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Levels of Acetyl Coenzyme A, Reduced and Oxidized Coenzyme A, and Coenzyme A in Disulfide Linkage to Protein in Dormant and Germinated Spores and Growing and Sporulating Cells of *Bacillus megaterium*

BARBARA SETLOW AND PETER SETLOW*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

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Dormant spores of *Bacillus megaterium* were found to contain ~850 pmol of coenzyme A (CoA) per milligram of dry weight. Of this total, less than 1.5% was acetyl-CoA, 25% was CoA-disulfide, 43% was in disulfide linkage to protein, and the remainder was the free thiol. Dormant spores of *Bacillus cereus* and *Clostridium bifermentans* contained 700 and 600 pmol of CoA per milligram of dry weight, respectively; in both species ~45% of the CoA was in disulfide linkage to protein. During germination of spores of all three species, >75% of the CoA-protein disulfides were cleaved. In *B. megaterium*, cleavage of these disulfides during spore germination did not require exogenous metabolites and occurred at about the same time as the initiation of germination. Much of the CoA was converted to acetyl-CoA at this time. Dormant spores also contained reduced nicotinamide adenine dinucleotide-dependent CoA-disulfide reductase at levels higher than those in other stages of growth. The level of total CoA in growing cells was two- to three-fold higher than in spores. This level remained constant throughout growth and sporulation, but <2% of the total cellular CoA was in disulfide linkage to protein until late in sporulation. The CoA-protein disulfides accumulated exclusively within the developing spore at about the time when dipicolinic acid was accumulated.

Dormant bacterial spores carry out no endogenous metabolism (8, 27) or macromolecular biosynthesis (13, 19) and are extremely resistant to heat and ultraviolet radiation (16); these properties of the dormant state can be maintained for long periods of time. However, if spores initiate the process of germination, metabolism and macromolecular biosynthesis begin, and the resistant properties are lost (13, 16, 27). It was suggested a number of years ago that dormant-spore proteins contained a large amount of disulfide bonds, and that many of these disulfides were cleaved early in spore germination (6, 30). It was further suggested that the high level of these disulfide bonds in the dormant spore might be important in the dormant and resistant properties of the spore (6, 30). Although it was subsequently shown that there was no extensive cleavage of protein disulfide bonds early in spore germination, this report did not rule out the possibility of cleavage of a small number of disulfide bonds in germination (26). Indeed, a nonspecific pyridine nucleotide-dependent disulfide reductase has been identified in spores of *Bacillus cereus* (5).

Two recent reports have suggested that it might be worthwhile reinvestigating the question of disulfide bond cleavage during bacterial spore germination. The first was the demonstration that germination of spores of the fungus *Neurospora crassa* is accompanied by increases in the content of reduced pyridine nucleotides and also by cleavage of the small amounts of both oxidized glutathione (GSSG) and glutathione in disulfide linkage to protein that accumulated during spore formation (9, 21). It was suggested that the increased level of disulfides in the dormant state might contribute to the dormancy and/or resistance of these fungal spores (20). A second report (22) demonstrated that dormant bacterial spores contained no reduced pyridine nucleotides, although the oxidized forms were present. Both reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were generated in the first minutes of spore germination (22).

These data suggested that it might be interesting to investigate the oxidation-reduction state of a specific sulfhydryl compound in grow-

ing and sporulating cells and dormant and germinated bacterial spores. In particular, the study of a low-molecular-weight sulfhydryl compound was suggested, since the oxidation-reduction state of such compounds is often coupled directly to the oxidation-reduction state of the pyridine nucleotide pool. The compound we chose to study was coenzyme A (CoA), which is present in rather high levels in bacteria (11) and for which there is a sensitive and specific assay (1). Preliminary results indicated that dormant spores of *Bacillus megaterium* contained high levels of CoA in disulfide linkage to protein and that these disulfides were cleaved during spore germination (23). This report presents more extensive studies of the levels of these CoA-protein disulfides as well as other forms of CoA during growth and sporulation in the dormant spore and during spore germination of *B. cereus* and *Clostridium bifermentans*. A preliminary account of this work has appeared (B. Setlow and P. Setlow, Fed. Proc. 34:923, 1977).

MATERIALS AND METHODS

Reagents and enzymes. The thiol form of CoA (CoA-SH), the disulfide form of CoA (CoA-S-S-CoA), reduced glutathione (GSH), GSSG, NAD, NADH, NADPH, cystine, dithiothreitol (DTT), acetylphosphate, *N*-ethylmaleimide (NEM), trypsin, pig heart malic dehydrogenase, yeast glutathione reductase, and yeast citrate synthase were from Sigma Chemical Co. [^{3}H]CoA (1 mCi/μmol) and [^{14}C]cystine (250 mCi/mmol) were from New England Nuclear Corp. Phosphotransacetylase was from the Boehringer-Mannheim Corp.

Growth of cells and spores. The majority of work described here was done with *B. megaterium* QM B1551, originally obtained from Hillel Levinson (U.S. Army Research Laboratories, Natick, Mass.). Unless otherwise noted, spores of this organism were prepared by growth in supplemented nutrient broth at 30°C, washed, lyophilized, and stored as previously described (26). For isolation of developing forespores, cells were also grown in supplemented nutrient broth, and cells were harvested and treated with lysozyme to convert them into sonic treatment-sensitive protoplasts; forespores were then obtained (>85% recovery) as previously described (28). Cells were also grown at 30°C in the medium of Spizizen (29) supplemented with 0.1% Casamino Acids, and spores were also prepared in this medium. *B. cereus* T was originally obtained from Harlyn O. Halvorson (Rosenstiel Basic Research Center, Waltham, Mass.), and spores of this organism were prepared in supplemented nutrient broth. Spores of *C. bifermentans* were the generous gift of William M. Waites (Food Research Institute, Agricultural Research Council, Norwich, Great Britain). All spore preparations used in this study contained >95% refractile forms when viewed in a phase-contrast microscope and were free of vegetative cells and cell debris.

Spore germination. Germination of *B. megaterium* spores was preceded by a heat shock (60°C, 10 min) of spores (25 mg [dry weight]/ml) in water. After cooling in ice, the spores (2.5 mg [dry weight]/ml) were germinated at 30°C either in 50 mM KBr and 10 mM KPO_4 (pH 7.4) (KBr medium) or in 100 mM glucose and 50 mM KPO_4 (pH 7.4) (glucose medium). The initiation of germination was monitored by measuring the optical density of the culture (27).

Germination of spores of *B. cereus* and *C. bifermentans* was carried out as previously described (22).

Extraction and analysis of CoA. Samples (2 ml) of growing cells or spores were added without prior centrifugation to 8 ml of ethanol at 75°C (cells) or 80°C (forespores and dormant and germinated spores). Other reagents were present as noted. The mix was immediately made 5 mM in ethylenediaminetetraacetic acid (EDTA) and ~25 mM in KPO_4 (pH 7.0). After incubation at either 75°C (cells) or 80°C (forespores or spores) for 15 min, the mix was chilled in ice and centrifuged. The supernatant fluid was made to 2 mM in DTT and flash evaporated, and the residue was redissolved in 500 μl of water. This 15-min treatment at 80°C extracts >98% of the dipicolinic acid from all dormant spores tested and >98% of the free adenine nucleotides from spores of *B. megaterium* (B. Setlow and P. Setlow, unpublished data). This first extraction also removed acyl-CoA's, CoA-SH, and CoA-S-S-CoA (see Results) (1, 23). CoA bound in disulfide linkage to protein was then extracted from the pellet by a second treatment at 75°C (cells) or 80°C (spores) in 5 ml of 80% ethanol, 2 mM KPO_4 (pH 7.0), and 4 mM DTT (23). After 10 min, this mix was chilled and centrifuged; then the supernatant fluid was flash evaporated, and the residue was dissolved in 500 μl of water. Unless otherwise noted, these two extraction procedures were used in all determinations of total cellular CoA and CoA in disulfide linkage to protein.

To obtain only acyl-CoA's, DTT (2 mM) was also present in the first extraction; after 15 min at 80°C, the suspension was made 5 mM in NEM and incubation was continued for an additional 10 min (1). The suspension was chilled and centrifuged, and the supernatant fluid made 5 mM in DTT and then flash evaporated. This procedure inactivated all exogenous CoA-SH added to spores (data not shown) and also inactivated all CoA in dormant spores (see Results), indicating that only acyl-CoA's survive this treatment (1). Since dormant spores contained no acetyl-CoA (see Results), we were also able to determine CoA-S-S-CoA in these spores by carrying out the first extraction in the presence of NEM (2 mM) to inactivate CoA-SH. After 15 min at 80°C, the suspension was chilled and centrifuged, and the supernatant fluid made 2 mM in DTT and flash evaporated.

In a few cases, dormant spores were broken in the dry state in a dental amalgamator (Wig-L-Bug), using glucose crystals as the abrasive as previously described (18, 27). The dry powder from this treatment was extracted as described above.

Separation of soluble and insoluble protein. Spores (~50 mg [dry weight]) were dry ruptured as

described above, and the resulting powder was suspended at 15 mg of dry spores per ml in 25 mM KPO_4 (pH 7.4), 5 mM EDTA, and 5 mM NEM. After 30 min at 4°C, the mix was dialyzed for 20 h against two changes of the above buffer without NEM. The sample was then centrifuged (10 min, 10,000 × *g*), the pellet was suspended in an equal volume of buffer, and both the supernatant fluid and the suspended pellet were extracted for protein-bound CoA.

Analysis of CoA. CoA was determined fluorimetrically, using the cycling assay described by Allred and Guy (1). This assay does not detect CoA precursors or acyl-CoA's longer than acetyl-CoA, but reacts equally well with CoA-SH, CoA-S-S-CoA, and acetyl-CoA (1).

Assay of disulfide reductase. Assays for disulfide reductase were carried out in 900 μl of 50 mM KPO_4 (pH 7.4) with 1 mM disulfide, 1 mM NADH or NADPH, and 0.1 to 0.5 mg of protein from spore or cell extracts. After 30 min at 37°C, 100 μl of 50% trichloroacetic acid was added, and the mix was chilled and centrifuged. The addition of the trichloroacetic acid caused only a slight reoxidation of GSH. A sample (300 μl) of the supernatant fluid was then added to 700 μl of a solution of 1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 1 mM dithiobisnitrobenzoic acid. After 5 min at room temperature, the absorbance of this solution was read at 412 nm and disulfide reduction was quantitated with reference to a standard curve constructed using GSH. Blanks without NADH, NADPH, or substrate were subtracted. When extracts from different stages of growth were mixed and assayed, no growth stage-specific inhibitors or activators were found. Extracts of spores and cells for enzyme assays were prepared by sonic treatment in the presence of glass beads (25), and protein was determined by the procedure of Lowry et al. (14).

Other methods. GSH, GSSG, cysteine, cystine, and their protein-bound disulfide derivatives were extracted as was CoA. Glutathione was determined by the method of Fahey et al. (9). Cysteine and cystine were measured by amino acid analysis after performic acid oxidation (26). Dipicolinic acid was analyzed by the method of Rotman and Fields (17).

Spore coat proteins were removed by incubation of spores at 5 mg/ml in 0.1 M glycine buffer (pH 11.0) containing 1.5% sodium dodecyl sulfate (SDS) and 0.1 M DTT. This procedure removes coat proteins from spores of several species (4, 24). After 2 h at 37°C, the spores were centrifuged, and the supernatant fluid was made 0.1 M in KCl to remove SDS before analysis of CoA.

Trypsin treatment of spore protein was carried out as previously described (23). Descending chromatography of [^{3}H]CoA was carried out on Whatman no. 1 paper with a solvent of 1-butanol–acetic acid–water (5:2:3) for 21 h at 24°C.

RESULTS

Levels of various forms of CoA in dormant spores of *B. megaterium*. Analysis of dormant spores of *B. megaterium* revealed that they contained a significant amount of CoA. However, less than 1.5% of the total CoA was present as acetyl-CoA, a finding consistent with the known absence of common high-energy compounds in dormant bacterial spores (Table 1) (22, 27). Significantly, 43% of the

TABLE 1. *Levels of various forms of CoA in dormant spores of B. megaterium*[a]

CoA determined for:	Form of CoA (pmol/mg of dry spores)				
	Acetyl-CoA	CoA-SH	CoA-S-S-CoA	Total soluble CoA	CoA in disulfide linkage to protein
1. Intact spores	<10 (<1.5)	260 (32)	200 (25)	460	340 (43)
2. Intact spores – heated for 10 min at 60°C	–[b]	–	–	485	370
3. Intact spores – prepared in Spizizen medium	–	–	–	390	425
4. Intact spores – no DTT in second extraction	–	–	–	500	<20
5. Intact spores – 1 or 10 mM NEM in first extraction	–	–	210	–	370
6. Dry ruptured spores	–	–	–	510	405
7. Dry ruptured spores – dialyzed and not treated with trypsin[c]	–	–	–	<25	390
8. Dry ruptured spores – dialyzed and treated with trypsin[c]	–	–	–	315	<20

[a] Spores were extracted, treated, and analyzed as described in the text. Values in parentheses are the percentage of total CoA.

[b] –, Not determined.

[c] Spores were dry ruptured and suspended at 20 mg/ml in 25 mM KPO_4 (pH 7.4), 5 mM EDTA, and 5 mM NEM. After 1 h at 4°C, the suspension was dialyzed for 24 h at 4°C against the same buffer without NEM. The mix was then analyzed for soluble and protein-bound CoA before or after trypsin treatment (100 μg of trypsin, 30 min, 37°C).

dormant spore's CoA was in disulfide linkage to protein as previously reported (23), and 25% of the total CoA was present as CoA-S-S-CoA (Table 1). Approximately 45% of the total CoA was also present as CoA-protein disulfides in heat-activated spores and in spores prepared in Spizizen medium (Table 1).

Since it seemed possible that the extraction procedure itself might have generated these oxidized forms of CoA, in particular the CoA-protein disulfides, we performed a number of controls to show that this was not the case. (i) The amount of CoA-protein disulfide in dormant spores was similar when spores were extracted with or without prior disruption of the spores in a dental amalgamator (lines 1 and 6, Table 1). (ii) Inclusion of NEM (1 or 10 mM) in the first extraction with ethanol had no effect on the yield of CoA in the second extraction (lines 1 and 5, Table 1). (iii) When small amounts (~10 pmol) of [^{3}H]CoA were present with the spores during the first extraction, <5% of the CoA became bound to the spores, and >85% of the added counts were recovered in CoA as shown by paper chromatography (data not shown). Other experiments also demonstrated that: (i) extraction of CoA from CoA-protein disulfides required a disulfide reducing agent (lines 1 and 4, Table 1); and (ii) the putative CoA in disulfide linkage to protein was solubilized by protease digestion (lines 7 and 8, Table 1). These controls, plus the facts that (i) the extraction conditions remove all small molecules from dormant spores, (ii) we saw no CoA-protein disulfides in growing cells (see below), and (iii) CoA-protein disulfides disappeared during spore germination (see below) strongly indicate that the observation of CoA-protein disulfides in dormant bacterial spores is not an artifact.

Cleavage of CoA-protein disulfides during spore germination. Although dormant spores of *B. megaterium* contain no reduced pyridine nucleotides, these compounds are known to be generated in the first minutes of spore germination (22). Since many enzymes are known that catalyze pyridine nucleotide-dependent disulfide reduction, it seemed likely that the CoA-protein disulfides might be cleaved early in germination. Indeed this was the case, and the cleavage of these disulfides occurred in the first minutes of germination at or slightly before the time of the fall in optical density, denoting the initiation of spore germination (Table 2, Fig. 1 and 2). In Fig. 1 and 2, note that the disulfide cleavage is presented as percentage of maximum amount of disulfide cleaved (see below). Although these results indicate that there is cleavage of disulfide

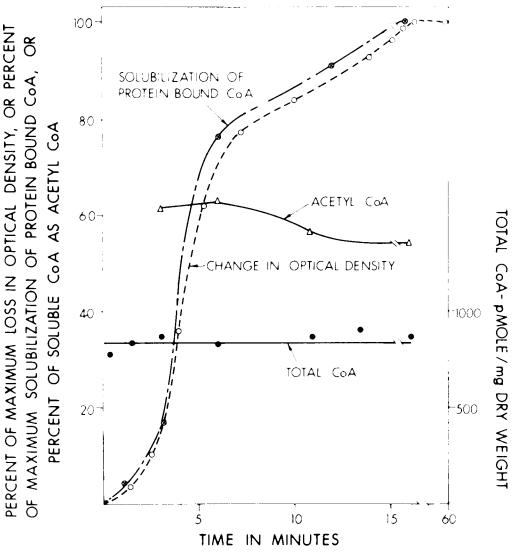

FIG. 1. *Levels of acetyl-CoA, protein-bound CoA, and total CoA during germination of B. megaterium spores in the glucose medium. Spores were germinated in the glucose medium, and the percentage of germination was determined from the fall in optical density as described in the text. Samples were taken and extracted either for acetyl-CoA, CoA-SH, and CoA-S-S-CoA, followed by extraction of CoA in protein disulfide linkage, or for acetyl-CoA followed by extraction of CoA in disulfide linkage in protein. Samples were treated and analyzed as described in the text. Total CoA is the sum of acetyl-CoA, CoA-SH, CoA-S-S-CoA, and CoA in disulfide linkage to protein. In this experiment, 83% of the CoA-protein disulfides present in the dormant spore were cleaved by 60 min of germination.*

bonds early in spore germination, the CoA-protein disulfides cleaved amount to less than 2% of the total disulfide content in the dormant spore (26).

Cleavage of the CoA-protein disulfides occurred even during germination in the KBr medium with no exogenous source of energy or reducing power present (Fig. 2). This indicates that endogenous reserves of reducing power can suffice for this disulfide cleavage. Previous work has also shown that endogenous reserves can be used to generate NADH and NADPH early in spore germination (22). Interestingly, only 85% of the CoA-protein disulfides were cleaved even when germination was extended for 60 min (Fig. 1 and 2, Table 2). The CoA-protein disulfides that were not cleaved were found primarily in the insoluble protein fraction (Table 3). This is in contrast to the total CoA-protein disulfides in the dormant spore, which were found primarily in the soluble protein fraction (see below).

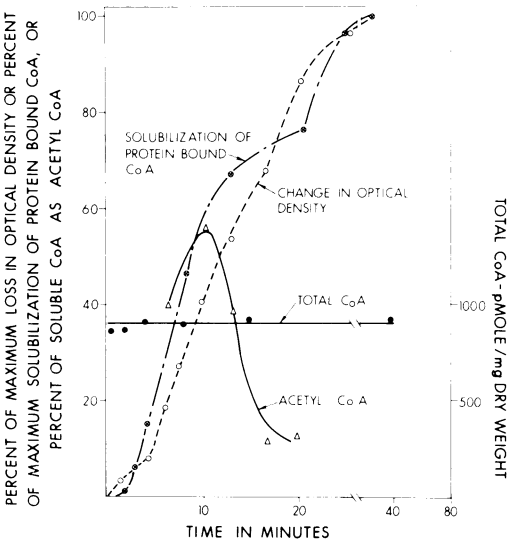

FIG. 2. *Levels of acetyl-CoA, protein-bound CoA, and total CoA during germination of B. megaterium spores in the KBr medium. Spores were germinated in the KBr medium; samples were taken, treated, and analyzed; and calculations were performed as in the legend to Fig. 1. In this experiment, 85% of the CoA-protein disulfides present in the dormant spore were cleaved by 60 min of germination.*

TABLE 2. *Levels of total CoA and CoA in disulfide linkage to protein in dormant and germinated spores of several species*[a]

Spores from:	Total CoA (pmol/mg [dry wt])	CoA-protein disulfides (% of total CoA)
B. cereus, dormant	710[b]	45 (47)
B. cereus, germinated for 25 min[c]	700	8[d]
B. megaterium, dormant	820	43 (44)
B. megaterium, germinated for 60 min[c]	835	7
C. bifermentans	610	55 (49)
C. bifermentans, germinated for 25 min[c]	580	16[d]

[a] Spores were extracted and analyzed as described in the text. Values in parentheses are for spores extracted initially with 1 mM NEM present.

[b] Removal of spore coat proteins (see text) extracted <5% of the total CoA.

[c] Germination >95% as determined by phase-contrast microscopy.

[d] This value was not lowered by longer germiation.

The early minutes of spore germination were also accompanied by formation of significant amounts of acetyl-CoA even in spores germinating in the KBr medium (Fig. 1 and 2). In the latter medium, however, the acetyl-CoA level fell rapidly as endogenous reserves of activatable acetyl groups were exhausted. Preliminary work has indicated that during germination in the KBr medium the acetyl moiety of acetyl-CoA is derived at least in part from metabolism of the depot of 3-phosphoglyceric acid stored in the dormant spore (27) (B. Setlow and P. Setlow, unpublished data). In both media tested, the total amount of CoA remained constant through at least 40 min of germination. Values for soluble CoA in germinated spores resistant to NEM with or without pretreatment with DTT were identical (data not shown), indicating that there is little CoA-S-S-CoA in germinated spores.

TABLE 3. *Location of CoA-protein disulfides in dormant and germinated spores of B. megaterium*[a]

CoA determined for:	CoA removed from spores by extraction of coat proteins	CoA in disulfide linkage to soluble protein	CoA in disulfide linkage to insoluble protein
Intact dormant spores	<40 (<5)[b]	–[c]	–
Disrupted dormant spores	–	385 (43)	80[d] (10)
Disrupted germinated spores	–	18 (2)	65 (8)

[a] Spores were treated, extracted, and analyzed as described in the text. Values in parentheses are percentages of total CoA. Amounts of CoA are expressed as picomoles per milligram (dry weight).

[b] <10% of exogenously added CoA (~100 pmol/mg of dry spores) was destroyed by the procedure for extraction of spore coat proteins.

[c] –, Not determined.

[d] Similar amounts of CoA were found in this fraction of disrupted spores when samples were not dialyzed before centrifugation and analysis.

Location of the CoA-protein disulfides in the dormant spore. It has been reported that spores of *B. cereus* contain significant amounts of non-protein cysteine in disulfide linkage to coat proteins (3). However, the CoA-protein disulfides in spores of *B. megaterium* are not in the spore coat, since no CoA was removed by treatment at pH 11 with SDS and DTT (Table 3), a treatment previously shown to solubilize spore coat protein (4, 27). Indeed, the CoA appeared to be bound primarily to soluble proteins presumably located in the spore core (Table 3).

Levels of various forms of CoA in dormant and germinated spores of several species. The high levels of CoA-protein disulfides in dormant spores of *B. megaterium* and their dramatic cleavage during germination made it of obvious interest to examine spores of several

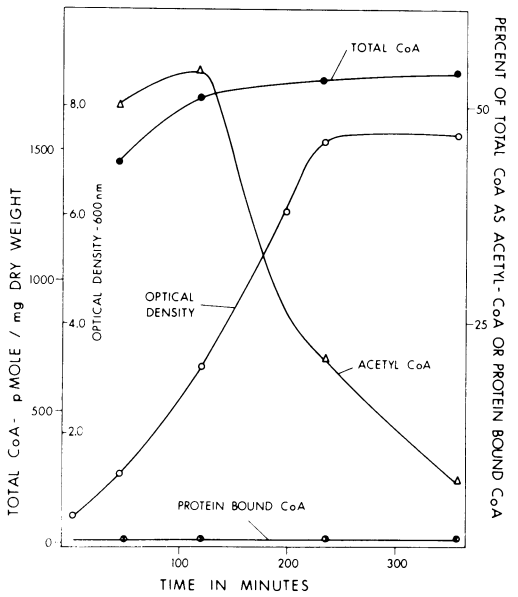

FIG. 3. *Levels of acetyl-CoA, protein-bound CoA, and total CoA during log- and stationary-phase growth of B. megaterium. Cells were grown at 30°C in Spizizen medium containing 0.1% Casamino Acids, and samples were taken, treated, and analyzed as described in the legend to Fig. 1.*

other species for this phenomenon. Dormant spores of both *B. cereus* and *C. bifermentans* also contained significant amounts of CoA, with close to 50% of the total as CoA-protein disulfides (Table 2). As with spores of *B. megaterium*, inclusion of 1 mM NEM in the first extraction step did not lower the yield of CoA-protein disulfides, and no CoA was removed by procedures that remove spore coat protein (Table 2). The majority of the CoA-protein disulfides in spores of *B. cereus* and *C. bifermentans* were also cleaved during spore germination, although as found with *B. megaterium*, some CoA-protein disulfides remained (Table 2). Germination of spores of *B. cereus* and *C. bifermentans* is also known to be accompanied by rapid generation of NADH and NADPH (22).

Changes in various forms of CoA during growth and sporulation of *B. megaterium*. With data on the level of various forms of CoA in dormant and germinated spores of *B. megaterium*, it was of interest to determine the levels of the various forms of CoA in growing and sporulating cells, in particular the time of appearance and the location (mother cell and/or forespore) of the CoA-protein disulfides destined for the dormant spore. Cells growing in Spizizen medium contained levels of total CoA similar to those in spores, but less than 2% of the CoA was in disulfide linkage to protein (Fig. 3). Similarly, <5% of the total CoA was CoA-S-S-CoA (data not shown). During log-phase growth, ~50% of the total CoA was acetyl-CoA, but this value fell dramatically during stationary-phase growth. The known rapid operation of the tricarboxylic acid cycle during stationary-phase growth of *Bacillus* species (31) may have been the cause of the lowered steady-state level of acetyl-CoA at this time.

When cells were allowed to sporulate in supplemented nutrient broth and the whole cell (mother cell plus forespore) and the developing forespore were analyzed separately, the majority of the cell's CoA was initially found in the mother cell compartment (Fig. 4A, B). The CoA level in the forespore increased about 30% early in its development and then remained constant (Fig. 4B), whereas the level in the whole cell fell almost twofold (Fig. 4A). Similar findings have been obtained on adenine nucleotide levels in the whole cell and the forespore late in sporulation (28).

At the earliest time isolated, forespores contained no detectable CoA-protein disulfides. However, these moieties appeared slightly after the time that dipicolinic acid accumulated within the forespore (Fig. 4B). After reaching a value of ~45% of the total CoA, the level of CoA-protein disulfides remained constant even when sporulating cultures were shaken for an additional 5 days (Fig. 4B). In contrast to the high levels of CoA-protein disulfides in the forespore, the level of CoA-protein disulfides in the whole sporulating cell could be almost completely accounted for by the CoA-protein disulfides within the forespore (compare Fig. 4A and B; note that values for CoA-protein disulfides are given as percentage of total CoA).

Absence of significant levels of other low-molecular-weight sulfhydryl compounds in spores and cells of *B. megaterium* and *B. cereus*. One might expect that the dramatic changes in the oxidation-reduction state of CoA during sporulation and germination of *B. megaterium* would be exhibited by other low-molecular-weight sulfhydryl compounds. However, we were unable to detect significant levels of cystine, cysteine, GSSG, GSH, or cysteine and GSH in disulfide (but not peptide) linkage to protein in dormant spores of *B. megaterium*.

GSSG, GSH, and protein-bound GSH were also not detected either in mid-log-phase cells of *B. megaterium* growing in either supplemented nutrient broth or Spizizen medium or in dormant spores and mid-log-phase cells of *B. cereus*. In all cases, the level of total gluta-

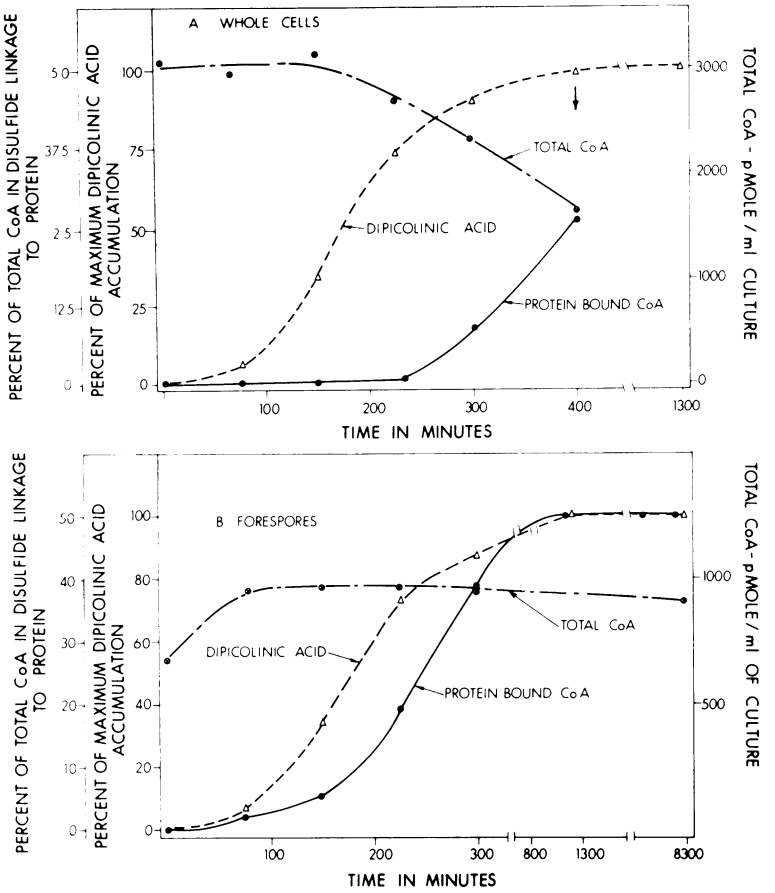

FIG. 4. *Levels of total CoA and protein bound CoA in (A) lysozyme-treated cells and (B) forespores of sporulating cells of B. megaterium. Cells were grown in supplemented nutrient broth and treated with lysozyme to convert them into sonic treatment-sensitive protoplasts. (A) Lysozyme-treated cells were extracted and analyzed as described in the legend to Fig. 1. (B) Forespores were prepared by sonic treatment of lysozyme-treated cells, and these were extracted and analyzed as described above. At the time denoted by the arrow in (A), no cell lysis could be observed in the phase-contrast microscope.*

thione (as GSH residues) or cysteine/cystine (as cysteine residues) was less than 5 pmol/mg (dry weight), a value two orders of magnitude lower than the CoA level.

Levels of disulfide reductases in various stages of growth of *B. megaterium*. The evidence that large amounts of CoA-protein disulfides are cleaved early in spore germination made it of obvious interest to identify an enzyme capable of such cleavage. As yet we have not identified an enzyme in any stage of the life cycle capable of direct cleavage of CoA-protein disulfides, using either other thiols or reduced pyridine nucleotides as a source of reducing power. However, dormant spores did contain an NADH-dependent CoA-S-S-CoA reductase (Table 4). Significantly, this enzyme was present at its highest level in the dormant spore and there was little NADH-dependent GSSG or cystine reductase in any stage of the life cycle (Table 4). *B. megaterium* also contained one or more NADPH-dependent disulfide reductases showing similar activity in GSSG, cysteine, and CoA-S-S-CoA. The level of this enzyme(s) was relatively constant in all stages of growth, with highest levels present in growing cells (Table 4).

DISCUSSION

The formation of CoA-protein disulfides within the developing spore late in sporulation and their rapid cleavage early in spore germination are further examples of the dramatic changes that occur in these periods of development. Previous work has indicated: (i) that this period in sporulation is also characterized

TABLE 4. *Levels of pyridine nucleotide-dependent disulfide reductases in various stages of growth of B. megaterium*[a]

Disulfide	Reduced pyridine nucleotide	Disulfide reductase activity (nmol/min per mg of protein)		
		Dormant spore	Log-phase cell[b]	Stationary-phase cell[c]
GSSG	NADPH	0.73	1.42	1.5
GSSG	NADH	0.15	0.12	0.08
Cystine	NADPH	0.92	1.50	1.27
Cystine	NADH	0.16	0.21	0.18
CoA-S-S-CoA	NADPH	0.60	1.03	1.02
CoA-S-S-CoA	NADH	1.50	0.6	0.47

[a] Spores and cells were extracted and assayed for disulfide reductase activity as described in Materials and Methods.

[b] Cells were grown in supplemented nutrient broth and harvested in mid-log phase.

[c] Cells were grown in supplemented nutrient broth and harvested ~2.5 h after the end of log phase.

by the loss of adenosine triphosphate (ATP) and NADH from the forespore (but not the mother cell) (28); and (ii) that this period in germination is accompanied by rapid formation of ATP, NADH, and NADPH (22, 28). The facts that (i) dramatic changes in NADH and NADPH levels occur during the same periods as formation and cleavage of the CoA-protein disulfides and (ii) an NADH-dependent CoA-reductase has been identified in spores suggest that formation and cleavage of these CoA-protein disulfides may be linked to the oxidation-reduction state of the spore pyridine nucleotide pool. Since we have been unable to identify a spore enzyme capable of cleavage of CoA-protein disulfides, the cleavage of these disulfides during germination may proceed via thiol-disulfide interchange between CoA-protein disulfides and CoA-SH. The latter may be formed in part via pyridine nucleotide-dependent reduction of CoA-S-S-CoA early in germination. Similarly, the formation of CoA-protein disulfides during sporulation may proceed via disulfide interchange between protein sulfhydryl groups and CoA-S-S-CoA formed due to the loss of NADH and NADPH from the developing forespore. Clearly there are a number of unanswered questions about these processes. For instance, we do not know why only 45% of the total spore CoA is in disulfide linkage to protein in all dormant spores tested, nor do we know why all of these disulfides are not cleaved during germination.

Despite the lack of knowledge of the mechanisms of formation and cleavage of spore CoA-protein disulfides, the fact that they are present at such high levels in spores of all species tested certainly suggests that they may have some function in the dormant spore. As recently proposed for the protein-glutathione disulfides in dormant fungal spores (21), two likely functions might be: (i) inactivation of key spore enzymes via formation of a mixed disulfide with a critical sulfhydryl group, thus causing and maintaining the metabolic dormancy of the spore; and (ii) contribution to the heat and radiation resistance of spore enzymes by protecting enzyme sulfhydryl groups. CoA-protein disulfides do appear to be located primarily in the spore core, presumably the site of most spore enzymes. It will be of interest to learn what spore proteins contain CoA in disulfide linkage.

One striking finding in this work was that CoA was the predominant low-molecular-weight sulfhydryl compound in spores and cells of *B. megaterium*, since levels of GSH, GSSG, cysteine, and cystine were <1% those of CoA. Glutathione has been reported in cells of *B. cereus* (7, 12, 15). However, the level of this compound in *B. cereus* was <1% of the CoA level, indicating that glutathione is a dispensable molecule for *Bacillus* species. Indeed, mutants of *Escherichia coli* have been identified that lack glutathione yet grow normally (2, 10). Possibly whatever function is served by glutathione (for example, maintenance of enzyme sulfhydryl groups in a reduced state) can also be fulfilled by CoA-SH, since the concentration of total CoA in growing *B. megaterium* is significant (0.45 mM; calculated from data in Fig. 3 and assuming 4 ml of cell water for each gram of dry weight).

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