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Highlights of the most significant research finding in the last few years are: 1) α/β-type SASP have been shown in vitro to be a novel group of non-specific, double-strand DNA binding proteins which slow DNA depurination, block hydroxyl-radical cleavage of the backbone, and block UV induced pyrimidine dimer formation, while promoting spore photoproduct formation; 2) the effects of α/β-type SASP in vitro are also exerted in vivo as these proteins are important factors in spore heat and hydrogen peroxide resistance, and the major cause of spore UV resistance; 3) studies of the regulation and processing of the SASP specific protease have strongly suggested that the processing of the zymogen form of this enzyme during sporulation is an autocatalyzed event triggered by changes in the forespore (very likely dehydration) which will block attack of the active enzyme on SASP. In the first minutes of spore germination spore core rehydration then allows rapid SASP degradation.

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Studies on Bacterial Spore Ultraviolet Light Resistance and Regulation of the Activity of a Spore Protease

Final Technical Report

December 8, 1993

U.S. Army Research Office

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University of Connecticut Health Center

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Final Technical Report

1.1 The abstract and outline of studies to be undertaken, as given in the initial grant application, were as follows:

1.2 Abstract and Statement of Work

Analysis of B. subtilis mutants with deletions in genes coding for the two major, α/β-type small, acid-soluble spore proteins (SASP) has shown that these proteins are required for spore ultraviolet light (UV) resistance and for the novel UV photochemistry of spore DNA in vivo. The direct involvement of α/β-type SASP in spore UV resistance has been further shown by immunoelectron microscopic localization of these proteins on DNA in UV resistant forespores. Recent in vitro experiments have shown that association of α/β-type SASP with DNA causes a change in DNA conformation from the B → A form, as well as an apparent large change in DNA topology. The major objective of this work is to continue studies on SASP-DNA interactions in attempts to achieve a detailed understanding of the mechanism(s) of spore UV resistance. Specific aims toward this goal are: 1) Using highly purified α/β-type SASP we will determine key binding parameters (i.e. - Kd, stoichiometry) for SASP with DNA. Specific variables to be tested for their effect on binding include: pH, ionic strength, divalent cations (Mg^{++} and Ca^{++}), phosphate, and the type (RNA vs. DNA, single stranded vs. double stranded DNA), conformation (linear, supercoiled, A form, B form), and base composition of the nucleic acid. 2) With the data from the above experiments giving us optimum conditions for formation of SASP-DNA complexes in vitro, we will form complexes using ^{3}H-thymidine labeled DNA, and determine the UV photoproducts formed in such complexes with the hope of duplicating spore UV photochemistry in vitro. 3) We will form specific SASP-DNA complexes in vitro and use DNA conformation specific ligands to probe the DNA conformation in SASP-DNA complexes. 4) We will study SASP-DNA complexes with the objective being to elucidate specific SASP-DNA and SASP-SASP interactions in these complexes. These analyses will include use of chemical crosslinking agents as well as assessment of protection of specific bases and bonds in SASP-DNA complexes from chemical and enzymatic attack. 5) We will initiate attempts to crystallize an α/β-type SASP by itself, and complexed with an appropriate oligonucleotide. Eventual determination of the crystal structure of the latter complex should provide insight into the mechanism whereby SASP effect DNA structure. 6) A few specific mutant SASP will be prepared by site directed mutagenesis of a cloned gene. The ability of these mutant SASP to provide UV resistance to spores, and to interact productively with DNA in vitro will then be tested. The mutant SASP produced will have only single amino acid changes and only in residues conserved in these proteins throughout evolution.

A second objective of this project will be the detailed understanding of the mechanism and regulation of processing of the SASP-specific protease. This enzyme is made in sporulation as an inactive 46,000 dalton precursor termed P_{46}, which is processed ~ 2 hr later to a form (termed P_{41}) which is active in vitro but not in vivo. During spore germination P_{41} is further converted to P_{40}. Since the processing of this enzyme takes place as spores become UV and then heat resistant, understanding of the requirements for protease processing may give us insight into conditions inside the developing spore at this time. Specific aims are to: 1) Determine the nature of the processing reactions in going from P_{46} to P_{41} to P_{40} by analysis of the various forms of the enzyme. 2) Purify large amounts of the P_{46} and P_{41} precursors from overproducing strains. 3) Use the purified P_{46} and P_{41} to study their processing in vitro with the aim of establishing the conditions for this processing, other gene products (if any) involved in these reactions, and their regulation.

During the three year period covered by this report we have made significant progress on the work outlined above, successfully achieving a number of the specific aims, and initiating several new lines of investigation. Highlights of the research achievements in the past three year period are summarized below.

1) Characterization of α/β-type SASP/DNA interaction in vitro. We have shown that purified α/β-type SASP from both Clostridium and Bacillus species interact similarly with and have the same effects on DNA. These proteins are non-specific double-stranded DNA binding proteins which bind to all DNAs (although GC rich DNA is preferred) with a stoichiometry of one protein/5 bp. This binding does not alter the DNA's backbone length, but does straighten and stiffen the backbone considerably. SASP binding prevents backbone cleavage by nucleases and hydroxyl-radicals, greatly slows depurination, blocks UV-induced pyrimidine dimer and pyrimidine-pyrimidine adduct formation, promotes UV-induced spore photoproduct formation, and converts DNA to an A-like conformation. α/β-type SASP with mutations in residues conserved in these proteins throughout evolution no longer bind DNA. These results indicate that α/β-type SASP are a new group of DNA binding proteins, with novel effects on DNA.

2) Effect of α/β-type SASP in vivo. The results of the studies noted above strongly suggested that α/β-type SASP might be significant contributors to spore resistance to heat, hydrogen peroxide, and UV radiation. Studies of the properties of spores which lack the majority of α/β-type SASP have proven this, and have shown that α/β-type SASP prevent DNA damage by UV, heat and hydrogen peroxide in vivo as well as in vitro. While α/β-type SASP are only one factor contributing to the resistance of bacterial spores to heat and hydrogen peroxide, these proteins are the major, and possibly the only cause of spore UV resistance.

3) Regulation of the SASP-specific protease GPR. Using the cloned and sequenced gpr genes from B. subtilis and B. megaterium, we have over-expressed them at least 100 fold during sporulation in B. subtilis. Strikingly, there was no effect on sporulation, SASP accumulated essentially normally, and the inactive GPR zymogen (termed P_{46}) was processed normally to the potentially active form (termed P_{41}) late in sporulation. Use of a variety of spo mutants showed that the $P_{46} \rightarrow P_{41}$ conversion was blocked only in mutants which did not accumulate dipicolinic acid. Analysis of the sequence cleaved in the $P_{46} \rightarrow P_{41}$ conversion indicated it was extremely similar to the sequence recognized and cleaved in SASP by GPR. This work has led to the suggestion that processing of P_{46} to P_{41} is self-catalyzed, with this process triggered by the uptake of DPA by the developing forespore. Presumably the conditions when DPA uptake takes place (i.e. - spore core dehydration) prevent the action of the active P_{41} generated on its SASP substrates. This model to explain how active P_{41} can be generated late in sporulation yet not attack SASP until spore germination when the spore core is rehydrated is currently being tested through a number of experiments, and to date all data are consistent with it.

4) Cloning and nucleotide sequence of genes coding for SASP from Clostridium perfringens. We analyzed SASP from C. perfringens and found that spores of this organism lack a γ-type SASP, but do have the highly conserved α/β-type SASP. Three genes coding for these proteins were cloned and sequenced, and the proteins coded for were extremely homologous to those of Bacillus species. This work significantly extended the evolutionary time over which α/β-type SASP sequences can be compared.

5) Nucleoid condensation during sporulation. We made a fortuitous observation when doing some fluorescence microscopy using a DNA stain, that the forespore nucleoid becomes quite ($\geq$ 2-3 fold) condensed early in sporulation. Analysis of this event has indicated it takes place approximately coincident with asymmetric septation. However, to date neither the mechanism nor the significance of this nucleoid condensation is clear.

6) Dipicolinic acid (DPA) sensitizes spore DNA to UV. As was suggested a number of years ago we obtained definitive evidence that DPA sensitizes spore DNA to UV light, both in vivo and in vitro. Thus, while DPA undoubtedly plays an important role in spores, it is actually deleterious as far as survival after UV irradiation is concerned.

1.3 Papers Published

a) Reviews - supported by ARO grant and NIH grant

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Fairhead, H., B. Setlow and P. Setlow, Prevention of DNA damage in spores and in vitro by small, acid-soluble proteins from Bacillus species, J. Bacteriol. 175, 1367-1374 (1993).

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