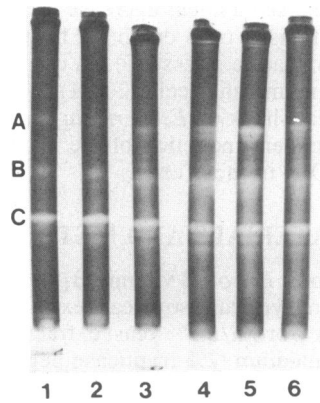

FIG. 2. Effect of chloramphenicol on induction of MnSOD following heat shock. Midlogarithmic cultures of *E. coli* were either exposed to 48°C or maintained at 37°C for 1 hr. Chloramphenicol (150 μg/ml) was then added where indicated, and the cultures were allowed to recover at 37°C for 1 hr. Extracts of these cells at 70 μg of protein per gel were subjected to electrophoresis and staining for SOD activity. Electromorphs A, B, and C were as identified in the legend of Fig. 1. Gels: 1, control 1 hr past midlogarithmic phase; 2, control, 2 hr past midlogarithmic phase; 3, control with addition of chloramphenicol; 4, 48°C for 1 hr past midlogarithmic phase; 5, 48°C for 1 hr then recovery at 37°C for 1 hr; 6, conditions as in gel 5 but with addition of chloramphenicol.

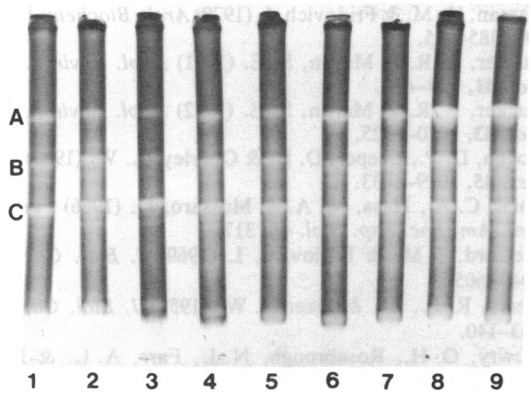

FIG. 3. Effects of heat shock on SOD electromorphs (TPY medium). Manipulations were as described in the legend of Fig. 1 except that TPY medium was used throughout. Gels: 1 and 2, control 1 hr past midlogarithmic phase without (gel 1) or with (gel 2) addition of 100 μM Mn(II); 3 and 4, control 2 hr past midlogarithmic phase, without (gel 3) and with (gel 4) addition of 100 μM Mn(II); 5, control 1 hr past midlogarithmic phase with addition of 0.25% glucose; 6, 48°C for 1 hr past midlogarithmic phase; 7, 48°C for 1 hr past midlogarithmic phase with addition of 100 μM Mn(II); 8, 48°C for 1 hr with recovery at 37°C for 1 hr; 9, as in gel 8 but with addition of 100 μM Mn(II).

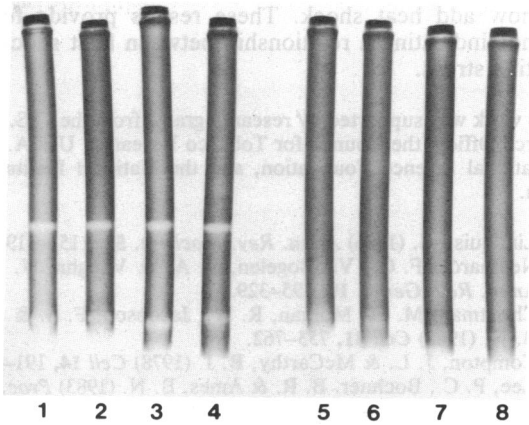

FIG. 4. Effect of anaerobiosis on response to heat shock in TSY medium. *E. coli* B were grown anaerobically (Coy chamber) at 37°C to midlogarithmic phase (A_{600} = 0.5). Heat shock (48°C for 1 hr) and recovery (37°C for 1 hr) were also performed anaerobically. Cell extracts were subjected to electrophoresis at 70 μg of protein per gel and were stained for SOD activity. Gels: 1 and 5, control 1 hr past midlogarithmic phase; 2 and 6, control 2 hr past midlogarithmic phase; 3 and 7, 48°C for 1 hr past midlogarithmic phase; 4 and 8, 48°C for 1 hr past midlogarithmic phase followed by 37°C for 1 hr. Gels 5–8 were treated with cyanide and H_2O_2 as described prior to staining for SOD activity.

Response of a Plasmid-Encoded MnSOD to Heat Shock. *E. coli* (AB 2463 + pDT1-2) bears a multicopy plasmid that contains the gene for MnSOD (32). This plasmid-borne gene, like the chromosomal gene, appears to be susceptible to repression/derepression, since this strain does not produce MnSOD under anaerobic conditions and overproduces MnSOD in response to aerobic paraquat. We explored the response of this strain to heat shock in the hope that it would show an exaggerated induction of MnSOD in response to this stress. Exposure of a midlogarithmic culture in TPY to 48°C for 1 hr resulted in a 1.7-fold increase in total SOD. This was not increased further by subsequent recovery at 37°C for 1 hr (Table 3). This heat-induced increase in total SOD was entirely due to an increase in MnSOD, as determined by polyacrylamide gel electrophoresis and activity-staining (data not shown). Table 3 also shows that the catalase content of this strain declined sharply following heating to 48°C.

DISCUSSION

E. coli responds to elevated temperatures by increasing the rate of synthesis of at least 17 polypeptides, referred to as heat shock proteins (2). This response may be part of a general cellular adaptation to stress, since other stimuli, including several oxidants, cause the induction of heat shock proteins in a variety of species (5, 8, 33, 34). The similarity of protein induction following either heat shock or exposure to oxidizing agents has led to the proposal that heat shock is a form of oxidative stress (5, 35). Lee *et al.* (5) and Bochner *et al.* (6) have related heat shock to oxidative stress by demonstrating that both of these conditions, applied to *Salmonella typhimurium*, elicit synthesis of a family of adenylylated nucleotides which they have named alarmones, and they have proposed that these nucleotides act as the primary signal for oxidation stress (5, 6).

We have now shown that heat shock induces increased biosynthesis of MnSOD by *E. coli*. This appears to be a response to increased intracellular production of O_2^- during heating because it does not occur in the absence of dioxygen and because the fraction of total respiration that was cyanide-insensitive was increased 2-fold when the temperature was raised from 37°C to 48°C. Thus, it appears that heating increases O_2^-- and H_2O_2-producing autoxidations within *E. coli*, possibly by disruption of the electron transport assemblies of the plasma membrane, and that increased O_2^- production elicits increased biosynthesis of MnSOD. Induction of MnSOD in *E. coli*, in response to increased O_2^- production, has been seen under a variety of circumstances to which we

Table 2. Effect of heat shock on growth, SOD, and catalase content of *E. coli* B in TPY medium

Growth conditions past midlogarithmic phase*	A_{600}	Specific activity, units/mg	
		SOD	Catalase
Control at 37°C			
1 hr	0.95	19.5	93.0
1 hr + Mn(II)	0.96	19.8	97.0
2 hr	1.11	24.5	107.0
2 hr + Mn(II)	1.08	23.8	97.0
Heat shock at 48°C			
1 hr	0.74	17.3	24.8
1 hr + Mn(II)	0.76	16.3	25.3
1 hr + 37°C 1 hr	0.75	22.8	26.8
1 hr + Mn(II) + 37°C 1 hr	0.71	20.5	27.8

*Conditions were as described in Fig. 3. Mn(II) was at 100 μM.

Table 3. Heat shock induction of SOD in *E. coli* AB 2463 with plasmid pDT1-2

Growth conditions past midlogarithmic phase	A_{600}	Specific activity, units/mg	
		SOD	Catalase
Control at 37°C			
1 hr	0.85	156.3	21.9
2 hr	1.00	138.3	30.0
Heat shock at 48°C			
1 hr	0.83	264.3	2.5
1 hr + 37°C 1 hr	0.80	251.5	6.4

E. coli AB 2463 with pDT1-2 was grown at 37°C in TPY medium containing 10 μM $MnSO_4$ and 30 μg of ampicillin per ml. At midlogarithmic phase, cells were shifted to 48°C for 1 hr or were maintained at 37°C (control). Where indicated, cells were recovered at 37°C for 1 additional hour.

may now add heat shock. These results provide further evidence indicating a relationship between heat shock and oxidative stress.

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