THE INHIBITION OF PHOSPHORIBOSYL-PYROPHOSPHATE AMIDOTRANSFERASE ACTIVITY BY CORDYCEPIN MONOPHOSPHATE

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SUMMARY

1. Cordycepin monophosphate has been shown to inhibit the activity of phosphoribosyl-pyrophosphate amidotransferase (ribosylamine-5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14) obtained from both pigeon liver and Bacillus subtilis.

2. Evidence is presented for the existence of a phosphorylated derivative of cordycepin in Bacillus subtilis cells which were grown in the presence of cordycepin.

3. These results, accompanied by previous evidence, suggest that the inhibition of this enzymatic reaction may be the mechanism by which cordycepin inhibits the growth of Bacillus subtilis.

INTRODUCTION

Previous studies have shown that the inhibition of Bacillus subtilis growth in the presence of cordycepin can be prevented by the addition of purines or 5-amino-4-imidazolecarboxamide. The incorporation of radioactive formate into the purine ring of the nucleic acids of this organism was suppressed by cordycepin, while in the same system the labeling of nucleic acid purines by radioactive 5-amino-4-imidazolecarboxamide was not affected.

Formation of formylglycinamide ribonucleotide, which accumulated in the presence of diazo-oxo-L-norleucine, was decreased in the presence of cordycepin. These results, coupled with the failure to demonstrate an accumulation of glycaminamide ribonucleotide by cordycepin, suggested the possibility that this nucleoside was interfering with one of the first two reactions in purine biosynthesis.

Wyngaarden and Ashton demonstrated that the activity of phosphoribosyl-pyrophosphate amidotransferase (ribosylamine-5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14) purified from pigeon liver was inhibited by certain purine nucleotides and postulated that such action might be metabolically important as a feedback regulator of purine biosynthesis. The possibility

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that cordycepin, as its phosphorylated derivative, could inhibit this reaction seemed worthy of investigation.

The data in this report show that cordycepin phosphate can inhibit the activity of phosphoribosyl-pyrophosphate amidotransferase purified from pigeon liver and *B. subtilis*. Evidence is presented also for the existence of cordycepin phosphate in this organism.

**MATERIALS AND METHODS**

*B. subtilis* ATCC No. 10783 was grown in the chemically defined medium of Herrriott which was supplemented with 0.2% casein hydrolysate. Cordycepin was isolated from culture filtrates of the mold *Cordyceps militaris* according to a published procedure. The synthesis and characterization of cordycepin monophosphate have been described.

L-Glutamate dehydrogenase (EC 1.4.1.2) (Boehringer and Soehne) was purchased from Calbiochem as the ammonium sulfate-free suspension in glycerol. Deoxyribonuclease (EC 3.1.4.5) was obtained from Nutritional Biochemicals. Deoxyadenosine 5'-phosphate was purchased from Calbiochem. Adenosine 5'-phosphate, the magnesium salt of 5-phosphoribosyl 1-pyrophosphate and the 3-acetylpyridine analog of DPN were products of the Pabst Brewing Company. L-Glutamine was purchased from Sigma Chemical Company.

Protein concentration was determined by absorbancy measurements at 260 and 280 m\(\mu\) (ref. 7).

**Inhibition of pigeon-liver phosphoribosyl-pyrophosphate amidotransferase by cordycepin monophosphate**

We carried out our initial studies with the enzyme purified from pigeon liver as described by Wyngaarden and Ashton. Enzymatic activity was determined by measuring the glutamate formed in Reaction 1 by coupling this reaction with glutamate dehydrogenase (L-glutamate:DPN oxidoreductase (deaminating), EC 1.4.1.2) and observing the reduction of the 3-acetylpyridine analog of DPN in a Gilford Multiple Sample Absorbance Recorder.

\[
\text{Mg}^{2+} + \text{Glutamate} + \text{Phosphoribosyl-pyrophosphate} + H_2O \rightarrow \text{Glutamate} + \text{5-Phosphoribosyl-} \\
\text{amine} + \text{Pyrophosphate} \quad (1)
\]

A typical reaction mixture contained in a final volume of 1 ml; 50 \(\mu\)moles Tris-HCl buffer (pH 8.0), 3 \(\mu\)moles MgCl\(_2\), 0.25 \(\mu\)mole 5-phosphoribosyl 1-pyrophosphate, 1.0 \(\mu\)mole glutamine, 0.6 \(\mu\)mole 3-acetylpyridine DPN, 0.05 ml of a 1:10 dilution of commercially available glutamate dehydrogenase, and sufficient quantity of the pigeon-liver enzyme to cause a change in absorbancy of 0.05 units per min. The reaction was initiated by the addition of 5-phosphoribosyl 1-pyrophosphate. The course of the reaction is indicated in curve A of Fig. 1. The first three steps in the purification of this enzyme from pigeon liver yielded preparations which could not be assayed in this coupled system. Therefore it is not possible to indicate an overall purification.

The rate of this reaction in the presence of cordycepin monophosphate is also...
shown in Fig. 1. The lag period, which extends for 5–8 min after 5-phosphoribosyl 1-pyrophosphate addition, was also observed by Wyngaarden and Ashton and is without explanation. The linear portion of the curve was used in the calculation of the apparent $K_m$ and $K_i$ and consequently does not represent a true initial velocity.

The apparent $K_m$ for 5-phosphoribosyl 1-pyrophosphate was determined from the data shown in Fig. 2 and was found to be approx. $3 \times 10^{-4}$ M. Furthermore, Fig. 2 shows that the inhibition by cordycepin monophosphate appears to be competitive. The $K_i$ for cordycepin monophosphate was calculated to be approx. $7 \times 10^{-4}$ M. Adenosine 5'-phosphate also inhibited the activity of this enzyme, as was first
demonstrated by Wyngaarden and Ashton. Fig. 3 demonstrates that cordycepin monophosphate and adenosine 5'-phosphate are equally effective as inhibitors. Although it is not shown in the figure, free cordycepin was without effect.

The 2' and 3' phosphoric acid esters of adenosine as well as deoxyadenosine 5'-phosphate have been shown by Wyngaarden and Ashton to be ineffective as inhibitors of phosphoribosyl-pyrophosphate amidotransferase activity. We also found that deoxyadenosine 5'-phosphate was not inhibitory when present at concentrations similar to those used for cordycepin monophosphate and adenosine 5'-phosphate.

Fig. 3. Comparison of the inhibition of phosphoribosyl-pyrophosphate amidotransferase by cordycepin phosphate and adenosine 5'-phosphate. The reaction mixture is described in the text. The concentration of cordycepin phosphate (×) was 1.9 μmoles/ml. The concentration of adenosine 5'-phosphate (Δ) was 2.0 μmoles/ml.

Partial purification of phosphoribosyl-pyrophosphate amidotransferase from B. subtilis

Since the intent of this work was to study a possible mechanism of inhibition of cordycepin in B. subtilis, it was desirable to show the effect of cordycepin phosphate on phosphoribosyl-pyrophosphate amidotransferase derived from this organism.

Attempts to demonstrate the activity of this enzyme in crude extracts of B. subtilis using the coupled assay system were not successful. The crude extract was capable of rapidly reducing the DPN analog in the absence of substrate. Dialysis of the extracts or passage over Sephadex G-25 eliminated most of the endogenous reduction but the preparation did not catalyze 5-phosphoribosyl 1-pyrophosphate-dependent reduction of the DPN analog. If an extract was fractionated with ammonium sulfate followed by dialysis in the presence of 2-mercaptoethanol a preparation was obtained that was able to catalyze the desired reaction.

B. subtilis cells were grown into the early log phase at which time they were harvested by centrifugation at 5°C. The cells were resuspended in 0.9% saline and again harvested by centrifugation. The packed cells were weighed and suspended in 5 vol. of buffer (0.05 M potassium phosphate, pH 7.5, containing 0.01 M 2-mercaptoethanol). Usually 10 g wet wt. of bacteria were used in this preparation. The cell

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suspension was sonicated for two 45-sec intervals with intermittent cooling in an ice-salt bath.

DNAase solution (0.2 ml, containing 0.2 mg of crystalline DNAase) was added to the sonicated solution and the material was stirred as it was allowed to approach room temperature over a 20-min period. The crude material was frozen at -20°. This was the only stage in the purification procedure in which the enzyme preparation was stable to freezing. Furthermore, the freezing seemed to be a necessary part of the procedure as the omission of this step gave preparations which were inactive. After thawing, the extract was centrifuged at 30,000 × g for 45 min. Subsequent steps were all carried out at 4° unless indicated otherwise.

The supernatant fluid was treated with a saturated ammonium sulfate solution (enzyme grade, purchased from Mann Research Laboratories) adjusted to pH 7 with NH₄OH. The protein which precipitated between 40 and 55% saturation was dissolved in 8 ml of the buffer used in the initial extraction and dialyzed for 6 h against 2 l of the same buffer. 8 liters of fresh buffer were added in a continuous manner over the 6-h period. The dialyzed fraction was the first stage in purification in which enzymatic activity could be demonstrated.

2% protamine sulfate was added to precipitate nucleic acids and some unreactive protein. Approx. 0.2 mg of protamine sulfate per mg of protein precipitated more than 95% of the nucleic acids, as determined by absorption at 260 μm, and some protein without significant loss of enzymatic activity. The mixture was stirred in an ice bath and the precipitate removed by centrifugation. The supernatant fluid was adjusted to pH 4.8 with 1 N acetic acid and the resulting precipitate removed by centrifugation. The pH of the supernatant fluid was quickly readjusted to 7.5 with NaOH. This neutralized supernatant fluid was placed on a Sephadex G-25 column 20 cm high × 3 cm which had been prepared by extensive washing with buffer (0.05 M phosphate, pH 7.5 and 0.01 M mercaptoethanol). The enzyme was eluted from the column with the same buffer and the yellow protein band was followed visually and collected as one fraction.

A summary of the partial purification is given in Table I. An accurate determination of the overall enzyme purification was impossible as the activity could not be determined until after dialysis.

Inhibition of B. subtilis phosphoribosyl-pyrophosphate amidotransferase activity by cordycepin monophosphate

The same method of assay was used as was employed in the pigeon-liver system, differing only in the concentration of reagents. The reaction mixture contained in 1 ml; 100 μmoles Tris–HCl buffer (pH 8.0), 6.0 μmoles MgCl₂, 0.5 μmole 5-phosphoribosyl 1-pyrophosphate, 6.0 μmoles glutamine, 0.6 μmole of the 3-acetylpyridine analog of DPN, 0.05 ml of a 1:10 dilution of commercially available glutamate dehydrogenase and sufficient enzyme to cause a change in absorbancy of 0.05 units per min. The reaction was followed spectrophotometrically as previously described.

Mercaptoethanol, which was a necessary component during enzyme purification did not seem to add significantly to its stability after purification. The enzyme was extremely unstable, losing half its original activity after standing in an ice bath for 4 h.

TABLE I

PURIFICATION OF PHOSPHORIBOSYL-PYROPHOSPHATE AMIDOTRANSFERASE FROM B. subtilis

1 unit of activity is defined as 1 μm mole of acetylpyridine DPN reduced per min.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Activity (units/ml)</th>
<th>Protein (mg/ml)</th>
<th>Purity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.40-0.55 ammonium sulfate</td>
<td>5.2</td>
<td>2.4</td>
<td>2.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>4.9</td>
<td>4.5</td>
<td>8.4</td>
<td>94</td>
<td>3.8</td>
</tr>
<tr>
<td>pH 4.8 supernatant</td>
<td>4.1</td>
<td>4.9</td>
<td>8.4</td>
<td>94</td>
<td>3.8</td>
</tr>
<tr>
<td>Sephadex</td>
<td>3.6</td>
<td>3.0</td>
<td>1.2</td>
<td>54</td>
<td>5.5</td>
</tr>
</tbody>
</table>

The activity of this partially purified enzyme was inhibited by cordycepin monophosphate (Fig. 4). The $K_i$ for cordycepin monophosphate (estimated as approx. $10^{-4}$ M) was similar to that obtained with the pigeon-liver enzyme. However, precise determinations of $K_m$ and $K_i$ could not be made because of the extreme lability of the enzyme, as well as the fact that initial velocities were not used in the calculation.

As was true with the enzyme obtained from pigeon liver, the activity of this enzyme was inhibited by adenosine 5'-phosphate while cordycepin and deoxyadenosine 5'-phosphate, when tested at similar concentrations, were without effect.

The occurrence of a phosphorylated derivative of cordycepin in B. subtilis

The previous enzymatic studies only have meaning with respect to cordycepin inhibition if there is evidence for cordycepin monophosphate existence in cordycepin-inhibited B. subtilis cells.

The direct isolation of cordycepin monophosphate from such a source was not achieved, but evidence for its existence was obtained. Presumably, the amount of cordycepin phosphate present in a cellular extract might be quite small in comparison to the large pool of other nucleotides. Therefore the ribonucleotides were destroyed by oxidation with periodate. This led to the production of many contaminating

![Fig. 4. Inhibition of B. subtilis phosphoribosyl-pyrophosphate amidotransferase by cordycepin phosphate. The components of the reaction mixture are given in the text. Curve A, complete system. Curve B, complete system plus cordycepin phosphate (1.9 μmoles/ml). Curve C, complete system minus 5-phosphoribosyl 1-pyrophosphate.]
fluorescent compounds with properties sufficiently similar to cordycepin phosphate so as to make their separation by conventional paper and column chromatography difficult. Furthermore, deoxyadenosine 5'-phosphate was not destroyed by periodate and the separation of this compound from cordycepin phosphate was not successful. Because of these complications, the following approach was used to demonstrate the existence of cordycepin phosphate in cordycepin-inhibited cells.

*B. subtilis* cells were grown to the log phase at which time the cells were quickly sedimented by centrifugation and resuspended in one-tenth the original volume of fresh media. After 2 h of growth, cordycepin was added to a final concentration of 200 μg/ml and the culture was incubated an additional 3 h. The cells were harvested by centrifugation and resuspended in a small volume of water. The suspension of cells was sonicated for 2 min and immediately treated with an equal volume of cold 10% trichloroacetic acid. Following centrifugation, the precipitate was extracted three more times with 5% trichloroacetic acid. The extracts were combined and extracted 5 times with ether to remove the trichloroacetic acid. The total extract was obtained from approx. 100 g wet wt. of bacteria and contained 7500 absorbancy units at 260 μm.

The ultraviolet absorbing compounds were adsorbed on norite and subsequently eluted with ammoniacal ethanol. The eluate was evaporated to dryness and the resulting material (3800 absorbancy units) treated with 2.5 mmol of sodium metaperiodate and 0.3 mmol of sodium acetate (pH 5.0). After 3-h incubation at room temperature 5 mmol of glucose were added to destroy the remaining periodate and the solution was incubated for 2 h. 1.7 mmol of glycine buffer (pH 10.5) were added and further incubation was carried out at pH 10 for 15 h at 37°C. A few drops of CHCl₃ were added to prevent bacterial growth. The ultraviolet absorbing material was again adsorbed on norite, eluted, and the eluate evaporated to dryness. This material, dissolved in a few ml of water, was placed on a Dowex-I-formate column 18 cm high × 1 cm and eluted with 0.01 M formic acid until the absorbancy of the eluate was less than 0.1. Free cordycepin was eluted in this fraction. Elution with 0.1 M formic acid, which under similar conditions had previously been shown to elute cordycepin monophosphate, gave two ultraviolet absorbing fractions. The nucleotides in these fractions were adsorbed on norite and subsequently eluted. The following procedures were carried out for the nucleotides obtained from both fractions.

The nucleotides were treated with crude snake venom phosphomonoesterase to obtain the corresponding nucleosides. The reaction mixture was placed on a Dowex-I-formate column and the column was eluted with water. The nucleosides were collected in the water eluate, leaving the contaminating fluorescent material on the resin. Following adsorption on norite, the nucleosides were eluted with 20% aqueous pyridine. The eluate was evaporated to dryness several times after the addition of water to insure complete pyridine removal. Dowex 50-H⁺ was used to hydrolyze the nucleosides and liberate the free sugars. This hydrolysis was necessary because we were unable to resolve deoxyadenosine and cordycepin by paper chromatography but their component sugars could be separated.

Aliquots of the solutions containing the free sugars were chromatographed on Whatman No. 1 filter paper in three solvent systems using deoxyribose and cordycepose as standards. The three solvents used were: (1) 2,4,6-collidine saturated with water, (2) n-butanol-acetic acid-water (4:1:5, v/v), and (3) isopropanol-pyridine-acetic acid-water (40:40:5:20, v/v). The chromatograms were visualized with AgNO₃ and

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alcoholic NaOH. Cordycepose was shown by each solvent system to be present in the material from the second fraction obtained in the initial elution of the Dowex-I-formate column with 0.1 M formic acid. Deoxyribose, prepared in the same manner as cordycepose, was rather unstable but could be shown to migrate at a rate different from that of cordycepose in all three solvent systems.

Even though the direct isolation and characterization of cordycepin monophosphate was not achieved as such, it appears to be present in *B. subtilis* cells inhibited by cordycepin.

These enzymatic studies, namely the inhibition of phosphoribosyl-pyrophosphate amidotransferase activity are in accord with our earlier work dealing with the effect of cordycepin on *B. subtilis* growth.

Presumably, as the level of intra-cellular cordycepin phosphate increases, phosphoribosyl-pyrophosphate amidotransferase recognizes this compound as a constituent of the nucleotide pool and further synthesis of purine nucleotides is diminished. Such an explanation would satisfy all the data we have obtained on cordycepin inhibition of this organism. However, a necessary corollary to this hypothesis is the presumption that cordycepin phosphate cannot substitute for natural nucleotides in the biosynthesis of nucleic acids and has no other metabolic fate.

As has been stated earlier, Wyngaarden and Ashton reported that phosphoribosyl-pyrophosphate amidotransferase prepared from pigeon liver was inhibited by certain purine nucleotides. More recently, Hartman, using several different methods of assay, was not able to demonstrate this phenomenon with enzyme prepared from chicken liver. He postulated that either an uninhibitable form of the enzyme may have been isolated under his condition of fractionation, or that the enzymes from these two avian sources were basically different in this respect.

We have not carried out any work with the chicken-liver enzyme, but we have been able to show AMP and cordycepin phosphate inhibition of phosphoribosyl-
pyrophosphate amidotransferase purified from both pigeon liver and *B. subtilis*. Unfortunately these results do little to explain the failure to observe this effect with the enzyme obtained from chicken liver.

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REFERENCES
