Elevation of Superoxide Dismutase in *Halobacterium halobium* by Heat Shock†

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**Exposure of Halobacterium halobium** to 50°C for 2.5 h in an aerobic environment resulted in a greater than twofold increase in the activity of the manganese-containing superoxide dismutase. Nondenaturing polyacrylamide gels stained for enzymatic activity did not reveal any additional isozymes of superoxide dismutase induced by the heat shock. The maximal effect was observed at 50°C, and the elevated levels of activity remained constant during 5 h of recovery at 40°C. The induction of enzymatic activity was sensitive to protein synthesis inhibitors. The results are discussed relative to heat shock and stress-related proteins as well as alterations in metabolism brought about by elevated temperatures.

Following exposure to elevated temperatures, many organisms rapidly synthesize a highly conserved set of proteins termed heat shock proteins (19). Enhanced synthesis of heat-shock proteins has been found in eubacteria and archaeabacteria as well as eukaryotes (1, 7, 20), and their induction appears to correlate with the organism's adaptation to hyperthermal stress (1, 19). However, the appearance of heat shock proteins is not confined to thermal stress conditions. Oxidative stress, either in the form of exposure of cells to oxygen after brief anaerobiosis or the exposure to substrates which generate active oxy-intermediates, also elicits the synthesis of many heat shock proteins (4, 6, 10, 19). Therefore, it would appear that organisms might respond in a similar fashion to heat and oxidative stress.

Additional evidence in support of this hypothesis comes from the observation that a variety of oxidative stresses as well as heat shock induce in bacteria the rapid accumulation of alarmones (4, 10, 19). A relationship between oxidative stress and heat shock response can be found in the report that *Neurospora crassa* induces higher levels of peroxidase upon exposure to hyperthermal conditions (9). Finally, a recent report by Privalle and Fridovich (16) has clearly shown that exposure of *Escherichia coli* to moderately high temperatures caused greater levels of a manganese-containing superoxide dismutase activity.

At this point, a direct relationship between heat shock and superoxide dismutase induction is not completely clear. Whereas elevated activities of superoxide dismutase have been found in *E. coli* (16) and in some mammalian cells (11), decreases in superoxide dismutase activity have been observed in *Staphylococcus aureus* upon transient exposure to elevated temperatures (5). During the course of our studies on superoxide dismutase and superoxide dismutase-like genes in the archaeabacterium *Halobacterium halobium*, we observed that a moderate heat shock caused a twofold stimulation in the amount of activity of the organism's manganese-containing superoxide dismutase. These studies confirm earlier reports of induced superoxide dismutase in some organisms and lend supporting evidence to a relationship between heat shock, metabolic alterations, and oxidative stress.

Reagent chemicals were of the highest analytical grade and were obtained, unless otherwise stated, from Sigma Chemical Co. and Fisher Scientific Co. Tryptone was obtained from Difco Laboratories, Detroit, Mich. *H. halobium* NRL was grown in 1-liter flasks containing a basal salt-peptone medium described previously (14). Culture flasks were shaken at 150 rpm in a water bath maintained at 40°C. At the middle of the log phase of growth, flasks were removed and transferred to a water bath set at 50°C or another designated temperature. After shaking at 150 rpm for 2.5 h at the designated temperature, the flasks were removed and placed once more in the shaking water bath set at 40°C. Cells were then allowed to recover for an additional 5 h. Where indicated, various concentrations of inhibitors were added to the culture medium just prior to the initiation of the heat shock. For experiments on cells in still culture, flasks were maintained in a water bath set at 40°C. After the cells reached mid-log phase, the culture flasks were transferred to a 50°C water bath and were maintained there for an additional 2.5 h. Thereafter, the cultures were allowed to recover in a 40°C water bath for 3 h.

Aliquots (50 ml) of each culture were taken at the initiation of heat treatment, after the termination of heat treatment, and during phases of recovery. Cells were spun at 7,500 × g for 15 min at 4°C and washed twice with 50 mM potassium phosphate (pH 7.0) containing 2 M NaCl. The cells were suspended in 5 ml of the wash buffer and then frozen and thawed five times. To further ensure maximal cell disruption, cells were subjected to sonication at full power on a Branson Sonifier. Sonic disruption at 4°C was performed for 20 s, and then the power was shut off for 2 min in order to prevent excessive heating. After a total of 3 min of sonication, the homogenate was spun at 10,000 × g for 10 min at 4°C. The pellet was removed, and the supernatant was assayed for enzymatic activity.

Superoxide dismutase activity was determined by the cytochrome c spectrophotometric method of McCord and Fridovich (13). Controls were run to ensure that the high-salt concentrations of the extract did not interfere with the assay. Enzymatic activity was also determined by nondenaturing polyacrylamide gel electrophoresis followed by a photochemical activity stain employing riboflavin and nitroblue

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tetrazolium (2). Catalase was monitored spectrophotometrically by the disappearance of H$_2$O$_2$ (3). Protein was measured by the method of Lowry et al. (12).

As seen in Fig. 1, a transient exposure to 45°C resulted in elevated enzymatic activity. However, the maximal activity was observed with a 10°C transient temperature elevation (50°C). Exposure to 55°C for 2.5 h resulted in a greatly diminished effect, whereas exposure at 60°C abolished superoxide dismutase induction. Also seen in Fig. 1 is the effect of the time of recovery. Heat-induced increases in superoxide dismutase activity remained high for 5 h in cells exposed transiently to 45 and 50°C. Superoxide dismutase activity increased with time of heat exposure and reached a maximal level following 2.5 h at 50°C (data not shown). Cells continued growth during the heat exposure as well as during the recovery phase for all temperatures. Figure 2 shows a photograph of a gel stained for superoxide dismutase activity. Bacteria which had been transiently exposed to 50°C showed approximately twofold more activity. There was only one isozyme of superoxide dismutase apparent. This corresponds to the manganese-containing enzyme (17, 18). Growth of the bacteria in a medium supplemented with 1 mM MnCl$_2$ did not result in further increases in superoxide dismutase activity over those levels observed with the standard medium.

In order to maximize the aerobic environment, bacteria used in the experiments shown in Fig. 1 and 2 were shaken vigorously. This served to increase the oxygen available for potential reduction to the superoxide free radical. When similar experiments were performed on cells grown in still culture (in which the oxygen content would be expected to be lower), the level of superoxide dismutase activity at 40°C was lower, and the overall enhancement by heat shock at 50°C was only 20% (data not shown). It would appear that both oxidative metabolism and heat are necessary to ensure superoxide dismutase induction.

An examination of catalase activity showed that there was essentially no difference between cells which were heat shocked and those which were maintained at 40°C (data not shown). This is similar to the finding of Privalle and Fridovich for heat shock effects in E. coli (16).

The effect of various protein synthesis inhibitors was examined. Figure 3 shows the effect of differing concentrations of antibiotic on the heat-induced elevation of superoxide dismutase. Chloramphenicol at a concentration of 100

![FIG. 2. Effects of heat shock on superoxide dismutase activity. H. halobium cells were grown at 40°C to mid-logarithmic phase and then exposed to either 40 or 50°C for 2.5 h. Cell extracts were prepared and applied to polyacrylamide gels at 490 μg of protein per gel. After electrophoresis, the gels were stained for superoxide dismutase activity (2).](http://jb.asm.org/)

![FIG. 3. Effect of chloramphenicol on heat shock induction of superoxide dismutase. Mid-logarithmic cultures of H. halobium were either exposed to 50°C (hatched bar) or maintained at 40°C (solid bar) for 2.5 h. Chloramphenicol was added at the initiation of the 2.5-h incubation. Extract preparation and assay conditions were as described in the text. Each bar represents the average of five determinations.)
µg/ml was effective in abolishing the heat induction of superoxide dismutase activity. Kanamycin and gentamicin at concentrations of 500 µg/ml also inhibited the heat-induced superoxide dismutase activity (data not shown).

Archaeabacterial heat shock proteins have been grouped into the following apparent molecular weight ranges: 21,000 to 28,000; 44,000 to 45,000; and 75,000 to 105,000 [7]. In this paper, we have shown that exposure of the archaeabacteria H. halobium to a transient 10°C temperature elevation resulted in a twofold elevation of the manganese-containing superoxide dismutase. The subunit molecular weight of the halobacterial manganese-containing superoxide dismutase is in the range of 20,000 [18]. Our findings of a twofold increase in superoxide dismutase activity are in agreement with the data of Privalle and Fridovich [16], who observed that E. coli responds to heat stress by enhancing its levels of manganese-containing superoxide dismutase activity. Catalase activity was not affected by transient exposure to elevated temperatures.

Whereas E. coli responds to heat shock within 1 h, H. halobium required 2.5 h to reach the maximal inductive effect. This is most likely due to the slower growth of the halophile. Under our conditions, the doubling time in log phase was in the range of 10 h. It should be noted that the appearance of heat shock proteins in archaeabacteria, as detected by radiolabeled bands on nondenaturing gels, reached maximal levels after 75 min [7].

In eukaryotic cells, an invariant nucleotide sequence has been suggested to be a controlling element for heat shock induction of gene expression. The sequence is located 80 to 150 bp upstream of the RNA transcription start site and is composed of an inverted repeat of the sequence CNNGAA NNTTCNNG [15]. It is of interest to note that in the H. halobium gene coding for the manganese-containing superoxide dismutase there is a sequence CACGAAACATTAAAC approximately 40 bp upstream of the RNA transcription start site [17].

Our confirmation that heat stress elicits the enhanced synthesis of an enzyme whose function is to scavenge oxygen free radicals demonstrates an association between heat and oxidative stress. However, the relationship might not necessarily be through the induction of a series of heat shock proteins. Rather, the relationship might involve an enhanced metabolism at higher temperatures.

Intensified metabolism at elevated temperatures might augment oxy-intermediate production. This might suffice to elicit the induction of superoxide dismutase. The negligible effect of heat at low O₂ tensions observed by us as well as by Privalle and Fridovich [16] supports this hypothesis. Additional support comes from the fact that there is a 25% increase in total respiration and a 50% increase in CN⁻-inhibitable respiration at the higher temperature. This latter phenomenon has been used as an indicator of oxy-intermediate generation [8, 16]. The increased O₂ uptake at 50°C might lead to elevated fluxes of superoxide or other oxy-intermediates, thereby inducing superoxide dismutase.

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REFERENCES