Superoxide Dismutases of \textit{Escherichia coli}: Intracellular Localization and Functions

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\textit{Escherichia coli} B contains two superoxide dismutases which differ with respect to their localization within the cell, the nature of their prosthetic metals, their responses to changes in \(\cdot \text{O}_2\), and their functions. One of these enzymes, which was liberated from the cells by osmotic shock and which was therefore presumed to be localized in the periplasmic space, is an iron-containing superoxide dismutase. The amount of this iron enzyme did not vary in response to changes in \(\cdot \text{O}_2\) during growth. In contrast, the other superoxide dismutase was not solubilized by osmotic shock, was a manganese-protein, and was found in greater amounts in cells which had been grown at high \(\cdot \text{O}_2\). \textit{E. coli}, which had low levels of the iron-enzyme and high levels of the manganese-enzyme, as a consequence of growth in iron-deficient aerated medium, was killed by exposure to an exogenous flux of \(\text{O}_2^-\) which was generated either photochemically or enzymatically. The addition of bovine superoxide dismutase to the suspending medium protected these cells against this stress. On the other hand, \textit{E. coli}, which had high levels of the iron-enzyme and low levels of the manganese-enzyme, as a consequence of growth in iron-rich anaerobic medium, was resistant to exogeneous \(\text{O}_2^-\). On the basis of these and of previously reported results (4a, Yost, F. J. and I. Fridovich, J. Biol. Chem., 1973, in press), it appears that the iron superoxide dismutase, of the periplasmic space, serves as a defense against exogenous \(\text{O}_2^-\), whereas the manganese-superoxide dismutase, in the matrix of these cells, serves to counter the toxicity of endogenous \(\text{O}_2^-\).

It has become increasingly clear that the univalent reduction of oxygen, which is favored by its electronic structure (12), is a commonplace biological event (4). The enzyme, superoxide dismutase (10), appears to be an important component of the defenses which have evolved to minimize the potential cytotoxicity of this radical (11). \textit{Escherichia coli} B has been found to contain two electrophoretically distinct superoxide dismutases (1). The first of these to be isolated was found to be a manganese-enzyme (7), whereas the second was found to contain iron (Yost, F. J. and Fridovich, I., J. Biol. Chem., 1973, in press). The manganese-superoxide dismutase of \textit{E. coli} has been shown to be induced by oxygen and to play a role in imparting resistance towards hyperbaric oxygen and towards the antibiotic, streptonigrin (4a). It appeared possible that the iron-containing superoxide dismutase, of these cells, fulfilled some special need. We will now report results which indicate that the iron enzyme is localized in the periplasmic space of \textit{E. coli} and that its diminution, by nutritional means, renders the cells susceptible to the deleterious effects of exogenous \(\text{O}_2^-\).

MATERIALS AND METHODS

Cytochrome \(c\), type III, xanthine and adenosine were obtained from Sigma Chemical Co. Trypticase-soy broth and yeast extract were obtained from the Baltimore Biological Labs. Ultrapure salts were obtained from Heico Inc. and metals at 99.99% purity were purchased from the Alfa-Ventron Corp. Xanthine oxidase was prepared from unpasteurized cream (Brady, F. O., Ph.D. thesis, Duke University, 1969). Superoxide dismutase was prepared and assayed as previously described (10). Electrophoresis was performed on 10% acrylamide gels by the method of Davis (3). Superoxide dismutase activity on acrylamide gels was localized by the photochemical methods previously described (1) and was estimated through the use of a Gilford gel scanner. Adenosine deaminase was assayed spectrophotometrically (2).

\textit{E. coli} B was grown to late log phase at 37 C either in a rich medium composed of 30 g of trypticase-soy
broth per liter and 5 g of yeast extract per liter or in a minimal medium whose composition was gram per liter: glucose, 40.5; NH₄Cl, 5.0; Na₂HPO₄, 6.0; KH₂PO₄, 3.0; MgSO₄·7H₂O, 0.2; NaCl, 0.2; Mn (NO₃)₂, 0.06; and Fe (NO₃)₃, 0.08. This chemically defined medium was rendered iron-deficient by omitting the Fe (NO₃)₃. The salts used were purchased at the ultrapure grade or were prepared by dissolving the pure metals in nitric acid. Cells were grown either under the relatively anaerobic conditions of deep, still cultures or under vigorous aeration. Growth was monitored turbidometrically (8). Cells were harvested by centrifugation and cell extracts were prepared by sonic disruption of cells suspended in 0.05 M potassium phosphate, 0.001 M ethylenediaminetetraacetic acid (EDTA) at pH 7.8, at ice-bath temperature. A Branson model W185 sonifier was operated at a power setting of 90 W for 3 min, per batch of cells. Cell debris was then removed by centrifugation at 37,000 × g for 16 min.

RESULTS

Localization of superoxide dismutase in E. coli. Cells were grown in a rich medium under vigorous aeration. Approximately 1 g of cells was washed three times with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.03 M NaCl, pH 7.3, and were then osmotically shocked by incubation in 80.0 ml of 20% sucrose, 0.001 M EDTA, and 0.03 M Tris at pH 7.3 for 5 min, and then transferred to 80.0 ml of distilled water. Enzymes of the periplasmic space were released into solution by this treatment, whereas enzymes of the matrix space were retained (5, 6). Adenosine deaminase, which is known to be a matrix enzyme in E. coli (5), was used to detect the small amount of cell lysis which accompanies this procedure. The osmotically shocked cells were separated from the shock fluid by centrifugation and were then completely extracted by sonic disruption. The shock fluid and the sonic extract of the shocked cells were then assayed for their content of both of the superoxide dismutases and of adenosine deaminase (Fig. 1). Adenosine deaminase, which is given as the percentage of the amount present in whole cells, was 98% retained by the cells during osmotic shock. This indicates that the procedures used in preparing the shock fluid caused only 2% lysis of cells and consequently little release of matrix enzymes. The shock fluid was markedly enriched with respect to the iron-superoxide dismutase, whereas in comparison the shocked cells were enriched with respect to the manganese-superoxide dismutase. Thus, 85% of the superoxide dismutase activity of the shock fluid was due to the iron enzyme and only 15% to the manganese enzyme. Since the shock fluid contained a total of 80 U of superoxide dismutase, of which 12 U were due to the manganese enzyme and 68 U were due to the ferri-enzyme, whereas a sonically disrupted preparation of this amount of whole cells contained 846 U of the manganese enzyme and 565 U of the ferri-enzyme, it is clear that 2% lysis during osmotic shock would have released 17 U of the manganese enzyme. It is clear that all of the manganese enzyme in the shock fluid can be accounted for in terms of 2% of all lysis. In contrast, only 40% of the activity retained in the shocked cells was due to the iron enzyme and 60% was due to the manganese enzyme. The relatively large retention of iron-enzyme, by the shocked cells, could easily have been due to incomplete solubilization of the iron-superoxide dismutase of the periplasmic space. Under the conditions used, approximately 4% of the total soluble proteins of E. coli were released by the osmotic shocking.

Nutritional modification of the levels of superoxide dismutase. E. coli was grown anaerobically in the complete minimal medium or aerobically in this medium without the addition of iron. Cell extracts of these E. coli were
subjected to electrophoresis on acrylamide gels and the gels were treated to visualize the bands of iron and manganese superoxide dismutases. The amount of each activity was estimated by use of a gel scanner at 400 nm. There was a linear relationship between the area under a given peak and the amount of enzyme placed on the gel (Table 1). It is clear that cells grown in the iron-deficient medium contained much less of the iron-superoxide dismutase than did cells grown in the iron-rich medium. It is also clear that, as seen previously (4a), the manganese enzyme was induced by O$_2$ so that the cells grown aerobically had more of the manganese-superoxide dismutase than did the cells which had been grown anaerobically.

The effects of iron-deficiency were reversible. Thus, cells harvested from iron-deficient medium and which contained a low level of the iron enzyme were transferred to the iron-rich anaerobic medium. After growth for 12 h they were harvested and found to contain the high level of the iron-enzyme. Similarly, the addition of Fe(NO$_3$)$_2$ to an iron-deficient culture at mid-log phase yielded cells, at late-log phase, which contained a high level of the iron enzyme. It was thus possible, by nutritional means, to modify the amounts of the iron and manganese superoxide dismutases present in E. coli.

**Effects of exogenous O$_2$.** Photochemically reduced flavins spontaneously react with oxygen to generate O$_2^-$ (9). This fact is the basis of the photochemical assay of superoxide dismutase (1). This photochemical method was used to generate a flux of O$_2^-$ in suspensions of E. coli. Thus, E. coli grown anaerobically in iron-rich medium or aerobically in iron-deficient medium, were suspended, to a density of 10$^7$ cells per ml, in 4% glucose containing 5 x 10$^{-4}$ M riboflavin and 0.015 M methionine. These suspensions were exposed to a 15-W fluorescent lamp at a distance of 5 cm, under continuous stirring at 25 C. At intervals samples were withdrawn and diluted and placed onto nutrient agar for the counting of viable cells (Fig. 2). Line 1 shows that cells grown aerobically in iron-deficient medium, and therefore containing a low level of the iron-enzyme and a high level of the manganese enzyme, were rapidly killed under these conditions. Line 3 demonstrates that shielding these cells from light, and therefore eliminating the photochemical production of O$_2^-$, prevented cell mortality. Line 4 demonstrates that 13 μg of bovine superoxide dismutase per ml protected these cells against mortality in the light. When cells, which had been grown anaerobically in iron-rich medium, were tested, they were found to be resistant to

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<th>Growth conditions</th>
<th>Enzyme*</th>
<th>Total enzyme*</th>
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<tr>
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<tr>
<td>Iron-rich, anaerobic</td>
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* Cells were grown in iron-deficient, aerated medium or in iron-rich anaerobic medium. Cell extracts were then prepared and the superoxide dismutases were separated by electrophoresis on acrylamide gels. The bands of activity were developed photochemically (1) and the gels were then scanned at 400 nm. The areas under the peaks, drawn by the gel scanner, were estimated by use of a planimeter. The identity of each band of superoxide dismutase activity rests upon comparisons of electrophoretic mobilities with those of the iron and manganese enzymes previously isolated from this organism (1, 11).

* Units of enzyme activity per milligram of protein.

**Fig. 2.** Effects of photochemically generated O$_2^-$. E. coli B grown either in iron-deficient, aerated, or in iron-rich anaerobic medium, was diluted to 10$^7$ cells per ml in 4% glucose, 5 x 10$^{-4}$ M riboflavin, and 0.015 M methionine, and then incubated at 25 C under the following conditions: line 1, iron-deficient cells incubated in the light; line 2, iron-rich cells incubated in the light; line 3, iron-rich or iron-poor cells in the dark; line 4, iron-rich or iron-poor cells in the light, but in the presence of 13 μg of bovine superoxide dismutase per ml. At intervals, samples were withdrawn, diluted, and plated onto nutrient agar and after 16 h of incubation at 37 C in the dark, colonies were counted.
photochemically generated $O_2^-$ (line 2). It is clear that resistance to an exogenous flux of $O_2^-$, generated photochemically, correlated with a high content of the iron-superoxide dismutase and appeared unrelated to the manganic-superoxide dismutase. These effects of iron deficiency could be reversed by growing the deficient cells for a few cell divisions in iron-rich medium. Thus, cells grown to mid-log phase in the iron-deficient medium and found to be rapidly killed by the photochemical flux of $O_2^-$, regained resistance towards this stress when iron was added to the medium and growth was allowed to continue to late-log phase.

In view of the ability of bovine superoxide dismutase to protect the iron-deficient cells against the photochemical mortality, it appeared unlikely that some chemical species other than $O_2^-$ was, in fact, the agent of the lethality observed. However, because of the potential complexity of photochemical reactions, it appeared important to repeat the experiments shown in Fig. 2 with a different source of $O_2^-$. This was done by using the xanthine oxidase reaction as the generator of $O_2^-$. Thus, *E. coli* B grown in iron-deficient or iron-rich media, as already described, were diluted to $10^7$ cells per ml in a stirred and aerated solution containing xanthine and xanthine oxidase at 25°C and at intervals samples were removed for dilution and plating onto agar.

Figure 3 presents the results of this experiment. As shown by lines 1 and 2, cells grown on iron-deficient medium were rapidly killed, whereas cells grown in iron-rich medium were quite resistant to the flux of $O_2^-$. Line 3 shows that bovine superoxide dismutase at 26.7 µg/ml completely protected these cells against the lethality of exposure to the aerobic xanthine oxidase reaction. It is again clear that exogenous $O_2^-$ is damaging to *E. coli* B and that resistance to this attack correlates with the iron-containing superoxide dismutase and not with the manganic-superoxide dismutase.

**DISCUSSION**

The iron-containing superoxide dismutase of *E. coli* B was found to be preferentially solubilized by osmotic shock, whereas the corresponding manganese-enzyme remained associated with the shocked cells and could be liberated therefrom by sonic disruption. It appears likely, on this basis, that the iron enzyme is localized in the periplasmic space, whereas the manganese enzyme resides in the matrix of these cells. It has already been shown that the amount of the manganese enzyme in these cells is increased by exposure to $O_2$ and that high levels of this enzyme correlated with enhanced resistance towards hyperbaric $O_2$ and towards streptonigrin.

Streptonigrin is an antibiotic whose lethality is markedly enhanced by $O_2$ and whose action has been supposed to involve the generation of $O_2^-$, within affected cells, by alternate reduction and reoxidation of this complex para quinone (13, 14). Both hyperbaric $O_2$ and streptonigrin may be presumed to lead to enhanced rates of generation of $O_2^-$ in the interior of these cells. The protective action of the manganic enzyme would then depend upon its ability to intercept and effectively scavenge this endogenous $O_2^-$. The periplasmic localization of the iron-superoxide dismutase suggested that it might serve to protect against exogenous $O_2^-$ and the ability to manipulate the level of this enzyme by nutritional means, provided the conditions needed to test this proposal were met.

*E. coli* B which contained low levels of the iron-enzyme and high levels of the manganese-enzyme were killed by exogenous $O_2^-$, whereas cells which had high levels of the iron-enzyme
and low levels of the manganese-enzyme were not. This was true whether the \( \text{O}_2^- \) was generated in the medium by photochemical or enzymatic reactions. Bovine superoxide dismutase protected the iron-deficient cells against the lethality of exogenous \( \text{O}_2^- \). It appears likely, on this basis, that the iron-containing superoxide dismutase does serve as a defense against exogenous \( \text{O}_2^- \).

It remains possible that iron deficiency causes some other change which is actually the cause of the observed enhanced sensitivity towards \( \text{O}_2^- \). The possibility could be tested by creating mutants specifically defective in the iron-enzyme and in the mangano-enzyme, respectively. The resistance of such mutants towards endogenous and exogenous \( \text{O}_2^- \) could then be examined. Such experiments are currently being pursued.

LITERATURE CITED