Microscopic and Thermal Characterization of Hydrogen Peroxide Killing and Lysis of Spores and Protection by Transition Metal Ions, Chelators, and Antioxidants

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Killing of bacterial spores by H2O2 at elevated but sublethal temperatures and neutral pH occurred without lysis. However, with prolonged exposure or higher concentrations of the agent, secondary lytic processes caused major damage successively to the coat, cortex, and protoplast, as evidenced by electron and phase contrast microscopy. These processes were also reflected in changes in differential scanning calorimetric profiles for H2O2-treated spores. Endothermic transitions in the profiles occurred at lower temperatures than usual as a result of H2O2 damage. Thus, H2O2 sensitized the cells to heat damage. Longer exposure to H2O2 resulted in total disappearance of the transitions, indicative of major disruptions of cell structure. Spores but not vegetative cells were protected against the lethal action of H2O2 by the transition metal cations Cu2+, Cu2+, Co2+, Co2+, Fe2+, Fe3+, Fe3+, Mn2+, Ti3+, and Ti4+. The metal chelator EDTA was also somewhat protective, while o-phenanthroline, citrate, deferoxamine, and ethylenehydroxydiphosphonate were only marginally so. Superoxide dismutase and a variety of other free-radical scavengers were not protective. In contrast, reducing agents such as sulfhydryl compounds and ascorbate at concentrations of 20 to 50 mM were highly protective. Decoating or demineralization of the spores had only minor effects. The marked dependence of H2O2 sporicial activity on moderately elevated temperature and the known low reactivity of H2O2 itself suggest that radicals are involved in its killing action. However, the protective effects of a variety of oxidized or reduced transition metal ions indicate that H2O2 killing of spores is markedly different from that of vegetative cells.

Hydrogen peroxide is used extensively for sterilization and disinfection, especially in aseptic processing and packaging of materials. The effects of factors such as pH and temperature on the sporidal action of H2O2 have been reviewed by Block (8). Spore killing is greater at acid than at neutral or alkaline pH and is highly temperature dependent. H2O2 is only a weak sporicide at room temperature but is very potent at higher temperatures. Industrial processes commonly make use of concentrated peroxide solutions, e.g., 30% by weight, at temperatures of about 85°C.

Mechanisms of spore killing by H2O2 were investigated initially by King and Gould (13). They found that H2O2 at high concentrations actually caused lysis of spores, indicated by loss of microscopic image and loss of light scattering by suspensions of treated spores. Primary lytic damage is to the spore coat, and H2O2 in concentrated solutions induces total dissolution of isolated coats. Further damage involves oxidative cortex hydrolysis or germination-like changes due to activation of cortex lytic enzymes (10). Decoated spores have been found to undergo such changes when exposed to H2O2-generating systems (1).

In contrast, H2O2 at lower concentrations, less than 1%, also kills spores but causes neither germination-like changes nor lysis (13). Thus, such H2O2 killing seems similar to heat killing in that dead spores remain intact and fully refractile. However, H2O2 killing is dissimilar to killing by pressure, which initially induces germination and then kills the resulting forms (14).

The generally proposed lethal action of H2O2 involves its breakdown to yield radicals such as the hydroxyl radical (OH·), which damage nucleic acids, proteins, and lipids (12). OH· production may involve so-called Fenton chemistry, typically initiated with reduction of Fe3+ (or other transition metal ions) to Fe2+ by agents such as the superoxide radical (O2· -). Fe2+ then reacts with H2O2 to yield hydroxyl anion, a hydroxyl radical, and Fe3+. Attempts to enhance radical production for sporidal action by addition of transition metal ions, especially Cu2+, to H2O2-treated spore suspensions have been successful for some spores but not for others (1, 3, 13, 19). Cu2+ appears to be more effective for enhancing lysis than for killing without lysis.

In this paper, we describe an extension of previous characterizations of H2O2 killing of bacterial spores by the use of electron microscopy and differential scanning calorimetry (DSC) and show that transition metal ions can actually protect against rather than enhance sporidal action.

MATERIALS AND METHODS

Spore production. Spores of Bacillus megaterium ATCC 19213, Bacillus subtilis subsp. niger, and Bacillus stearothermophilus ATCC 7953 were prepared as described previously (7). Spores of B. megaterium ATCC 33729 were prepared by the procedures of Belliveau et al. (6). Harvested spores were differentially centrifuged repeatedly in water to remove debris and stored in 95% ethanol until needed. Spores of B. megaterium ATCC 19213 were demineralized by acid titration and remineralized by means of base titration in the presence of Ca2+ by the procedures of Bender and Marquis (7). The spores were decoated by extraction with dithiothreitol and sodium lauryl sulfate by the procedures of Aronson and Horn (2).

Spore killing. Suspensions of clean spores in water contain-
H₂O₂ KILLING OF SPORES

Microscopic and thermal characterization of spore killing by H₂O₂. The electron micrographs in Fig. 1 show the progressive effects of 15% H₂O₂ at 60°C on spores of B. megaterium ATCC 33729. After 0.5 h of treatment, there were no observable changes in the fine structure of the spores (Fig. 1A) compared with untreated spores (see Fig. 2A in reference 6). However, nearly all of the spores were killed by this exposure,
as indicated by a drop in the viable-cell count from $5.0 \times 10^9$ to $9.3 \times 10^8$ CFU/ml. Furthermore, there was no significant loss in optical density of the suspensions or change in the phase microscopic appearance of the spores. Thus, killing preceded lysis. After 2 h of treatment, major gaps in the coat-outer membrane complex and partial lysis of the cortex appeared (Fig. 1B). After 8 h, decoating and cortex lysis appeared to be nearly complete (Fig. 1C). After 24 h, extensive damage to the protoplast as well as to the cortex and coat was readily apparent (Fig. 1D).

At lower concentrations or lower temperatures, H$_2$O$_2$ killed the spores without lysis or loss of refractility, even after prolonged incubation. For example, 1% H$_2$O$_2$ solution at 50°C was lethal for spores of B. megaterium ATCC 19213, as indicated by a nearly 100,000-fold reduction in viable-cell counts (from ca. $10^{10}$ to $10^8$ CFU/ml) but with almost no loss in optical density of the suspension or change in the phase microscopic appearance of the spores.

Figure 2 shows DSC scans of spores of B. megaterium ATCC 33729 exposed to 15% H$_2$O$_2$ at 60°C. The top scans show DSC profiles for untreated spores, which are like those described by Belliveau et al. (6). As shown by the broken line, the scan after heating to 150°C is essentially flat, indicating that the transitions detected by the initial scan are irreversible. The peaks and valleys of the initial scan are labeled by letters corresponding to those used and identified by Belliveau et al. (6). Endothermic peak a is associated with heat activation for germination, peak b is coincident with heat killing, peak c is identified with the coat-outer membrane complex, and exothermic valley d is most likely a reflection of aggregation or coagulation of denatured macromolecular constituents.

![DSC profiles of spores of B. megaterium ATCC 33729 after treatment with 15% H$_2$O$_2$ at 60°C for different times](image)

**FIG. 2.** DSC profiles of spores of *B. megaterium* ATCC 33729 after treatment with 15% H$_2$O$_2$ at 60°C at 0 h (uppermost profile set), 0.25 h (middle set), and 4 h (bottom set). In each set, the rescans are shown by a broken line. The peaks and valley are labeled with letters corresponding to those used by Belliveau et al. (6).

The middle scans of Fig. 2 show DSC profiles obtained after treatment with H$_2$O$_2$ for 15 min. The two major effects are a reduction in peak and valley amplitudes and a shift to the left, i.e., to lower temperatures. The bottom scans of Fig. 2 show DSC profiles obtained after 4 h of H$_2$O$_2$ treatment, resulting in complete elimination of the endothermic peaks and a temperature extension of the exothermic valley, which disappeared after the scan reached 145°C.

**Hierarchy of spore resistance.** Previously, for studies of heat resistance, strains of spores with wide ranges of heat resistance were used (7). Similarly, in this study, strains were selected to determine ranges of resistance to H$_2$O$_2$. As shown by the data in Fig. 3, there were significant differences in H$_2$O$_2$ resistance among the four standard strains chosen to represent a range in heat resistance, from spores of the relatively heat-sensitive ATCC 19213 and ATCC 33729 strains of *B. megaterium* (Fig. 3A) to those of the more resistant strain of *B. subtilis* subsp. *niger* and the very resistant ATCC 7953 strain of *B. steatorrhombus* (Fig. 3B). Because of the differences in resistance, it was necessary to use 1% H$_2$O$_2$ to kill the latter spores and...
Citrate or o-phenanthroline added without a protective metal cation had at best only a small protective effect against killing by 0.1% H2O2, while 20 mM EDTA alone showed significant protection (Fig. 6). However, when 10 mM Fe2+ was added with the chelators, full protection against killing by 0.1% H2O2 was evident. Deferoxamine and ethanehydroxy-

0.1% to kill the former. The data indicate a D value (time for killing of 90% of the population) of about 3 h in 1.0% H2O2 for B. stearothermophilus, compared with a value of about 0.67 h for B. megaterium in 0.1% peroxide.

Protective effects of transition metal ions, chelators, and antioxidants. Because of their low level of resistance to H2O2, spores of B. megaterium ATCC 19213 were used to assess the protective effects of antioxidants. The addition of transition metal ions in the reduced form effectively protected spores exposed to 0.1% H2O2 at 50°C and pH 7, as shown for Fe2+ in Fig. 4A and for Cu2+ in Fig. 4B. The chelator o-phenanthroline, which is protective against killing by organic peroxides (17), somewhat enhanced protection by Fe2+ but markedly reduced protection by Cu2+.

The protection by Fe2+ was concentration dependent, as shown by the data in Fig. 5. At a concentration of 1 mM, Fe2+ was only marginally protective against 0.1% H2O2. Under the same experimental conditions, 5 mM Fe2+ was nearly completely protective and 20 mM Fe2+ was only slightly more protective.

FIG. 5. Titration of protective effect of Fe2+ for spores of B. megaterium ATCC 19213 against killing by 0.1% H2O2 at 50°C and pH 7. Data are shown for spores exposed to only the peroxide (□) or the peroxide plus 1 mM (●), 5 mM (■), or 20 mM (×) FeCl2.

FIG. 6. Effects of EDTA or citrate on oxidative killing of spores of B. megaterium ATCC 19213 with or without 10 mM FeCl2. Data are shown for spores exposed to 0.1% H2O2 at 50°C at pH 7 (□) and for similarly exposed suspensions containing 10 mM citrate (●), 20 mM EDTA (▲), 20 mM EDTA plus 10 mM FeCl2 (■), or 10 mM citrate plus 10 mM FeCl2 (×).
The results of previous work with tertiary butyl hydroperoxide (17) indicated that only the reduced forms of transition metal ions are protective against oxidative killing. However, with 0.1% H$_2$O$_2$ at 50°C and pH 7, the oxidized forms Fe$^{3+}$, Cu$^{2+}$, and Mn$^{2+}$ at 10 mM were nearly completely protective against killing. Other oxidized transition metal ions (Co$^{2+}$, Co$^{3+}$, Ti$^{3+}$, and Ti$^{4+}$) were also found to be protective. Vegetative cells could not be protected against the lethal action of 0.1% H$_2$O$_2$ at room temperature by Fe$^{2+}$ or Fe$^{3+}$ or by EDTA or α-phenanthroline. In fact, the mineral ions enhanced the lethal action of H$_2$O$_2$ for vegetative cells (data not shown).

The minerals within spores appeared to be only somewhat protective against killing by H$_2$O$_2$, and only small differences in sensitivity to H$_2$O$_2$ were obtained for native spores, spores demineralized by acid titration, and spores demineralized and then remineralized with Ca. In addition, decoating the spores prior to exposure to H$_2$O$_2$ had little or no effect on their sensitivity.

Attempts to protect spores against H$_2$O$_2$ killing with the radical scavengers l-histidine, butanol, alpha-tocopherol, manitol, or superoxide dismutase were unsuccessful. However, sulfhydryl compounds were protective, as shown by the representative data in Fig. 7 for a variety of sulfhydryl compounds. Although high levels (50 mM) were required, dithiothreitol, cysteine, or glutathione fully protected the spores against 0.1% H$_2$O$_2$ at 50°C. Thioglycollate was only weakly protective at this concentration. l-Ascorbate at concentrations greater than 20 mM was highly protective also (data not shown).

**DISCUSSION**

The results of the DSC analyses presented here show that H$_2$O$_2$ damage to spores is extensive and results in loss of all molecular interactions that are the basis for cooperative heat denaturation processes, reflected by endothermic peaks or exothermic valleys in DSC profiles. The disappearance of the endothermic peaks precedes that of the exothermic valley. The progressive shifts of the transitions to the left, i.e., to lower temperatures, indicate that H$_2$O$_2$ damage to spore components affects heat-induced cooperative molecular interactions.

Setlow (16) has found that small, acid-soluble spore proteins protect DNA in vivo against damage by H$_2$O$_2$, just as they protect against UV irradiation. However, small, acid-soluble spore proteins would not likely protect proteins or lipids against H$_2$O$_2$ damage. Part of the resistance of spores to H$_2$O$_2$ develops during the stationary phase of the culture cycle prior to sporulation (9). Then, as Setlow and Setlow (15) have shown, still greater resistance develops in several stages during sporulation. One stage coincides with synthesis of small, acid-soluble spore proteins, and resistance is then augmented in later stages of sporulation.

The hierarchy of resistance to H$_2$O$_2$ determined in the present study suggests that heat resistance and H$_2$O$_2$ resistance are correlated among various spore strains but that the range of resistance to heat is much broader.

A reasonable view of spore killing by H$_2$O$_2$ is that the agent itself is not reactive but is converted to radicals, such as the hydroxyl radical, and the radicals then react with spore components. H$_2$O$_2$ itself in aqueous solution does not oxidatively modify nucleic acids, lipids, or proteins in the absence of catalysts for radical formation (11). Presumably, the need for radical formation is a major basis for the very strong temperature dependence of H$_2$O$_2$ killing described by Toledo et al. (18) and by others and found also in this study.

However, the findings reported here do not fit well with known modes of radical production. In Fenton reactions, reduced but not oxidized transition metal ions react with H$_2$O$_2$ to produce OH·. In our experiments, both oxidized and reduced forms were protective. These findings contrast also with our previous ones (17) with organic hydroperoxides, which showed that only the reduced ions were protective. Thus, the pathways for the formation of organic peroxy radicals appear to differ from those for the formation of radicals from H$_2$O$_2$. The findings for H$_2$O$_2$ do not indicate that radical formation is unimportant in oxidative damage but simply that the pathway for radical formation is probably not via standard Fenton reactions.

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**REFERENCES**