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Differential Analysis of Sulfhydryl and Disulfide Groups of Intact Spores

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ABSTRACT

BLANKENSHIP, L. C. (Eastern Utilization Research and Development Division, Washington D.C.), AND M. J. PALLANSCH. Differential analysis of sulfhydryl and disulfide groups of intact spores. J. Bacteriol. **92**:1615–1617. 1966.—Fluorescence quenching of fluorescein mercuric acetate in alkaline medium (1 N NaOH) was found to be an accurate, sensitive method for differential analysis of sulfhydryl and disulfide groups of intact bacterial spores. Disulfide content of three species of spores was found to be slightly higher than reported by other workers. A significant increase in sulfhydryl group concentration was observed after physiological germination, indicating participation of a disulfide-reducing system in the germination process.

Sulfhydryl and disulfide groups play important roles in the physiological and structural functions of proteins in biological systems (3). Although bacterial spores have been shown to contain significantly higher concentrations of cystine than vegetative cells (6, 8, 9), no conclusive relationship between sulfhydryl and disulfide groups of dormant or germinating spores has been established. All studies of the disulfide content of spores, to date, have relied upon hydrolysis or hydrazinolysis procedures. Both of these procedures are prone to error, the hydrolysis method because of destruction of cysteine and cystine, and hydrazinolysis because it does not distinguish between cystine and cysteine sulfur, and thus cannot yield accurate estimates of the disulfide content of spores. Karush (5) described a method for estimating disulfide groups by fluorescence quenching of fluorescein mercuric acetate (FMA); this method is specific, extremely sensitive, and can be used to distinguish between sulfhydryl and disulfide groups. This report describes application of the FMA-quenching method to a differential analysis of sulfhydryl and disulfide groups in intact dormant and germinating spores.

MATERIALS AND METHODS

Culture procedures. Three species of spores were used in this study: *Bacillus megaterium* QM 1551, *B. cereus* NRS 804, and *B. subtilis* 15u. All spores were produced by the active culture technique in "G" medium as described by Hashimoto (2). Spores were extensively washed in demineralized water at 2 to 4 C, lyophilized, and stored in a desiccator at room temperature.

Spore counts were determined in a model A Coulter counter in 2% KCl. Viable counts were estimated by the plate count technique.

Germination procedures. Germination was performed by suspending 5.0-mg quantities of each spore species in a final 5.0-ml volume containing 50 mM potassium phosphate buffer (*p*H 7.0) and the appropriate germinants as follows: *B. megaterium*, 12 mM glucose and 5 mM L-alanine; *B. cereus*, 5 mM L-alanine and 5 mM adenosine; *B. subtilis*, 5 mM L-alanine and 20 mM glucose. Incubation temperature was 37 C. Germination was more than 90% complete in 30 min as judged microscopically by loss of refractility. *B. subtilis* and *B. megaterium* required heat activation at 95 and 65 C, respectively, for 30 min.

Spore rupture. Disintegrated spores were dry-ruptured by the technique of Sacks (7) with use of glass beads and a Wig-L-Bug dental amalgamator. Spores and glass beads were mixed in a 1:1 ratio and disintegrated for 5 min under a stream of Dry Ice-chilled air.

Chemical procedures. Disulfides and sulfhydryls were estimated according to the technique described by Karush (5). FMA (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared as a 10^{-3} M stock solution in 0.01 N NaOH and was stable for several weeks when stored in low actinic glassware. Appropriate volumes of aqueous spore suspensions were mixed with 0.05 ml of stock FMA, diluted to 10 ml with 1 N NaOH, and incubated 3 hr for disulfide measurements. Sulfhydryl groups were similarly measured, except that reactions were conducted in 0.10 N NH_4OH for

10 min. Fluorescence quenching was measured in an Aminco-Bowman Spectrophotofluorimeter, equipped with polarizing prisms to control light scattering, and with a small sample cell of 0.0625 cm^2 cross-sectional area. Instrument response was standardized as described by Fox (1). Activation wavelength was 499 mμ and fluorescence was measured at 520 mμ. Disulfide concentration was estimated by reference to a standard curve based on disulfide content of ribonuclease, Hirs component A (Calbiochem); sulfhydryl groups were determined by reference to a cysteine standard curve.

Oxidation of spores was conducted according to Hirs' technique (4). We mixed 25-mg quantities of spores with 9 ml of 88% formic acid and 1 ml of 30% H_2O_2, and then heated the mixture at 50 C for 1 hr. Suspensions were then lyophilized, and disulfide determinations were performed as described above.

Results

The results of disulfide determinations on three species of spores are given in Table 1. *B. subtilis* spores contained the highest concentration of disulfides. Extraction of spores with ethyl alcohol-ether (3:1) three times or treatment with lysozyme did not change disulfide estimates. Spore suspensions which had been oxidized with performic acid failed to quench FMA fluorescence.

Disintegrated spore samples were treated with FMA to check the effect of diffusion of the reagent into the spore interior. As noted in Table 1, disulfide content of ruptured spores compares favorably with intact spores.

The time course of the fluorescence quenching (Fig. 1) suggests that most of the disulfides are readily accessible to the reagent, whereas a small fraction is buried in less accessible locations.

All three species of spores were readily killed by suspension in 1 N NaOH, as indicated by comparison of Coulter counter cell counts and viable counts. Less than 1% of the spores survived after soaking for 1 hr, in agreement with Whitehouse's findings (10). Spores remained refractile, however, during extraction and subsequent washing, and they remained resistant to 10-min staining with 0.2% aqueous crystal violet.

The sensitivity and ability of the FMA method to distinguish between sulfhydryl and disulfide groups suggested that it could be used to determine the appearance of sulfhydryl groups after physiological germination. Table 2 shows a comparison of sulfhydryl content of the three spore specimens before and after germination. As noted in the table, there was a considerable rise in the sulfhydryl content after germination in all three species.

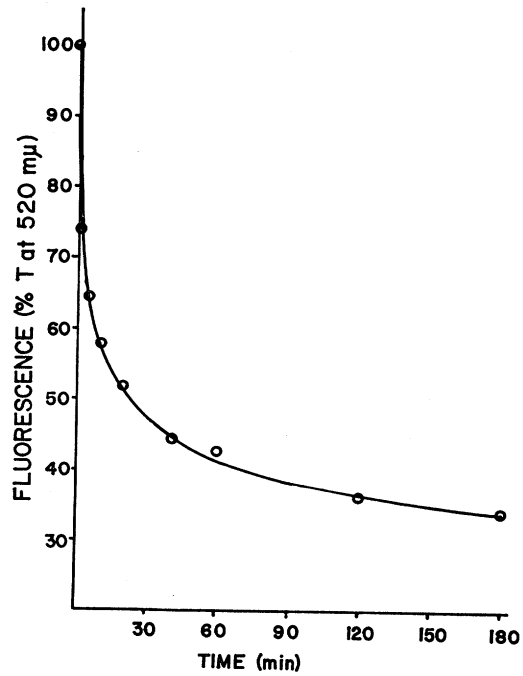

FIG. 1. *Fluorescence quenching of fluorescein mercuric acetate (FMA) by Bacillus subtilis 15u spores. Reaction mixture contained 0.3 mg of spores and 0.03 ml of 10^{-3} M FMA diluted to final volume of 10 ml with 1 N NaOH. Activation wavelength was 499 mμ and fluorescence was measured at 520 mμ.*

TABLE 1. *Disulfide content of intact and ruptured bacterial spores*

Specimen	Moles of disulfide/mg of spores × 10^{-8}		Per cent disulfide of spore dry weight
	Intact spores	Ruptured spores	
Bacillus megaterium QM 1551	7.67	8.25	0.49
B. cereus NRS 804	6.95	8.67	0.45
B. subtilis 15u	9.51	9.67	0.61

TABLE 2. *Comparison of sulfhydryl group content of bacterial spores before and after germination*

Specimen	Moles of sulfhydryl/mg of spores × 10^{-8}	
	Before germination	After germination
Bacillus megaterium QM 1551	0.65	2.2
B. cereus NRS 804	0.29	4.2
B. subtilis 15u	0.84	1.6

Discussion

Table 1 demonstrates that fluorescence quenching of FMA in alkaline medium can be used as a sensitive and reproducible method for differentially estimating the sulfhydryl and disulfide content of intact spores. Disulfide concentrations determined by this method are slightly higher than those reported by Vinter (8); however, the results compare favorably. Measurements made by the FMA method probably represent a more accurate estimate of spore disulfides, by virtue of its application to intact spores, than previously employed techniques of hydrazinolysis and hydrolysis with their inherent inadequacies.

The possibility that FMA was adsorbed onto spore surfaces was refuted by the finding that ruptured spores, which present a much larger surface area, gave disulfide values similar to those of intact specimens. Failure of oxidized spores to quench FMA fluorescence discounts the probability that FMA was reacting with some functional group other than disulfide groups and supports the specificity of the method. Failure to influence the disulfide estimates by organic solvent extraction, by lysozyme treatment, and by extraction with 1 N NaCl excludes the possibility that material externally adsorbed from the culture medium or that disintegrated sporangia were contributing to the observed disulfides.

The ability of the FMA method to distinguish between sulfhydryl and disulfide groups provides an excellent tool for studying the fate of disulfides during physiological germination. It has been suggested (9) that the cystine-containing protein structure of spores may play a dual role by stabilizing structure in the dormant spore and by contributing to the burst of physiological activity during germination. Such a dual role would most certainly involve the reduction of part of the spore disulfides. The results presented in Table 2 definitely demonstrate disulfide reduction and suggest that spores possess a disulfide-reducing system that is active during germination. This system is presently under investigation.

Acknowledgment

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