A high yield method for the preparative synthesis of coenzyme A by combination of chemical and enzymic reactions

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Dried cells of *Brevibacterium ammoniagenes* are a good enzyme source for the preparative synthesis of CoA from pantothenic acid, L-cysteine and ATP. A problem with this synthesis is that the CoA synthesis is repressed by negative feedback inhibition by CoA to pantothenate kinase, the first step enzyme for the biosynthesis of CoA, which catalyses phosphorylation of pantothetnic acid or pantetheine. As the inhibition operates only at this step, a further increased yield is possible if the enzymic phosphorylation step is replaced with chemical synthesis. Yields from phosphorylated substrates are more than 10-times higher than those from pantothenic acid or pantetheine (33 g/l from phosphopantothenic acid with a molar yield of 86%; 115 g/l from phosphopantetheine with a molar yield of 100%).

CoA *Pantothenic acid* *Phosphopantothenic acid* *Phosphopantetheine* *Enzymic synthesis* *Feedback inhibition*

1. INTRODUCTION

Coenzyme A (CoA) is biosynthesised from pantothenic acid, L-cysteine and ATP through 5 enzymic steps as shown in fig.1 [1]. We found that *Brevibacterium ammoniagenes* have all 5 enzymes necessary for the synthesis of CoA, all with high activities. Using cells of this organism as the catalyst, we established a novel process for the preparative synthesis of this coenzyme from the above substrates [2–4]. The advantages of this enzymic method are that the process is simple and rapid, and the yield is higher compared to chemical synthesis and extraction from microbial cells. Under the established conditions, the yield of CoA reached 2–5 g/l reaction medium. However, we could not attain a further increase in CoA yield. One of the possible reasons why we could not improve the yield is probably negative feedback inhibition of pantothenate kinase, which catalyzes the phosphorylation of pantothenic acid, by overproduced CoA itself [5,6]. Another route, by which phosphopantetheine is derived from pantetheine, also undergoes the feedback inhibition because the reaction is catalysed by the same enzyme as that for the phosphorylation of pantothenic acid [6]. A scheme in which the reaction is started from the condensation of phosphopantothenic acid and L-cysteine or the transadenylylation of phosphopantetheine seems promising for improving the CoA yield, since these routes skip phosphorylation of pantothenic acid or pantetheine by pantothenate kinase. Our results show

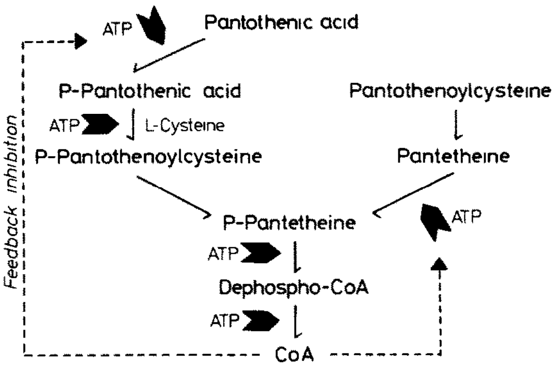

Fig.1. Pathway for the biosynthesis of CoA.

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that removal of the feedback inhibition by replacing the enzymic phosphorylation with chemical phosphorylation brings about a significantly higher yield of CoA.

2. MATERIALS AND METHODS

2.1. *Microorganism and preparation of dried cells*

Brevibacterium ammoniagenes IFO 12071 was used. Cultivation techniques and preparation of dried cells were as in [2].

2.2. *Chemicals*

CoA and pantethine were kindly donated by Kyowa Hakko (Tokyo) and Daiichi Pharmaceutical (Tokyo), respectively. Phosphopantothenic acid and phosphopantetheine (disulfide form) were prepared chemically [7]. All other reagents used in this study were as in [2].

2.3. *Analyses*

CoA was determined as in [8]. Pantothenic acid and pantetheine were measured microbiologically [9,10]. Phosphopantothenic acid and phosphopantetheine were measured as pantothenic acid and pantetheine after phosphatase digestion, respectively, and ATP was measured enzymically [11]. Other analyses were as in [2].

3. RESULTS AND DISCUSSION

3.1. *Inhibition of CoA synthesis by CoA*

As shown in table 1, there was almost completely no synthesis of CoA from pantothenic acid or pantetheine when 4 mM CoA was present in the reaction mixture. As either pantothenic acid or pantetheine was almost completely recovered without phosphatase digestion, the point of the inhibition by CoA was at the phosphorylation step for these substrates. On the other hand, CoA synthesis from the phosphorylated substrates was not inhibited by CoA at all. These results demonstrate that CoA inhibited only phosphorylation of pantothenic acid or pantetheine, and that the reactions following the phosphorylation were insensitive to CoA.

Based on the results in table 1, we investigated suitable reaction conditions for CoA synthesis from phosphorylated substrates and established the following conditions.

Table 1

Effect of CoA on the synthesis of CoA

Substrate	Addition of CoA	CoA found	Substrate recovered
Pantothenic acid	0.0	2.0	<0.01
	4.0	3.8	5.13
Phosphopantothenic acid	0.0	3.8	<0.01
	4.0	7.1	<0.01
Pantetheine	0.0	2.5	<0.01
	4.0	3.9	5.12
Phosphopantetheine	0.0	4.3	<0.01
	4.0	8.0	<0.01

The reaction mixture (1 ml) containing 15 μmol ATP, 10 μmol L-cysteine, 10 μmol $MgSO_4$, 200 μmol potassium phosphate (pH 6.5), 1 mg sodium laurylbenzenesulfonate, 100 mg dried cells and 5 μmol of each substrate as indicated was incubated for 5 h at 37°C with or without 4 μmol CoA. When pantetheine or phosphopantetheine was the substrate, L-cysteine was replaced with 20 μmol dithiothreitol. All values are given in μmol

3.2. *Synthesis of CoA from phosphopantothenic acid, L-cysteine and ATP*

A reaction mixture containing 2.5 mmol of phosphopantothenic acid (3 Li, pH 7.0), 3.25 mmol L-cysteine, 10 mmol ATP (2 Na, pH 6.5), 0.5 mmol $MgSo_4$, 10 mmol potassium phosphate (pH 6.5), 100 mg sodium laurylbenzenesulfonate and 5 g dried cells in a total volume of 50 ml was incubated at 37°C for 48 h with gentle shaking. At 12 and 20 h of reaction, 1 g dried cells was put into the mixture. Under these conditions, 2.15 mmol (1.65 g) of CoA was synthesised in the mixture, as shown in fig.2. In this reaction, 4 sequential enzymic steps are involved and 1 mol each of phosphopantothenic acid and L-cysteine and 3 mol ATP are required for the synthesis of 1 mol CoA, thus molar conversion ratios of these substrates to CoA were 86.0, 66.1 and 64.5%, respectively.

The lithium salts of CoA were obtained from the reaction mixture by the method involving treatment with charcoal and Dowex 1 × 2 as in [2,12] (yield, 1.13 g; purity, 92% [13]; adenosine:P:SH, 1:2.93:0.97).

3.3. *Synthesis of CoA from phosphopantetheine and ATP*

Fig.3 shows that the synthesis of CoA proceeded

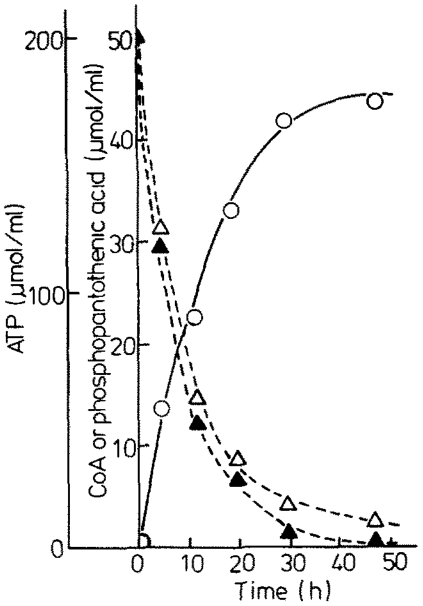

Fig.2. Synthesis of CoA from phosphopantothenic acid, L-cysteine and ATP. For reaction conditions see text: (—○—) CoA; (---△---) phosphopantothenic acid; (---▲---) ATP.

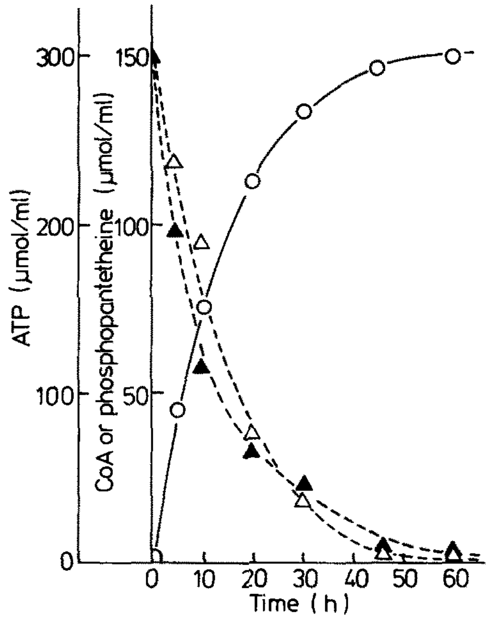

Fig.3. Synthesis of CoA from phosphopantetheine and ATP. For reaction conditions see text: (—○—) CoA; (---△---) phosphopantetheine; (---▲---) ATP.

stoichiometrically, when phosphopantetheine and ATP were the starting substrates. In this reaction, 1 mol phosphopantetheine is incorporated into 1 mol CoA with 2 mol ATP. The reaction mixture containing 15 mmol phosphopantetheine (2 Li, pH 7.0), 15 mmol dithiothreitol, 30 mmol ATP (2 Na, pH 6.5), 1 mmol $MgSO_4$, 20 mmol potassium phosphate (pH 6.5), 200 mg sodium laurylbenzenesulfonate and 15 g dried cells in a total volume of 100 ml was incubated at 37°C for 60 h with gentle shaking. At 20 h of reaction, 4 g dried cells was put into the mixture. Under these conditions 15 mmol (11.5 g) of CoA was synthesised.

The lithium salts of CoA, weighing 8.03 g, were isolated from the reaction mixture by the same procedure as those above (purity, 93% [13]; adenosine:P:SH, 1:2.09:0.95).

The present method, using a combination of chemical and enzymic reactions, offers the most preferable route for the preparation of CoA because of its simplicity and high efficiency in conversion of substrates to the product.

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REFERENCES

[1] Brown, G.M. (1959) J. Biol. Chem. 234, 370–378.
[2] Shimizu, S., Tani, Y. and Ogata, K. (1979) in: Methods in Enzymology (McCormick, D.B. and Wright, L.D. eds) vol. 62, pp. 236–245, Academic Press, New York.
[3] Shimizu, S., Tani, Y. and Yamada, H. (1979) in: Immobilized Microbial Cells (Venkatsubramanian, K. ed) pp. 87–100, American Chemical Society, Washington DC.
[4] Yamada, H., Shimizu, S. and Tani, Y. (1980) in: Enzyme Engineering (Weetall, H.H. and Royer, G.P. eds) vol. 5, pp. 405–411, Plenum, New York.
[5] Shimizu, S., Kubo, K., Morioka, H., Tani, Y. and Ogata, K. (1974) Agric. Biol. Chem. 38, 1015–1021.
[6] Shimizu, S., Kubo, K., Tani, Y. and Ogata, K. (1973) Agric. Biol. Chem. 37, 2863–2870.
[7] Shimizu, M. (1970) in: Methods in Enzymology (McCormick, D.B. and Wright, L.D. eds) vol. 18, pp. 322–338, Academic Press, New York.

[8] Allred, J.B. and Guy, D.G. (1969) Anal. Biochem. 29, 293–299.

[9] Skeggs, H.R. and Wright, L.D. (1944) J. Biol. Chem. 156, 21–26.

[10] Snell, E.E. and Witle, E.L. (1957) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds) vol. 3, pp. 918–928, Academic Press, New York.

[11] Lamprecht, W. and Trautschold, I. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) vol. 4, 2nd edn, pp. 2101–2110, Academic Press, New York.

[12] Shimizu, S., Morioka, H., Tani, Y. and Ogata, K. (1975) J. Ferment. Technol. 53, 77–83.

[13] Bergmeyer, H.U., Holz, G., Klotzsch, H. and Lang, G. (1963) Biochem. Z. 338, 114–121.