PHOSPHORYLATION OF PANTOTHENIC ACID AND PANTETHINE BY AN ENZYME FROM PROTEUS MORGANII

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Before the nature of “bound” pantothenic acid was established, McIlwain and Hughes (1) showed that incubation of pantothenic acid with various streptococci or Proteus morganii led to its “inactivation” as a growth factor for these organisms. This inactivation reaction was dependent upon glycolysis, but independent of growth and the presence of oxygen, and was inhibited by pantoyltaurine (2). McIlwain (3) assumed that both glycolysis and pantothenic acid are necessary for formation of some substance necessary for growth.

It now seems probable that this inactivation reaction represented one or more reactions associated with conversion of pantothenic acid to coenzyme A. The latter product and each of the proposed intermediates between it and pantothenic acid are inactive as growth factors for yeast and many bacteria (4-6).

In a previous communication (7) it was stated that one such inactivation reaction in P. morganii depended upon the presence of adenosinetriphosphate (ATP) and cysteine, yielding a product identical in behavior to pantothenylcysteine. The latter product, though inactive for most bacteria (5-7), is highly active for Acetobacter suboxydans (5), and appears to be an intermediate in coenzyme A synthesis (5, 7, 8). During this work, acetone-dried cells of P. morganii also were observed to inactivate pantothenic acid as a growth factor for yeast in the absence of cysteine, provided ATP were present. Phosphatase treatment regenerated pantothenic acid inactivated by the cysteine-independent reaction, but not that inactivated by the cysteine-dependent reaction, thus permitting their ready differentiation.

The enzyme associated with this cysteine-independent inactivation of pantothenic acid has been obtained in cell-free extracts. Its partial purification and some of its properties are described below.

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Assay Procedures—Pantothenic acid was determined with Saccharomyces carlsbergensis 4228 (9), for which coenzyme A (4), pantethine (4), pantethenylcysteine (6, 7), and each of the phosphorylated forms of pantothenic acid (10, 11) are inactive. Pantethine was determined with Lactobacillus helveticus 80 (4); pantothenic acid, pantethenylcystine, and coenzyme A are essentially inactive for this organism, and 4'-phosphopantetheine is less than 20 per cent as active as pantethine (4, 6).

Source of Enzyme—P. morganii (A. T. C. C. No. 8019) was carried on nutrient agar slants with monthly transfer. Inocula were prepared by suspending growth from a 24 hour-old culture on such a slant in 5 ml. of sterile water; 2.5 ml. of the resulting suspension were used to inoculate each 500 ml. of basal medium. The basal medium was that described by Pelczar and Porter (12) with the alkali-treated peptone, nicotinic acid, and cystine replaced by 0.5 per cent yeast extract. 6 liter lots of this medium in 9 liter Pyrex jars were sterilized, cooled, inoculated, and incubated with aeration at 30° for 24 hours. Cells were harvested in a Sharples centrifuge and washed twice with water.

Enzyme Assay—Activity of the enzyme is measured as the difference between the pantothenic acid initially present and that remaining after an appropriate time interval. To avoid inaccuracies attendant upon this value being a small difference between two large numbers, the reaction must go substantially toward completion. For this reason, the amount of pantothenic acid added as substrate was suboptimal, and ATP was used in excess. After appropriate preliminary experiments, a total reaction volume of 4 ml. containing 62.5 μg (0.292 μmole) of calcium pantothenate, 13 mg. (22 μmoles) of ATP, 160 μg of MgSO₄·7H₂O, 0.25 μmole of tris-(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, and enzyme was adopted. 1 unit of enzyme activity was defined as that amount of preparation that would catalyze inactivation of 1 μg of calcium pantothenate in 90 minutes under these conditions. Variations from these conditions are specified with Tables I and II. Protein was determined turbidimetrically by the procedure of Stadtman et al. (13).

Preparation of Enzyme—Active preparations of the enzyme are obtainable from acetone- or vacuum-dried cells, but are less active than those obtained by sonic disruption of the freshly harvested cells (Table I). For the latter purpose, washed cells from 5 liters of medium were suspended in 50 ml. of 0.2 M phosphate buffer, pH 7.0, and subjected to 10 kc. vibrations in a Raytheon ultrasonic oscillator for 25 minutes. Cell débris was removed by high speed centrifugation in the cold. The combined extracts (55,000 units, 1.1 units per mg. of protein) from cells from 36 liters of medium were refrigerated at 4° for several days, and precipitated, inactive
proteins were removed by centrifugation. Further inactive protein precipitated on standing for 6 hours at 37° and was discarded. A small amount of additional inactive material was precipitated with protamine sulfate, as described for removal of nucleic acids (15). The supernatant solution from the latter precipitation (34,100 units, 7.2 units per mg. of protein), pH 6.9, was treated at room temperature with an equal volume of saturated ammonium sulfate solution, and the precipitate was collected by centrifugation and dissolved in one-fourth the original volume of water. It was then dialyzed overnight against two changes of distilled

### Table I

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Pantothenic acid inactivated</th>
<th>μmoles × 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetone-dried cells†</td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>2. Aqueous extract of (1)‡</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>3. Vacuum-dried cells§</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>4. Aqueous extract of (3)¶</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>5. Alumina-ground cells‖</td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>6. Sonically disrupted</td>
<td></td>
<td>6.7</td>
</tr>
</tbody>
</table>

* 40 mg. of cells, or extract prepared from them, were used in the assay procedure described in the text.
† Washed cells poured into 10 volumes of cold acetone (2°), stirred 15 minutes, filtered, washed with 10 volumes of ether, and dried over P₂O₅ in a vacuum.
‡ 40 mg. of dried cells extracted overnight at 4° with 1 ml. of 0.25 M Tris buffer, pH 7.0; cells removed by centrifugation.
§ Wet cells were dried in a vacuum over KOH for 24 hours.
¶ Method of McIlwain (14).

The final preparation (26,600 units, 23.9 units per mg. of protein) represented 48 per cent of the initial activity, purified approximately 22-fold over the initial extract.

Nature and Substrate Specificity of Inactivation Reaction—Under the assay conditions used here, a tremendous excess of ATP is required for the inactivation reaction (Fig. 1). This is due in part to the low concentration of pantothenate used (quantitative conversion to the product was achieved) and in part to the presence of an active ATPase in the enzyme preparation used. The specificity of the reaction with respect to ATP has not been investigated, however, and the possible participation of other nucleotide polyphosphates (16, 17), possibly present as a contaminant of the ATP used, is not excluded. That the reaction is a phosphorylation of pantothenic acid is shown by the quantitative recovery of pantothenic acid fol-
Fig. 1. The relationship of ATP concentration to the phosphorylation of pantothenic acid. The incubation mixture contained 0.048 mmole of calcium pantothenate (12.5 γ) and 46 units of kinase per 4 ml., and was incubated 3 hours at 37°. Other conditions are as in the text.

**TABLE II**

*Phosphorylation of Pantothenic Acid, Pantetheine, and Pantethine by Kinase and Hydrolysis of Products by Intestinal Phosphatase*

The values are given in micromoles \( \times 10^3 \) per 4 ml.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Substrate present in reaction mixture</th>
<th>Before reaction</th>
<th>After reaction</th>
<th>After reaction and intestinal phosphatase treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1†</td>
<td>Pantothenic acid</td>
<td>37</td>
<td>27.5</td>
<td>41</td>
</tr>
<tr>
<td>2‡</td>
<td>Mercury mercaptide of pantetheine$</td>
<td>17</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>3‡</td>
<td>Pantothenic acid</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pantetheine$</td>
<td>34</td>
<td>29.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mercury mercaptide of pantetheine$</td>
<td>34</td>
<td>29.8</td>
<td></td>
</tr>
</tbody>
</table>

* 10 mg. of Armour’s purified intestinal phosphatase were added to the reaction mixture which was incubated an additional 90 minutes at 37° before assay.
† 13 units of kinase per 4 ml.; incubated 90 minutes at 37°.
‡ 13 units of kinase per 4 ml.; incubated 90 minutes at 37°.
§ These products contain 2 equivalents of pantothenic acid per mole.
‖ The enzyme concentration was reduced to 0.3 unit per 4 ml.
lowing treatment with Armour's purified intestinal phosphatase (Table II). The product of the enzymatic phosphorylation was identified as 4'-phosphopantothenic acid with the aid of paper chromatography. Appropriate amounts of the enzymatic reaction mixture and control substances were spotted on separate strips of filter paper. These were developed overnight with n-propanol-ammonium hydroxide (33 per cent ammonia)-water (60:30:10 parts by volume), then dried, sprayed with a solution of Armour's intestinal phosphatase (4 mg. per ml. of 0.1 M Tris buffer, pH 7.5), and incubated in a closed container at 37° for 90 minutes. Zones were located by the bioautograph technique with S. carlsbergensis as the test organism. The enzymatic inactivation product ($R_F = 0.50$) migrated identically with a sample of 4'-phosphopantothenic acid kindly supplied by Dr. J. Baddiley.

From Table II it is apparent that the same enzyme preparation phosphorylates both pantethine and pantetheine at approximately the same rate as pantothenic acid. The amount of pantothenic acid phosphorylated was decreased by simultaneous addition of pantethine; i.e., the two products appear to be competitive substrates for the enzyme. In a quanti-
tative experiment of this nature, 0.1 μmole of calcium pantothenate and 600 units (≈ 0.1 μmole of pantetheine) of pantetheine were incubated with 43 units of enzyme for 90 minutes. During this time, 50 per cent of the pantothenic acid and 86 per cent of the pantetheine were inactivated by phosphorylation. In the absence of pantetheine, the pantothenic acid was completely phosphorylated. From this result, pantetheine would appear to be somewhat the preferred substrate. By analogy with the product formed from pantothenic acid, that from pantetheine is assumed to be 4'-phosphopantetheine.

The Michaelis constant for the kinase with pantothenic acid as substrate is approximately $2.5 \times 10^{-5}$ M (Fig. 2). It will be observed from Fig. 2 that the substrate concentration used for assay of the kinase, for reasons discussed earlier, is considerably below that necessary to saturate the enzyme.

Miscellaneous Properties—The kinase has a sharply defined pH optimum between 7.0 and 7.5 (Fig. 3).

A requirement for magnesium ion was demonstrable following dialysis of the purified enzyme. With 50 γ of calcium pantothenate and 55 units of dialyzed enzyme in the reaction mixture, 12 γ of calcium pantothenate were phosphorylated in the absence of added magnesium, whereas 31 γ were phosphorylated by the magnesium-supplemented enzyme.

Limited studies of heat stability showed no inactivation after 90 minutes at $37^\circ$, slightly over 75 per cent inactivation after 30 minutes at $50^\circ$, and complete destruction of enzymatic activity in 30 minutes at $60^\circ$.

**DISCUSSION**

The kinase described here resembles the pantetheine kinase from pigeon liver described by Levintow and Novelli (18) in pH optimum, in its Michaelis constant for its substrates, and in the high requirement for ATP relative to pantetheine required for maximal activity. The Proteus enzyme appears to be considerably more stable to heat than the enzyme from pigeon liver. The substrate specificity of the pigeon liver enzyme was not determined; whether it is specifically a pantetheine kinase or, like the enzyme from Proteus studied here, has a more general specificity, is not known. The presence of an apparently similar enzyme in *Lactobacillus arabinosus* has been noted without details by Baddiley et al. (19).

Phosphorylation of pantothenic acid by the enzyme raises the question of the possible importance of phosphopantothenate as an intermediate in pantothenate metabolism. Treatment of several natural products, and especially the spent growth medium of *Neurospora crassa*, with purified intestinal phosphatase results in enhanced pantothenic acid activity for *S. carlsbergensis*. Of the known bound forms of pantothenic acid, only phos-
phopantothenic acid is converted to a growth factor for yeast by this treatment. This compound might be visualized as an intermediate in coenzyme A synthesis by Route A.

(A) Pantothenate → phosphopantothenate → phosphopantothenylcysteine → phosphopantetheine → dephosphocoenzyme A → coenzyme A

which might be an alternative to Route B, all reactions of which have been shown to occur in cell-free extracts (7, 8, 18):

(B) Pantothenate → pantetheine → pantetheine → dephosphocoenzyme A → coenzyme A

As noted elsewhere (6), permeability relationships are such that the participation of phosphopantothenic acid in a scheme such as Route A (which might occur together with or as an alternative to Route B) cannot be discounted because of its inactivity in supporting growth. It is also possible, however, that phosphopantothenate formation by the kinase described here represents a metabolic cul-de-sac, and that the essential function of the enzyme is as a pantetheine kinase in accord with biosynthetic Route B.

Conversion of pantothenate by P. morganii to a product identical in growth-promoting characteristics with pantetheine cysteine has been noted previously (7). It appears beyond question that one or both of these two reactions are inhibited by pantoyltaurine, as observed originally by McIlwain (2).

SUMMARY

Preparation and partial purification of a kinase from Proteus morganii that phosphorylates pantothenic acid, pantetheine, and pantetheine with approximately equal facility are described. The Michaelis constant for pantothenic acid is approximately $2.5 \times 10^{-5}$ M; adenosinetriphosphate and magnesium ions are required for the reaction. The pH optimum lies between 7.0 and 7.5. The product formed from pantothenic acid was identified chromatographically as 4'-phosphopantothenic acid. The enzyme resembles in many respects the pantetheine kinase from pigeon liver (18), but is more stable to heat and is less specific with respect to substrate. The possible significance of phosphopantothenic acid as a metabolic intermediate is discussed briefly.

BIBLIOGRAPHY

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