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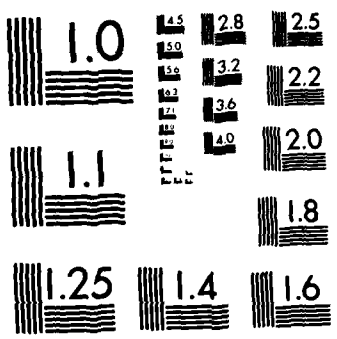

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MECHANISMS FOR REGULATION OF THE ACTIVITY OF KEY ENZYMES IN DEVELOPING DORMANT AND GERMINATED BACTERIAL SPORES

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Final Technical Report

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19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*
1-bacterial spores; 2-heat resistance; 3-dormancy; 4-thiol-disulfide interconversion; 5-regulation of metabolism; 6-intracellular pH.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*
Highlights of research findings in the past three years are: 1) A novel enzyme which uses NADH to reduce disulfides (such as oxidized Coenzyme A) containing 4-phosphopantethine residues has been purified to homogeneity from Bacillus megaterium spores and characterized; it is a new member of the class of pyridine nucleotide dependent disulfide reductases.. The enzyme is present in a number of gram positive spore formers, but not in gram negative bacteria or eukaryotic organisms. 2) The low pH (6.3-6.5) in the dormant spore core rises to ~ 7.5 in the first seconds of spore germination. This process is more rapid than

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dipicolinic acid release and is accompanied by a release of protons as well as other monovalent cations (K^+ and Na^+) into the growth medium. 3) Using a mutant of B. sphaericus which cannot form spore cortex we have shown that spore cortex is not necessary for acquisition of UV light resistance or low molecular weight basic proteins by the developing spore. 4) The sequence specific spore protease of B. megaterium has been purified and characterized. It is synthesized during sporulation as a zymogen which is only processed to an active form later in sporulation. Identification of the zymogen form of this enzyme explains in part the regulation of this enzyme! 5) Cyclic AMP was shown to be essentially absent from C. perfringens during all phases of its life cycle. Thus cAMP plays no role in regulating sporulation in this organism.

Final Technical Report

The objectives and abstract of studies to be undertaken as given in the orginal grant application were as follows:

OBJECTIVES: A guiding principle for much of the work we plan is that there is no single factor which by itself results in the dormancy of bacterial spores. Rather, there are a number of factors each of which contributes to some degree to the enormous reduction in activity of key spore enzymes in vivo, which in sum result in the phenomenon of bacterial spore dormancy. It is the objective of the work we propose to identify and understand fully these various factors. In short, we hope to understand in detail the mechanisms whereby key bacterial spore enzymes become inactive during spore formation and/or maturation, remain inactive in the dormantspore, and become active once again in the first minutes of spore germination. This knowledge will of itself be worthwhile, since it should give insight into the mechanisms whereby bacterial spore become dormant, remain dormant and then break dormancy. Understanding of the mechanism of spore dormancy should also have an added bonus, in that it should further our knowledge and understanding of the mechanisms of spore resistance to heat and radiation.

ABSTRACT: Studies will be undertaken to understand the mechanisms whereby key enzymes within bacterial spores become inactive during spore formation, remain inactive in the dormant spore, and become active in the first minutes of spore germination. Knowledge of these mechanisms should provide insight into bacterial spore dormancy as well as mechanisms for the high resistance of spores to heat and irradiation. Key spore enzymes to be examined will include the specific spore protease, phosphoglycerate mutase and arginase. Mechanisms which will be examined for regulation of these and other enzymes within spores will include: compartmentation of enzyme and substrate; low spore water content; formation of disulfides with key spore thiol groups; sequestration of metal ions essential for enzyme activity; and the low pH within the dormant spore ($\sim$ 6.3) as compared with that within the germinated spore ($\sim$ 7.5). With regard to the latter, we will also alter the internal pH of dormant spores, and determine if this affects the activity of spore enzymes in vivo as well as the spore's heat and radiation resistance and its long term survival.

During the three year period covered by this report, some of the studies outlined above have been completed, and others are still in progress. Several new areas of research have also been initiated and/or completed. Highlights of the achievements in the preceeding three year period are given below. Except as noted, all of the studies utilized Bacillus megaterium.

1) Absence of cAMP from Clostridium perfringens. In collaboration with Dr. L.E. Sacks we showed that C. perfringens contained no detectable cyclic AMP (cAMP) in any stage of growth. Thus, as has been shown previously for Bacillus species, cAMP is not present in and thus plays no role in regulating sporulation in Clostridia. This finding further indicates that the well documented stimulation of Clostridial sporulation by compounds which can inhibit cAMP phosphodiesterase is in fact not related to this inhibition!

2) Role of low molecular weight proteins in spore UV resistance (1) - We have used a mutant of B. sphaericus defective in spore cortex formation as well as its wild type parent in establishing the role of spore cortex in spore ultraviolet light (UV) resistance. We found that the defective spores formed in the cortexless mutant acquired UV resistance with the same kinetics and to the same level as its wild type parent. In addition, the cortexless mutant accumulated the spore specific low molecular weight proteins with the same kinetics and to the same level as its parent, and that these proteins were stable in the cortexless spores. These findings indicate that the spore cortex plays no significant role in spore UV resistance or the stability of the low molecular weight spore proteins.

3) Thiol-disulfide interchange in spore germination (2) - The disulfide reductase which we first identified in B. megaterium spores, has been purified and characterized. The enzyme has been purified 25,000 fold to homogeneity from spores and its native molecular weight ($\sim$ 110,000), subunit structure (2 identical) and cofactor composition (1 FAD per subunit) determined. The enzyme is most active with 4-phosphopantethine as a substrate with NADH as a reductant. The enzyme also reduces oxidized Coenzyme A, but has very high Km values (> 0.5 mM) for its disulfide substrates.

This enzyme was at only low levels in growing and early sporulating cells, but increased > 10 fold midway in sporulation. However, the enzyme was present in similar specific activities in both mother cell and forespore, and was also accumulated to a high level in an asporogenous mutant blocked early in sporulation. The high level of enzyme present in the spore was lost during germination with a t1/2 of $\sim$ 100 min.

We also found this enzyme in a number of other Bacillus and one Clostridial species, however, it was not found in any other organisms. Surprisingly, the enzyme level was much higher in B. cereus and B. sphaericus, but did not vary significantly in different stages of growth.

The enzyme described above is a new member of the pyridine nucleotide dependent disulfide reductases, with similar catalytic and physical properties. Its exact function in Bacillus species is not clear, but it may well serve to keep CoA in reduced form much as glutathione reductase keeps glutathione in reduced form in other organisms (glutathione is not found in Bacillus spores!). It may also be involved in the CoA - thiol disulfide transitions in sporulation and germination.

4) Changes in ions and pH during spore germination (3) - Previous work from our laboratory has shown that the pH in the core of bacterial spores is low (6.3-6.5), but that it rises to 7.5 upon germination. We have further shown that the change in spore core pH is an extremely early event in germination- even earlier than dipicolinic acid release. The rise in spore core pH is accompanied by a rapid excretion of protons as well as other monovalent ions including K^+ and Na^+. Approximately 80% of the latter two ions are released with spore core protons in a process that is more rapid than dipicolinic acid release. This massive rapid ion release early in germination indicates that there is a tremendous change in spore inner membrane permeability in the first seconds of spore germination.

We were also able to significantly elevate (by $\sim$ 1.5 units) the dormant spores core pH. However, this had no effect on spore dormancy, heat resistance or UV resistance.

5) Regulation of spore protease (4,5,6,7) - The major effort in the past years has been the study of the specific spore protease. This enzyme has been purified to homogeneity from spores. It is a tetramer of identical 40,000 molecular weight subunits, and only the tetramers are active. An antibody and a radioimmunoassay (RIA) have been developed against this enzyme.

The protease is synthesized during sporulation in the forespore, and approximately in parallel with its low molecular weight substrates. The enzyme is synthesized as an inactive zymogen which forms tetramers, butwith a subunit of 46,000 molecular weight (P_{46}). P_{46} is processed to a tetrameric form with 41,000 molecular weight subunits (P_{41}) late in sporulation in parallel with dipicolinic acid accumulation. P_{41} is active in vitro, but not in vivo. How P_{41} is regulated and how the P_{46} to P_{41} processing takes place is not clear. P_{41} is only processed to a form with 40,000 molecular weight subunits (P_{40}) in the first seconds of germination. This process is ATP independent, but subsequently P_{40} disappears completely in an ATP dependent process.

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