Presence of Cysteic Acid in the Sporangium and its Metabolic Pathway during Sporulation of *Bacillus subtilis* NRRL B558

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(Received 7 October 1980)

Cells of *Bacillus subtilis* NRRL B558 at early stages of sporulation contained considerable amounts of cysteic acid. The cell cysteic acid content began to increase at the onset of sporulation and was maximal when synthesis of dipicolinic acid commenced; it then decreased as sporulation advanced. Cysteic acid was synthesized from cysteine and metabolized to sulpholactate in the mother-cell cytoplasm, and the sulpholactate was then incorporated into the forespore. This metabolic change is considered to be a general phenomenon in spore-formers that contain sulpholactate in their spores.

**INTRODUCTION**

Identification of spore-specific materials and knowledge of their role during sporulation has been considered essential for studies on sporulation mechanisms. Studies of the chemical constituents of mature spores have revealed the existence of many spore-specific materials, such as dipicolinic acid (Powell, 1953), sulpholactate (Bonsen *et al.*, 1969), spore coat protein (Kadota & Iijima, 1965; Kondo & Foster, 1967; Sano *et al.*, 1975) and cortex materials (Warth & Strominger, 1968). It has been generally accepted that these spore-specific materials are newly synthesized in sporangia from substances derived from vegetative cell constituents, in which case precursors of spore constituents are presumably present in the cells at an early stage of sporulation. No detailed information has yet been obtained on the chemical nature or biological roles of such precursors. Chemical and biochemical studies on precursors of spore-specific materials may contribute to our understanding of sporulation mechanisms. The present paper describes the identification of cysteic acid as a precursor of sulpholactate and the metabolic pathway of cysteine to sulpholactate via cysteic acid.

**METHODS**

*Strain and culture conditions.* *Bacillus subtilis* NRRL B558 was used unless otherwise indicated. Bacilli from an overnight nutrient agar slope were suspended in Schaeffer's medium (Schaeffer & Ionesco, 1960) and incubated for 5 h at 37 °C. A sufficient volume of this culture was inoculated into fresh Schaeffer's medium to give an initial of 0.03 to 0.05, and the fresh culture was incubated with shaking (120 strokes min⁻¹, 3 cm amplitude) at 37 °C. Cells were harvested at various stages of sporulation and washed with chilled saline by centrifuging at 10000 rev. min⁻¹ for 10 min.

Replacement sporulation was carried out in a medium containing (per litre) 50 mmol acetic acid, 10 mmol glycine, 10 mmol serine, 1-5 g NaCl, 0-45 g KH₂PO₄, 0-58 g K₂HPO₄, 200 mg MgSO₄·7H₂O, 7H₂O, 7 mg MnSO₄·7H₂O, 15 mg FeSO₄·7H₂O and 73-5 mg CaCl₂·2H₂O (pH 6-8).

*Trichloroacetic acid (TCA) extraction and preparation of fractions.* Cells (100 mg dry wt) were treated in 15 ml 10% (w/v) TCA with stirring for 2 h at 0 °C, and the extract was centrifuged at 10000 rev. min⁻¹ for 20 min at 0 °C. The supernatant was extracted with 45 ml diethyl ether to remove the TCA and the water layer (TCA extract) was retained.
The TCA extract was applied to an Amberlite CG-120 (200 to 400 mesh, H+ form) column (1 × 7 cm) and eluted with deionized water. Cysteic acid was found in the first eluate (40 ml, fraction R-1). To prepare 2,4-dinitrophenyl (DNP) derivatives, fraction R-1 (40 ml) was treated with 2 ml 1-3% (w/v) 2,4-dinitro-l-fluorobenzene for 2 h at 40 °C (pH 9.0). The reaction mixture was washed three times with 15 ml diethyl ether until the ether layer became colourless. The ether layer was discarded and the reaction mixture was extracted three times with 10 ml water-saturated 1-butanol. The whole 1-butanol layer was evaporated and the residue (DNP-fraction R-1) was washed with deionized water through a membrane filter. The water-soluble fraction was concentrated to dryness and then dissolved in 0-1 ml deionized water (non-DNP-fraction R-1).

Analytical methods. Cysteic acid was determined as DNP-cysteic acid by high-pressure liquid chromatography (Shimadzu-DuPont LC-1) as described by Koshikawa et al. (1979). DNP-fraction R-1 was applied to a Zorbax ODS column and eluted with 0-01 M-sodium acetate buffer (pH 7-0). The column temperature was 50 °C and the column pressure 100 kgf cm-². Amino acids were analysed by a JEOL JLC-5AH amino acid analyser. Dipicolinic acid was assayed as described by Janssen et al. (1958).

Radioactivity measurement. Radioactivity was measured by liquid scintillation counting (Beckman LSC-150) using Bray’s fluid (Bray, 1960). [35S]Cysteic acid was measured as DNP-[35S]cysteic acid isolated from DNP-fraction R-1 by high-pressure liquid chromatography. [35S]Sulpholactate was measured after isolation by thin-layer chromatography on silica gel 60 PF254 (Merck) with a solvent system of 1-propanol/ammonia soln (sp.gr. 0-880)/water (6:3:1, by vol.). Bromocresol green was used to detect sulpholactate on thin-layer chromatograms.

Radiochemicals. [35S]Methionine (820 Ci mmol⁻¹, 30-3 TBq mmol⁻¹) and [35S]cysteine (97 mCi mmol⁻¹, 3-59 GBq mmol⁻¹) were obtained from The Radiochemical Centre, Amersham. [35S]Cysteic acid was prepared by performic acid oxidation of [35S]cysteine and purified by Amberlite CG-120 column chromatography. [35S]Sulpholactate was prepared from [35S]cysteic acid by deamination with nitrous acid as described by Bonsen et al. (1969).

RESULTS

Identification of cysteic acid

Cells cultured for 12 h in Schaeffer’s medium were extracted with 10% TCA and the TCA-free extract was run through an amino acid analyser. A significant ninhydrin-positive peak (compound I, elution time 25 min) eluted prior to aspartic acid (elution time 65 min); this peak remained intact even after the extract was hydrolysed with 6 M-HCl at 105 °C for 16 h. To identify this material, the extract was purified according to the procedure shown in Fig. 1. Purified crystalline DNP-compound I was finally obtained and this material showed a positive reaction with sodium nitroprusside. DNP-compound I was subjected to thin-layer and paper chromatography, high-pressure liquid chromatography and paper electrophoresis, and was compared with authentic substances (Table 1). From these results, DNP-compound I from B. subtilis NRRL B558 was identified as DNP-cysteic acid. Cysteic acid was also identified in extracts of sporangia of other strains of B. subtilis (see Discussion). It was not found when authentic cysteine was treated by the same procedure.

Changes in intracellular cysteic acid content

Fraction R-1 was prepared from cells at various stages of growth and the cysteic acid content was measured by high-pressure liquid chromatography (Fig. 2). The cysteic acid content began to increase at the onset of sporulation (after 5 h incubation), and was maximal when dipicolinic acid synthesis started. The cysteic acid content then decreased as sporulation advanced. Cysteic acid was not detected in culture supernatants analysed at various stages of sporulation. These results suggest that cysteic acid is metabolized during sporulation.

Distribution of cysteic acid in sporulating cells

Cells were harvested at various stages of sporulation and treated with lysozyme in phosphate buffer (pH 7-0) containing 10% (w/v) sucrose at 37 °C for 30 min (Kawasaki et al., 1967), to separate forespores from sporangia. The lysate was centrifuged at 9500 g for 20 min. The supernatant was evaporated to dryness in vacuo, and a fraction R-1 was prepared from the residue after initial extraction with 10% TCA. The insoluble residue from the lysate (forespore fraction) was resuspended in deionized water and disrupted in a cell mill (Buhler
Sporulating cells

10% TCA extraction (0 °C, 2 h, twice)

TCA extract

Diethyl ether extraction (3 times)

Water layer

Amberlite CG-120 column chromatography

Fraction R-1

Acid hydrolysis (6 M-HCl, 105 °C, 16 h)

Amberlite CG-120 column chromatography

Activated charcoal treatment

2,4-Dinitrophenylation (40 °C, pH 9.0, 2 h)

Diethyl ether extraction

Water layer

1-Butanol (water saturated) extraction (3 times)

1-Butanol layer

ECTEOLA cellulose column chromatography

Fraction R-3

Sephadex G-10 gel filtration

Recrystallization

DNP-compound I

Fig. 1. Procedure for purification of ninhydrin-positive materials from sporulating cells.

Table 1. Identification of DNP-compound I by chromatography and electrophoresis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Thin-layer chromatography*: Rp value</th>
<th>Paper chromatography†: Rp value</th>
<th>High-pressure liquid chromatography‡: elution time (min)</th>
<th>Paper electrophoresis§: mobility (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-compound I</td>
<td>0.81</td>
<td>0.53</td>
<td>2.1</td>
<td>6.5</td>
</tr>
<tr>
<td>DNP-cysteic acid</td>
<td>0.81</td>
<td>0.53</td>
<td>2.1</td>
<td>6.5</td>
</tr>
<tr>
<td>DNP-taurine</td>
<td>0.60</td>
<td>0.55</td>
<td>14.0</td>
<td>4.3</td>
</tr>
<tr>
<td>DNP-phosphorylserine</td>
<td>0.72</td>
<td>0.57</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DNP-homocysteic acid</td>
<td>0.55</td>
<td>0.90</td>
<td>2.6</td>
<td>—</td>
</tr>
</tbody>
</table>

* Gel, silica gel PF254; solvent, 1.5 M-phosphate buffer (pH 6.4).
† Paper, Toyo no. 51; solvent, 1-butanol/acetic acid/water (4:1:5, by vol.).
‡ Column, Zorbax ODS; solvent, 0.01 M-sodium acetate (pH 7.0); column temp. 50 °C; column pressure 100 kgf cm⁻².
§ Paper, Toyo no. 51; voltage, 10 V cm⁻¹; time, 2.5 h; buffer, 0.15 M-sodium acetate buffer (pH 5.0).

Vibrogen Cell Mill VCM-1). The resulting suspension was evaporated to dryness in vacuo and a fraction R-1 was prepared from the residue. The cysteic acid content of both fraction R-1 preparations was measured by high-pressure liquid chromatography (Fig. 3). Cysteic acid was found mainly in the lysozyme-soluble fraction, only a small amount being located in
the insoluble fraction. The cysteic acid content of the soluble fraction decreased as sporulation advanced. These results suggest that cysteic acid exists in the mother-cell cytoplasm and is further metabolized.

**Biosynthesis of cysteic acid in sporangia**

\(^{35}\text{S}\)Methionine (20 μCi per 100 ml culture) was added to a culture after 5 h incubation and incubation was continued for a further 5 h. The sporulating cells were then harvested and washed with chilled saline. The washed cells were extracted with 10% TCA, the extract was run through an amino acid analyser and the radioactivities of the eluate fractions were measured. The two major peaks of radioactivity were identified as cysteic acid and methionine; two minor peaks corresponded to methionine sulphone and cystine. The radioactivity of \(^{35}\text{S}\)cysteic acid was calculated to be more than 50% of the total radioactivity of the TCA extract. When a culture was incubated with \(^{35}\text{S}\)cysteine (10 μCi per 100 ml) in the same way, almost all the activity in the TCA extract was found in the cysteic acid fraction (Fig. 4). DNP-fraction R-1 was also prepared from sporulating cells grown in medium containing \(^{35}\text{S}\)methionine or \(^{35}\text{S}\)cysteine. In both cases, high-pressure liquid chromatography indicated that the radioactivity was present only in the DNP-cysteic
Cysteic acid metabolism in *B. subtilis* sporangia

0.4

P

0.2

CysSO₂H

Asp

Glu

Ala

Met

Fig. 4. Identification of ³⁵S-labelled compounds in TCA extracts of cells incubated with [³⁵S]cysteine. Cells were grown in Schaeffer’s medium for 5 h, [³⁵S]cysteine (10 μCi per 100 ml) was added to the cultures, and incubation was continued for a further 5 h. TCA extracts were run through an amino acid analyser and the radioactivity of the eluate fractions (3-1 ml) was measured.

acid fraction. These results suggest that cysteic acid is synthesized from cysteine and methionine.

*Biosynthesis of sulphotolactate in sporangia*

Non-DNP-fraction R-1 was prepared from both sporulating cells and spores grown in medium containing [³⁵S]methionine or [³⁵S]cysteine. The radioactive compounds in these fractions were analysed by thin-layer chromatography with radioactive reference compounds. In each case, only one bromocresol green-positive spot (Rₚ 0-35) was found on the silica gel thin-layer plate, and most of the radioactivity was associated with this spot. The Rₚ values of the reference compounds in the solvent system used were: DNP-cysteic acid, 0-68; cysteic acid, 0-45; methionine, 0-72; sulphotolactate, 0-35. The radioactive compound in non-DNP-fraction R-1 was therefore identified as [³⁵S]sulphotolactate, which suggests that sulphotolactate was derived from cysteine or methionine during sporulation.

Cells grown in Schaeffer’s medium for 5 h were transferred to sporulation medium containing [³⁵S]cysteic acid (5 μCi per 100 ml) and incubated at 37 °C. Sporulating cells and spores were harvested after 8 and 20 h incubation, respectively. Both cell samples were extracted by autoclaving and the extracts were analysed by thin-layer chromatography. It was found that sulphotolactate was also derived from cysteic acid (Fig. 5). From these results, we conclude that sulphotolactate is synthesized from cysteine or methionine via cysteic acid during sporulation.

Changes in the cellular distribution of cysteic acid and sulphotolactate were followed in cells harvested at various stages of sporulation. Cells were grown in Schaeffer’s medium and [³⁵S]cysteine was added at the initial stage of sporulation (after 5 h incubation). Cell samples were collected every 2 h and separated into soluble (mother-cell cytoplasm) and insoluble (forespore) fractions by lysozyme treatment. The radioactivity of cysteic acid and sulphotolactate was measured in both fractions after purification (Fig. 6). In the lysozyme-soluble fraction, the cysteic acid content reached its maximum after 8 h incubation, while the content of sulphotolactate reached its maximum after 10 h incubation. The content of both
Fig. 5. Conversion of cysteic acid to sulpholactate in vivo. Cells grown in Schaeffer's medium for 5 h were transferred to replacement sporulation medium containing [35S]cysteic acid (5 μCi per 100 ml). Autoclaved extracts were prepared of sporulating cells after 8 h incubation (a), and of spores after 20 h incubation (b). The extracts were analysed by thin-layer chromatography; the position of the sulpholactate spot on the chromatograms is indicated.

Fig. 6. Distribution of cysteic acid and sulpholactate in sporulating cells. Cells were grown in Schaeffer's medium, with [35S]cysteine (100 μCi per 100 ml) added after 5 h incubation (arrow). Cells harvested at various stages of sporulation were separated into mother-cell cytoplasm and forespore fractions by lysozyme treatment. ○, Growth; ●, cysteic acid content of mother-cell cytoplasm fraction; □, cysteic acid content of forespore fraction; ■, sulpholactate content of mother-cell cytoplasm fraction; □, sulpholactate content of forespore fraction.

compounds decreased as sporulation advanced. In the insoluble fraction, the sulpholactate content increased as sporulation advanced; cysteic acid was found in this fraction. These results suggest that cysteic acid, synthesized from cysteine, was metabolized to sulpholactate in the mother-cell cytoplasm and incorporated into the forespore.

In vitro synthesis of cysteic acid and sulpholactate

Sporulating cells were harvested from a culture incubated for 8 h in Schaeffer's medium and disrupted by sonication in the cold. The cell brei was centrifuged at 10000 rev. min⁻¹ for 15 min and the supernatant was used for the experiment. A 1 ml portion of the supernatant (8 mg protein ml⁻¹) was added to 1 ml 0-2 M-glycine/NaOH buffer (pH 9-0) containing [35S]cysteine (0-3 μCi) and incubated anaerobically at 37 °C for 30 min, followed by aerobic
incubation at 37 °C for 30 min. Then 0.5 ml 10% TCA was added to the reaction mixture and [35S]cysteic acid was determined (as its DNP derivative) by high-pressure liquid chromatography (Fig. 7a). Most of the radioactivity was found in the cysteic acid fraction. The percentage conversion of cysteine to cysteic acid was 5%. When the crude enzyme preparation was inactivated by heating before the experiment, no radioactivity was found in the cysteic acid fraction. A 1 ml portion of the same crude enzyme preparation was added to 1 ml 50 mM-Tris/HCl buffer (pH 7.8) containing 1 mM-2-oxoglutarate and [35S]cysteic acid (0.3 μCi) and the reaction mixture was incubated at 37 °C for 30 min. Non-DNP-fraction R-1 was prepared from the mixture and subjected to thin-layer chromatography. Radioactive sulpholactate was detected in this reaction mixture (Fig. 7b); the percentage conversion of cysteic acid to sulpholactate was 10%. When heat-inactivated crude enzyme preparation was used, no radioactive sulpholactate was found.

**DISCUSSION**

Our results show that cysteic acid, derived from cysteine, is present in the sporangium of *B. subtilis* NRRL B558 and is metabolized to sulpholactate. Cysteic acid was also present in the sporangia of *B. subtilis* 60015, *B. subtilis* K, *B. subtilis* H, *B. subtilis* var. *niger* and *B. subtilis* var. *natto* IAM 1071, but only a small amount of cysteic acid was found in the sporangia of other species of spore-formers. These results suggest that the presence of cysteic acid is confined to spore-formers that contain sulpholactate in their spores.

The cysteic acid content of the cells began to increase at the onset of sporulation. When chloramphenicol (Spudich & Kornberg, 1968) or glucose (Schaeffer et al., 1965) was added to the sporulation medium and thus sporulation was inhibited or delayed, the accumulation of cysteic acid was also inhibited or delayed. When cells in the exponential growth phase were suspended in manganese-deficient medium and growth was inhibited (Oh & Freese, 1976), the cysteic acid content did not increase, but when manganese was added to the medium, allowing sporulation to proceed, the cysteic acid content began to increase at the same time as sporulation was initiated. These results suggest that the biosynthesis of cysteic acid is closely
related to the sporulation mechanisms and that synthesis occurs as a consequence of the decomposition of cell constituents prior to spore formation.

Our results on the cellular distribution of cysteic acid and sulpholactate suggest that these substances are synthesized in the mother-cell cytoplasm and that sulpholactate is incorporated into forespores. Cysteic acid and sulpholactate start to accumulate in the cell constituents prior to spore formation. This conclusion is supported by the results of in vitro experiments with a crude enzyme preparation, in which the percentage conversion of cysteic acid to sulpholactate was twice as great as that of cysteine to cysteic acid.

Experiments in vivo with radioactively labelled compounds showed that cysteic acid is synthesized from methionine or cysteine and is metabolized to sulpholactate. The conversion rate of radioactivity from cysteine to cysteic acid was calculated to be four times greater than that from methionine to cysteic acid. This suggests that cysteine is preferred to methionine as a precursor. The experiments with a cell-free extract of sporangia suggest that enzymic conversion of cysteine to cysteic acid occurs in the mother-cell cytoplasm.

It has been reported that cysteic acid is synthesized from adenosine 3'-phosphate-5'-phosphosulphate and serine (Sass & Martin, 1972) or from cysteine via cysteinesulphinic acid by cysteine oxidase (Awapara & Doctor, 1955; Sakakibara et al., 1973; Söbro & Ewetz, 1965). As these results were obtained from experiments with animal tissues, they are not directly applicable to bacterial metabolism. However, of these two synthetic pathways, the latter would appear to be more in keeping with the results obtained in the present work, since cysteine was found to be a precursor of cysteic acid.

REFERENCES


Cysteic acid metabolism in B. subtilis sporangia
