MOST OF THE COENZYME A IN DORMANT SPORES OF BACILLUS MEGATERIUM IS IN DISULFIDE LINKAGE TO PROTEIN

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SUMMARY: Approximately 70% of the Coenzyme A in dormant spores of Bacillus megaterium is in disulfide linkage to protein. About 75% of these disulfides are cleaved during the first 15 min of spore germination. In contrast, less than 2% of the Coenzyme A in log phase or stationary phase cells is in disulfide linkage to protein.

INTRODUCTION: Dormant spores of the various Bacillus species are devoid of metabolic activity and contain no detectable reduced pyridine nucleotides (1,2,3). In the first minutes of germination metabolism begins and reduced pyridine nucleotides are generated (1,2,3). Since the state of reduction of a cell's low molecular weight sulfhydryl compounds may be related to the state of reduction of its pyridine nucleotide pool, we thought it would be worthwhile to examine the state of reduction of a low molecular weight sulfhydryl compound in dormant and germinated spores of a Bacillus species. CoA*, an important cofactor in metabolism, is a major low molecular weight sulfhydryl compound found in bacteria (4). For this reason, as well as the availability of a sensitive and specific assay (5), the compound we have chosen for study is CoA.

MATERIALS AND METHODS: ^{3}H-CoA was obtained from New England Nuclear. Citrate synthase was obtained from the Boehringer Mannheim Corporation. Acetyl phosphate, ADP, CoA, NAD, NEM, DTT, malic dehydrogenase and phosphotransacetylase were obtained from the Sigma Chemical Company.

All work was carried out with Bacillus megaterium QM B1551 originally obtained from Hillel Levinson (U.S. Army Development Center, Natick, Mass.). Spores of this organism were prepared by growth at 30° in supplemented nutrient broth, harvested, cleaned, lyophilized and stored as previously

* The abbreviations used are: CoA, Coenzyme A; DTT, dithiothreitol, and NEM, N-ethylmaleimide.

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described (6). All spore preparations contained > 95% refractile forms as seen in a phase contrast microscope, and were free of vegetative cells and cell debris. A single preparation of spores was used for all experiments described in this paper, since we have observed significant variations in the amount of protein-bound CoA in different spore preparations (Setlow, B. and Setlow, P., manuscript in preparation).

Spore germination was preceded by a heat shock (10 min, 60°) of a spore suspension (25 mg/ml) in water. After cooling in ice spores were germinated at 2.5 mg/ml and 30° in the medium of Spizizen (7) containing no casamino acids. Under these conditions > 90% of the spores germinated in 15 min. Cells were also grown at 30° in the medium of Spizizen (7) but with 0.1% casamino acids added.

CoA was extracted from cells and spores using hot ethanol. Dormant spores (25 mg/ml in 25 mM KPO_4, pH 7.0) or samples of cells or germinated spores were added without prior centrifugation to preheated (75°) ethanol (final concentration 75%). EDTA (final concentration 1 mM) was then added immediately. DTT (4 mM) or NEM (1 mM) were present, as noted. After 10 min at 75° the sample was cooled in ice and centrifuged. The supernatant was made 1 mM in DTT (5 mM if NEM had been added), flash evaporated and redissolved in 0.5 ml H_2O. This procedure extracted all small molecules including nucleotides from dormant and germinated spores (1,3). A second extraction (if carried out) was performed on the pellet fraction from the first extraction. When small amounts ($\sim$ 1-5 pmole) of ^{3}H-CoA were added to dormant spores immediately after addition of hot ethanol-EDTA, > 90% of the ^{3}H-CoA was recovered in the supernatant fraction. Dormant spores were also extracted as described above after prior disruption of dry spores in a dental amalgamator with glucose crystals as the abrasive as previously described (2,8). This procedure completely disrupted dormant spores allowing facile extraction of small molecules (2).

Acetyl CoA was extracted from intact spores as described above by incubation in ethanol-EDTA (5). After 10 min at 75° in the presence of 0.5 mM DTT the suspension was made 2 mM in NEM, incubation continued for another 10 min and the suspension cooled, and centrifuged. The supernatant fluid was made to 5 mM in DTT, flash evaporated, the residue dissolved in 0.5 ml of H_2O and analyzed for CoA. CoA in the thiol form added to spores just after their addition to the ethanol-DTT mixture described above was completely (> 98%) inactivated by the NEM under these conditions.

Pronase treatment of dormant spore protein was carried out on dry ruptured spores (25 mg) suspended in 1 ml of 25 mM KPO_4 (pH 7.4), 2 mM EDTA and 1 mM NEM. After incubation for 30 min at 4° the suspension was centrifuged (6000 x g, 3 min) to remove unbroken spores, and the cloudy supernatant fluid dialyzed in acetylated tubing against 1 liter of 25 mM KPO_4 (pH 7.4) and 2 mM EDTA at 4°. After 6 hours of dialysis aliquots (0.4 ml) were incubated in 25 mM KPO_4 (pH 7.4), 2 mM EDTA and 5 mM $CaCl_2$ with or without pronase (20 µg). After 20 min at 37° the solution was diluted 1/1 with 5% acetic acid, centrifuged, and the supernatant fluid lyophilized and redissolved in water. The acetic acid pellet was then extracted with ethanol plus 5 mM DTT as described above, the supernatant fluid flash evaporated, and the residue dissolved in water. Aliquots of both supernatant fluids were assayed directly for CoA.

CoA was determined using the cycling assay described by Allred and Guy (5). This assay measures the thiol form of CoA, CoA in disulfide linkage, and acetyl CoA but not longer chain acyl CoA derivatives.

TABLE I

Coenzyme A Distribution in Dormant Spores

Spores were extracted, incubated and analyzed as described in Materials and Methods.

	Spores extracted	Extraction Procedure		CoA in each extraction pmole/mg dry spores	
		First	Second	First	Second
1.	Intact	Ethanol + DTT	-	875	-
2.	Intact	Ethanol	Ethanol + DTT	302	658
3.	Intact	Ethanol	Ethanol	324	15
4.	Intact	Ethanol + NEM	Ethanol + DTT	36	700
5.	Dry ruptured	Ethanol	Ethanol + DTT	125	668
6.	Dry ruptured, NEM treated and incubated without pronase	Acetic acid	Ethanol + DTT	134	485
7.	Dry ruptured, NEM treated and incubated with pronase	Acetic acid	Ethanol + DTT	580	< 10

RESULTS AND DISCUSSION: Treatment of intact or dry-ruptured dormant spores of B. megaterium with hot ethanol in the absence of a reducing agent extracted a significant amount of CoA (first extraction, lines 2, 3 and 5; Table I). Although the hot ethanol treatment of dry ruptured and even intact dormant spores extracts all small molecules (see Methods), when ethanol extraction was carried out in the presence of DTT, about 3 times more CoA was extracted from dormant spores (line 1; Table I). Furthermore, a re-extraction with ethanol-DTT of spores which had first been extracted with ethanol alone, released about 2 times as much CoA as was released in the first extraction (lines 2, and 5; Table I). The DTT was necessary for solubilization of CoA in the second extraction (line 3; Table I). When dormant spores were extracted with ethanol plus NEM little CoA was found;

TABLE II

Coenzyme A Distribution in Spores and Cells

Spores and cells were prepared and extracted with ethanol followed by extraction with ethanol plus DTT as described in Materials and Methods. CoA in disulfide linkage to protein was calculated as the percentage of the total CoA which was extracted with ethanol plus DTT. Acetyl CoA was determined as described in Materials and Methods.

Stage of growth	CoA in disulfide linkage to protein % of total CoA	Total CoA pmole/mg dry wt[a]
Dormant spores[b]	69	960 (< 15)
Spores germinated 15 min	16	916 (405)
Mid-log phase cell	< 2	1450
Stationary phase cell - 3 hours after the end of log phase growth	< 2	1790

[a] Values in parentheses are the pmoles of acetyl CoA/mg dry wt.

[b] Similar results were obtained with heat shocked spores

if these spores were then re-extracted with ethanol-DTT, the second extraction released as much CoA as was found in experiments when NEM was not used in the first extraction (lines 2 and 4; Table I).

The data cited above suggest that about 30% of the spore's CoA is in the free thiol form, while about 70% of the CoA is present in disulfide linkage with a macromolecule which is insoluble in 75% ethanol. That this macromolecule is protein is suggested by the results of incubation of NEM-treated dry ruptured dormant spores with or without pronase. Without pronase treatment the CoA remained acid insoluble, while pronase digestion rendered > 90% of the CoA acid soluble (Table I).

Although the majority of the CoA in dormant spores is in disulfide

linkage to protein, this is not the case in growing cells or germinated spores. In either growing or stationary phase cells < 2% of the cellular CoA is in disulfide linkage to protein, while in germinated spores this value is 16%. Since the total CoA content is fairly constant in spores going from the dormant to germinated state, this suggests that the majority of the CoA-protein disulfides in the dormant spore are cleaved early in spore germination. Interestingly, approximately half of the total cellular CoA is present as acetyl CoA in germinated spores, while dormant spores contain no detectable acetyl CoA (Table II).

In view of the absence of reduced pyridine nucleotides from dormant bacterial spores (3), the finding that about 70% of the CoA in dormant spores of B. megaterium is in disulfide linkage to protein may not be surprising. This finding, coupled with the cleavage of these disulfides in the early minutes of spore germination, is another example of the dramatic changes which take place early in bacterial spore germination. Significantly the magnitude of the change in the state of reduction of CoA during spore germination is much larger than previously noted for changes in sulfhydryl/disulfide ratios during the breaking of dormancy in other systems. For example, the germination of spores of the fungus Neurospora crassa is accompanied by a significant decrease in the content of mixed disulfides between protein and glutathione (9). However, in the dormant fungal spore these disulfides account for < 1% of the total glutathione pool, of which 99% exists in the thiol form (9).

What is the function of the CoA-protein disulfides in dormant bacterial spores? As has recently been proposed for the glutathione-protein disulfides of dormant fungal spores (10), the CoA-protein disulfides in dormant bacterial spores might be involved in the metabolic dormancy and/or the high heat resistance of the spore. Late in sporulation the formation of a disulfide between CoA and a sulfhydryl group of an enzyme within the developing spore could inactivate this enzyme and contribute to the onset

and maintainance of metabolic dormancy. Similarly, the formation of such a mixed disulfide might contribute to the high heat resistance of enzymes within the dormant spore. The rapid cleavage of these CoA-protein disulfides in the first minutes of spore germination would then provide a simple mechanism for the activation of metabolism and loss of heat resistance which occur at this time.

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