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PURIFICATION AND PROPERTIES OF D-PANTOTHENATE KINASE FROM RAT LIVER*

YASUSHI ABIKO, SHIN-ICHIRO ASHIDA AND MASAO SHIMIZU
Research Laboratories, Daiichi Seiyaku Co., Ltd., Edogawa-ku, Tokyo 132 (Japan)

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SUMMARY

Rat-liver D-pantothenate kinase (ATP:D-pantothenate 4'-phosphotransferase, EC 2.7.1.33) was purified about 700-fold compared to crude extract and its properties were examined. The purification steps included protamine treatment of the crude extract of rat liver, pH-fractionation, DEAE-Sephadex A-50 chromatography and Sephadex G-200 gel filtration. The enzyme was very unstable and its purification was successful only in the presence of ATP and sucrose as stabilizers throughout the purification procedures. The purified preparation was actually free from ATPase and phosphatase activities, although it was not completely homogeneous.

The study of the magnesium requirement of the kinase indicated that an ATP–Mg^{2+} 1:1 complex was an active substrate. Apparent K_m values of the kinase were 0.011 mM for D-pantothenate and 1.0 mM for ATP–Mg^{2+} at the optimal pH of 6.1. D-Configuration of 2'-OH in the pantothenic acid molecule was found to be essential for functional interaction with the enzyme.

Pantothenate kinase from rat liver was inhibited to various extents by the intermediates of CoA biosynthesis, of which CoA itself and pantetheine 4'-phosphate were most effective.

INTRODUCTION

Pantothenate kinase (ATP:D-pantothenate 4'-phosphotransferase, EC 2.7.1.33) catalyzes the formation of pantothenate 4'-phosphate from pantothenate, the first step of CoA biosynthesis in rat liver and kidney and in those of other organisms[1,2]. Of five enzymes participating in the biosynthesis of CoA in rat liver, four enzymes, except pantothenate kinase, were purified and characterized in detail by Abiko and his co-workers[3–6]. However, studies on pantothenate kinase have been limited to those with crude enzyme preparations[2,7–10] and lability of this enzyme has hindered its detailed investigations.

We attempted the purification of this kinase with a favorable action of some stabilizers to obtain further information about the kinase. The present report deals with the purification of D-pantothenate kinase from rat liver and its properties,

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including kinetic characterization, substrate specificity and the effect of the intermediates of CoA biosynthesis. The role of this kinase in the control of CoA biosynthesis in rat liver is briefly discussed.

MATERIALS AND METHODS

Chemicals. Calcium D-pantothenate, Calcium L-pantothenate, 2'-ketopantothenic acid, 2',2''-diketopantethine, and the barium salts of pantothenic acid 4'-phosphate, pantothenoyl-L-cysteine 4'-phosphate, pantetheine 4'-phosphate, 2',2''-diketopantethine 4',4''-diphosphate, and the lithium salts of dephospho-CoA and CoA were synthesized in our laboratories[11–14]. The barium salts were treated with Amberlite IR-120 (H^+) to obtain the corresponding free acids. The calcium salt of D-pantothenic acid was equal to the sodium salt in activity as the substrate. ATP (disodium salt) was a preparation of our company. All the substrates and reactants were dissolved in 0.05 M Tris–maleate buffer (pH 6.4) and were used for the experiments. Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden. The basal medium used for the microbiological assay of pantothenate was a commercial preparation of Nissui Seiyaku Co., Ltd, Tokyo. Protamine sulfate from salmon sperm was perchased from Tokyo Kasei Co., Ltd, Tokyo. Other reagents used were of analytical grade of purity.

Assay of pantothenate. Pantothenate was microbiologically assayed using *Lactobacillus arabinosus* 17-5 as the test organism[15], for which 2'-ketopantothenic acid, 2'-ketopantetheine, pantothenoyl-cysteine 4'-phosphate, pantetheine 4'-phosphate, dephospho CoA and CoA were inactive. This method, however, was limited to the samples which did not contain large amounts of L-pantothenate (more than 5 nmoles per assay tube) or pantothenic acid 4'-phosphate (more than 0.5 nmole/assay tube), because these compounds promoted, to some extent, the growth of this microorganism. The range of assay for pantothenate was below 0.4 nmole/assay tube.

Assay of pantothenate kinase. Pantothenate kinase activity was assayed as described previously[2]. The reaction mixture contained 8 nmoles of D-pantothenate, 0.4 μmole of ATP, 0.4 μmole of $MgCl_2$ and the enzyme in a total volume of 0.2 ml (25 mM Tris–maleate buffer, pH 6.1). The mixture was incubated at 37 °C for 10–60 min. One enzyme unit is defined in this report as the amount which catalyzes the decrease of 1 nmole of pantothenate per min under the above conditions. Specific activity is represented as the units of activity per mg of protein. Protein was determined by the method of Lowry *et al.*[16].

Assay of ATPase. The ATPase activity in the kinase fraction was checked by measuring the release of inorganic phosphate from ATP. The reaction mixture contained 0.4 μmole of ATP, 0.4 μmole of $MgCl_2$ and the enzyme in a total volume of 0.1 ml (25 mM Tris–maleate buffer, pH 6.1). After incubation at 37 °C for 30 min, P_i released was determined by the method of Yanagita[17].

Assay of phosphatase. Contaminated phosphatase activity in the kinase fraction was checked by the *p*-nitrophenyl phosphate procedure[18] at pH 6.1. The phosphatase activity toward pantothenic acid 4'-phosphate was checked by measuring pantothenate released from the phosphate. The enzyme solution to be tested was dialyzed against 500 vol. of 0.02 M Tris–maleate buffer (pH 5.8) containing 0.1 M NaCl and 10% sucrose. The dialyzed solution was incubated at 37 °C for 30 min with 4 nmoles

of D-pantothenic acid 4'-phosphate and 0.2 μmole of $MgCl_2$ in a total volume of 0.1 ml (25 mM Tris–maleate buffer, pH 6.1). After incubation, the reaction mixture was heated in a boiling water bath for 1 min to stop the reaction and was microbiologically assayed for pantothenate released.

Paper chromatography. Paper chromatography for detection of phosphorylated products was carried out as previously described[2,10], using Toyo Roshi No. 51-A paper (Toyo Roshi Co., Ltd, Tokyo) with *n*-butanol–acetic acid–water (5:2:3, by vol.) and *n*-propanol–conc. NH_4OH–water (6:3:1, by vol.) as solvents. Phosphorylated products on paper chromatograms were detected by the Hanes-Isherwood-reagent[19].

RESULTS

Enzyme purification

Extraction and protamine treatment. All the steps of enzyme purification were carried out at 4 °C. The pH of buffers was measured at room temperature (23 °C).

Livers from male Wistar rats, weighing about 250 g, were homogenized with 3 vol. of chilled 0.02 M phosphate buffer (pH 7.2). The homogenate was centrifuged at 7500 × *g* for 40 min. The supernatant solution was again centrifuged at 77 500 × *g* for 1 h. To the resultant supernatant solution (Fraction 1) 0.035 vol. of 2% protamine sulfate solution was added dropwise with stirring and the precipitate formed was removed by centrifugation (7500 × *g* for 20 min). The supernatant solution was treated in a similar manner with an additional 0.1 vol. of the protamine sulfate solution and the precipitate was collected by centrifugation (7500 × *g* for 20 min). The gummy precipitate was washed once quickly with 0.01 M phosphate buffer (pH 7.0) containing 1 mM ATP and then extracted with the same buffer overnight. The residue was centrifuged down (12 000 × *g* for 30 min) and the clear solution was obtained (Fraction 2).

pH fractionation. Fraction 2 was adjusted to pH 5.0 with dil. HCl and the precipitate formed was removed by centrifugation (12 000 × *g* for 30 min). The supernatant solution was then adjusted to pH 5.8 with dil. NaOH (Fraction 3). This fraction contained most of the activity present in Fraction 2.

DEAE-Sephadex A-50 chromatography. 2.0 ml of Fraction 3 (16.2 mg protein) was diluted 1:1 with 0.04 M Tris–maleate buffer (pH 5.7) containing 0.1 M NaCl and 20% sucrose, and loaded onto a DEAE-Sephadex A-50 column (1 cm × 13 cm, stacked with Sephadex G-25 gel of 1 cm height on the top), equilibrated with 0.02 M Tris–maleate buffer (pH 5.8) containing 0.05 M NaCl and 10% sucrose. The column was washed with the equilibration buffer and the protein were eluted at a flow rate of about 10 ml/h with a linear gradient of NaCl of 0.05 to 0.55 M. The fractions of 1.5 ml each were collected in tubes containing 0.1 ml each of 20 mM ATP (pH 5.8) as a stabilizer. Thus Fraction 3 was processed rapidly in six batches in order to achieve better purification, because larger scale chromatography resulted in considerable loss of the activity. Fig. 1 illustrates a typical elution profile of the chromatography. The kinase activity was eluted at approx. 0.2 M NaCl. Fractions containing most of the activity were pooled. The fractions from six batches were combined (Fraction 4), concentrated by pressure filtration through an Amicon XM-50 ultrafiltration membrane (Amicon Corp., Lexington, Mass., U.S.A.) and then dialyzed against 0.02 M Tris–maleate buffer (pH 5.8) containing 0.1 M NaCl, 10% sucrose and

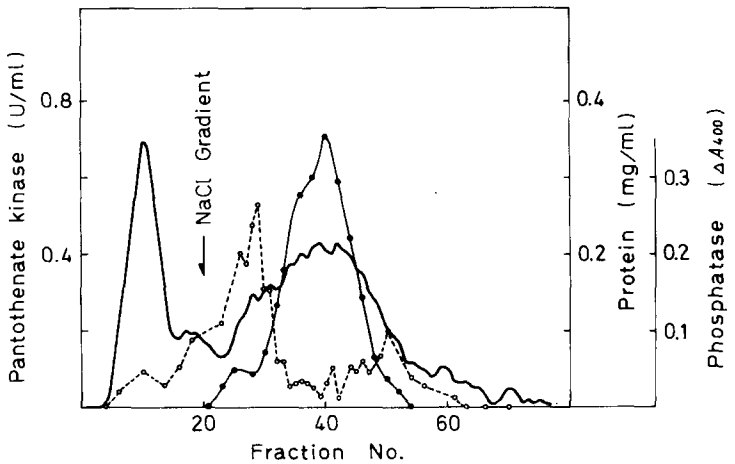

Fig. 1. DEAE-Sephadex A-50 chromatography of pantothenate kinase. Load: 16.2 mg protein of Fraction 3. Column size: 1 cm × 13 cm (0.02 M Tris–maleate–0.05 M NaCl–10% sucrose, pH 5.8). Proteins were eluted with a linear gradient of NaCl (0.05–0.55 M). Flow rate: 10 ml/h. Fractions of 1.5 ml each were collected in tubes containing 0.1 ml each of 20 mM ATP (pH 5.8). ———, protein; ●—●, pantothenate kinase activity; ○---○, *p*-nitrophenylphosphatase activity.

1 mM ATP. Insoluble material occurred during the concentration and the dialysis was removed by centrifugation (12 000 × *g* for 30 min). The specific activity of the concentrated kinase fraction (2.60) was almost the same as that of Fraction 4. Most of this fraction (about 5 ml, 3.25 mg protein/ml) was stored at −20 °C for the kinetic experiments of the kinase.

Sephadex G-200 gel filtration. A 1.0-ml aliquot of the concentrated A-50 eluate (3.25 mg protein) was passed through a Sephadex G-200 gel column (2 cm × 40 cm, stacked with Sephadex G-25 gel of 1 cm height on the top) equilibrated with 0.02 M Tris–maleate buffer (pH 5.8) containing 0.1 M NaCl, 10% sucrose and 1 mM ATP. Fig. 2 shows a typical elution pattern. The kinase activity was eluted in two peaks. Active fractions of the main peak were combined and concentrated by the use of a collodion bag (Sartorius-Membranfilter GmbH, Göttingen, Germany) (Fraction 5).

Polyacrylamide gel electrophoresis of this kinase preparation revealed contamination of several proteins. Refiltration on Sephadex G-200 gel of the kinase fraction obtained, however, resulted in a marked loss of the activity and in decrease of the specific activity even in the presence of ATP and sucrose. The attempt to further purify the kinase by the use of the isoelectric focusing technique[20] was unsuccessful because of insolubility of the kinase in a medium of low ionic strength.

Table I summarizes the purification of pantothenate kinase. The enzyme has been purified approx. 700-fold compared to the crude extract. Rat-liver pantothenate kinase was considerably unstable, independent of the degree of purity. ATP (1 mM) and sucrose (10%) stabilized the kinase and allowed its purification. Pantothenate kinase was found to be most stable at pH 5.8 in the presence of ATP and sucrose. The purified kinase of Fraction 4 could be stored without loss of the activity at protein concentrations of more than 3 mg/ml and at −20 °C for more than 1 week or at 4 °C for 4 days under the above favorable conditions. The preparations of pantothenate kinase, Fractions 4 and 5, were found to be free from ATPase activity

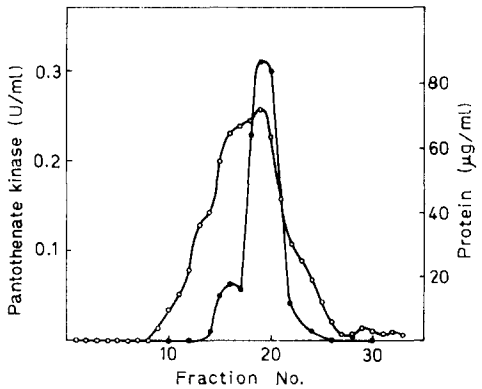

Fig. 2. Sephadex G-200 gel filtration of pantothenate kinase. Load: 3.25 mg protein of concentrated and dialyzed Fraction 4. Column size: 2 cm × 40 cm. Buffer: 0.02 M Tris–maleate–0.1 M NaCl–10% sucrose–1 mM ATP (pH 5.8). Flow rate: 10 ml/h. Fraction size: 5 ml. ○—○, protein; ●—●, pantothenate kinase activity.

TABLE I

SUMMARY OF PURIFICATION OF PANTOTHENATE KINASE FROM RAT LIVER

Fraction	*Vol. (ml)*	*Protein (mg)*	*Pantothenate kinase*		
			Specific activity (units/mg)	*Total activity (units)*	*Overall recovery (%)*
1. Crude extract*	100	3660	0.006	22.2	100
2. Protamine treatment	11.3	171	0.955	164	740**
3. pH fractionation	12.0	97.2	1.32	121	545
4. DEAE-Sephadex A-50	96	21.5	2.88	62	280
5. Sephadex G-200***	7.5	1.22	4.10	5.0	54

* From 50 g of livers.

** Anomalous excess yield of the activity in this step is probably due to removal of large amounts (more than 95% of those present in Fraction 1) of phosphatase activity (see text).

*** 3.25 mg of Fraction 4 was used for this step. Figure in the last column (54) represents the recovery of this gel filtration step.

and phosphatase activity toward pantothenate 4'-phosphate. In all experiments described below the enzyme preparation of the stock solution of Fraction 4 was used.

Kinetic characterization of the kinase

The pH optimum of the kinase reaction was found to be 5.9–6.3. The effect of Mg^{2+} on the enzyme activity was studied at pH 6.1. As shown in Fig. 3, a maximal rate was observed when the Mg^{2+} concentration was equal to the initial concentration of ATP. This result indicates that ATP–Mg^{2+} 1:1 complex is an active substrate, so that in the subsequent studies the ATP and $MgCl_2$ concentrations were varied simultanously to satisfy the 1:1 relationship.

The effect of the concentration of the substrates, ATP and D-pantothenate, on the reaction rate was examined. Double reciprocal plots of the reaction velocity

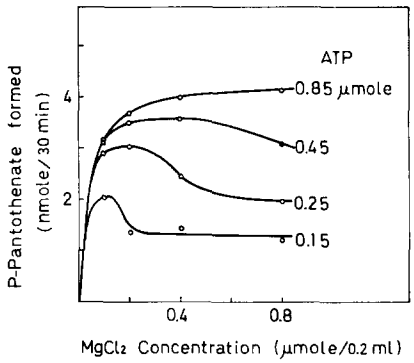

Fig. 3. Effect of Mg^{2+} concentration in relation to ATP concentration on the pantothenate kinase activity. The reaction mixture contained pantothenate kinase (0.12 unit), pantothenate (8 nmoles), ATP and $MgCl_2$ as indicated in a total volume of 0.2 ml (0.03 M Tris–maleate, 0.025 M NaCl and 2.5% sucrose, pH 6.1). The mixture was incubated at 37 °C for 30 min.

versus varing concentrations of one substrate at a fixed high concentration of the other substrate (4.5 mM ATP–Mg^{2+} or 0.16 mM D-pantothenate) gave apparent K_m of 0.011 mM for D-pantothenate and 1.0 mM for ATP–Mg^{2+}.

Substrate specificity of the kinase

Substrate specificity of pantothenate kinase was studied in respect to the configuration of 2'-OH in the pantothenic acid molecule, using L-pantothenate and 2'-ketopantetheine. 1 μmole of L-pantothenate or 2'-ketopantetheine (0.5 μmole 2',2''-diketopantethine *plus* 40 μmoles cysteine) was incubated with 18.1 units of pantothenate kinase (Fraction 2), 20 μmoles of ATP and 20 μmoles of $MgCl_2$ in a total volume of 4 ml (0.05 M Tris buffer, pH 7.0) at 37 °C for 2 h, and the reaction was stopped by heating. Under the same conditions, D-pantothenate was

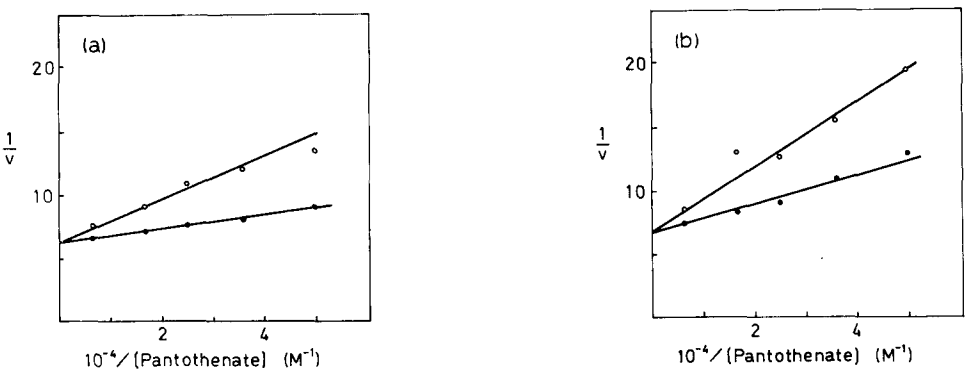

Fig. 4. Competitive inhibition of pantothenate kinase by L-pantothenate (a) and by 2'-ketopantetheine (b). (a) The reaction mixture contained varied amounts of D-pantothenate (2–16 nmoles), ATP–$MgCl_2$ (0.4 μmole) and pantothenate kinase (0.12 unit) with (○) or without (●) L-pantothenate (0.125 μmole) in a total volume of 0.1 ml (0.035 M Tris–maleate, 0.05 M NaCl and 5% sucrose, pH 6.1). (b) The reaction system was the same as in (a), except for pantothenate kinase (0.1 unit) with (○) or without (●) 2'-ketopantetheine (0.1 μmole) (0.05 μmole 2',2''-diketopantethine *plus* 0.5 μmole L-cysteine). The mixture was incubated at 37 °C for 10–50 min depending on the reaction rate. Velocity units are nmoles of D-pantothenate decreased per min.

completely phosphorylated. The detection of enzymatically phosphorylated products, if present, in the reaction mixtures was attempted by paper chromatography, because of the lack of suitable assay methods for L-pantothenate, 2′-ketopantetheine or their phosphates. The chromatography system, including the pretreatments of the reaction mixture, can detect phosphorylated products if more than 1.2% of the substrate is phosphorylated. Neither L-pantothenate 4′-phosphate nor 2′-ketopantetheine 4′-phosphate was detected in the reaction mixtures. This indicates that L-pantothenate and 2′-ketopantetheine can not be the substrate of the kinase.

On the other hand, pantothenate kinase was found to be inhibited by L-pantothenate and 2′-ketopantetheine. Double reciprocal plots of the kinase reaction in the absence and the presence of these compounds indicated that they were competitive inhibitors of the kinase with respect to D-pantothenate (Figs 4a and 4b). The apparent K_i values were 0.6 mM for L-pantothenate and 0.83 mM for 2′-ketopantetheine.

Inhibition by the intermediates of CoA biosynthesis

The effects of the intermediates of CoA biosynthesis on the kinase activity were studied. As shown in Table II, all the intermediates inhibited the enzyme at 1 mM. Among these, CoA, pantetheine 4′-phosphate and pantothenoylcysteine 4′-phosphate were found to be strong inhibitors, and the inhibitory effect of CoA was markedly dependent on its concentration.

TABLE II

INHIBITORY EFFECTS OF VARIOUS INTERMEDIATES OF CoA BIOSYNTHESIS ON THE PANTOTHENATE KINASE ACTIVITY

The inhibitory effects of various intermediates of CoA biosynthesis including CoA itself were measured in the standard assay system for the kinase described in Materials and Methods.

Intermediate	*Inhibition (%) at*		
	1.0 mM	*0.25 mM*	*0.05 mM*
Pantothenate 4′-phosphate			13.0
Pantothenoylcysteine 4′-phosphate	69.2		14.5
Pantetheine 4′-phosphate	76.6	53.1	27.5
Dephospho-CoA	28.0		
CoA	97.0	51.2	10.0

DISCUSSION

Rat-liver D-pantothenate kinase was unstable even in a crude preparation such as the pH 5 fraction, which lost more than 60% activity after 1 week of storage at 4 °C and at the most stable pH, 5.8. Lability of this enzyme further increased as the purification progressed. However, the kinase was found to be considerably stable in the presence of 1 mM ATP (one of the substrates of this enzyme) and/or 10% sucrose. The use of these stabilizers facilitated further purification of this enzyme as described above. Removal of phosphatase and ATPase activities from the enzyme preparation is essential for the kinetic studies on the kinase reaction. This was satisfactorily achieved by the use of the protamine treatment and DEAE-Sephadex A-50

chromatography. More than 95% of the phosphatase activity toward *p*-nitrophenylphosphate present in the crude extract was eliminated by the protamine treatment, and anomalously excess yield of the kinase activity in this step (Table I) seemed to be due to the removal of unfavorable action of the phosphatase activity. The residual phosphatase activity was effectively separated from pantothenate kinase fraction by DEAE-Sephadex A-50 chromatography (Fig. 1). It was confirmed that the kinase preparation of this fraction possessed no phosphatase activity toward pantothenate 4'-phosphate. The specific activity of ATPase of this fraction was 0.236 nmole P_i released from ATP/min per mg of protein, which corresponded to a hydrolysis of less than 0.05% of ATP in the standard kinase reaction system for 30 min. These results indicate reliability of kinetic studies with this kinase preparation.

The elution profile of the kinase activity from a Sephadex G-200 gel column revealed the existence of two molecular species of the kinase activity: a major component of a smaller molecular size and a minor of a larger molecular size (Fig. 2). The minor component seemed to be an artifact occurred during purification procedures, because the pooled major component again yielded a new minor component on refiltration of the same system, being accompanied by marked loss of the total activity.

A previous report from our laboratories demonstrated that rat-liver pantothenate kinase phosphorylated panteth(e)ine, pantothenoyl-L-cysteine and pantothenyl alcohol at similar rates to the rate with D-pantothenate[2,6,10]. The present study provided additional information about the substrate specificity of the kinase, especially stereospecificity. The purified rat-liver kinase did not phosphorylate L-pantothenate and 2'-ketopanthetheine within the limits of detection, indicating that, if these analogs were phosphorylated, the rate must be less than 1% of that with D-pantothenate. Although they were competitive inhibitors of the phosphorylation of D-pantothenate, their apparent K_i values were very high (0.6 mM for L-pantothenate and 0.83 mM for 2'-ketopantetheine) as compared with apparent K_m for D-pantothenate (0.011 mM). These findings indicate that the configuration of 2'-OH in the pantothenic acid molecule plays an important role in the interaction between pantothenate and the kinase. On the other hand, using L-CoA and 2'-keto-CoA, Shimizu *et al.*[21] demonstrated that the configuration of the pantoyl moiety in the CoA molecule did not show any essential requirement for recognition by a CoA-dependent enzyme, phosphotransacetylase.

The interesting finding presented in this report is inhibition of the kinase by the intermediates of CoA biosynthesis, especially by pantetheine 4'-phosphate and CoA itself. These two compounds inhibited the phosphorylation of pantothenate by this kinase by about 50% at the concentration of 0.25 mM (Table II). The inhibitory action of these two compounds seems very meaningful, although characterization of the mechanism of this inhibition requires further detailed investigation. Recently, Kuwagata[22] and Nakamura *et al.*[23] demonstrated that pantetheine 4'-phosphate was a sole intermediate of CoA biosynthesis having a considerable pool in rat liver: 219 nmoles/g (approx. 0.22 mM). They also reported that CoA level in rat liver was 363 nmoles/g (approx. 0.36 mM). These values are close to the effective concentration of these compounds for inhibiting pantothenate kinase, suggesting that the kinase is not fully active in the liver cells and that its inhibition by pantetheine 4'-phosphate and CoA may be involved partly in regulating CoA level in the cells. Pantetheine

4′-phosphate was also reported to exert product inhibition (K_i, 0.43 mM) toward the pantothenoylcysteine 4′-phosphate decarboxylase system[3]. The possibility that pantothenate kinase is a regulating enzyme in the biosynthesis of CoA was also presented by Karasawa *et al.**, using a crude preparation of the kinase.

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