ENZYMATIC SYNTHESIS OF THE COENZYME A ESTER OF *o*-SUCCINYLBENZOIC ACID, AN INTERMEDIATE IN MENAQUINONE (VITAMIN K_2) BIOSYNTHESIS

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1. Introduction

The quinonoid moiety of menaquinones (i.e., vitamin K_2) (V) is known to be biosynthetically derived from shikimic acid [1] and 4-(2'-carboxyphenyl)-4-oxobutanoic acid (i.e., *o*-succinylbenzoic acid, OSB) (I) [2] (fig.1). While the cell-free synthesis of (I) has not yet been demonstrated its enzymic conversion to 1,4-dihydroxy-2-naphthoic acid (DHNA) (III) was shown to require ATP and CoASH [3]. This conversion is catalyzed by 2 different enzymes, viz., *o*-succinylbezoyl-coenzyme A synthetase and dihydroxynaphthoate synthase [4]. It has been assumed [3] that in the course of this conversion the aromatic carboxyl group of (I) is activated with coenzyme A. The postulated CoA ester (II; R_1 = SCoA; R_2 = OH), however, has never been isolated. We now report on the detection of a CoA derivative of (I) in cell-free preparations of *Mycobacterium phlei*. The unstable CoA derivative, presumably a CoA ester, was isolated and shown to be converted to 1,4-dihydroxy-2-naphthoic acid (DHNA) (III) by an enzyme preparation from the same organism.

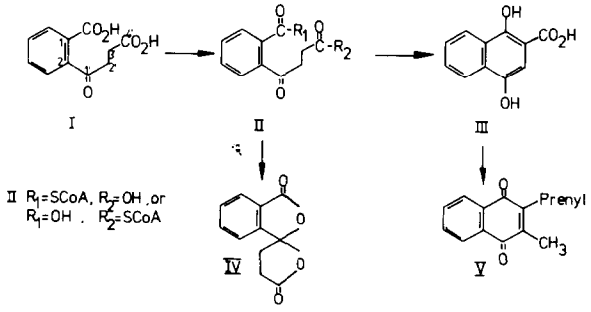

Fig.1. Formation and metabolism of the thioester (II) of *o*-succinylbenzoic acid (I).

2. Experimental

2.1. *Material*

The *M. phlei* strain was obtained from Dr J. Reiff, Institut für Mikrobiologie of this University. *o*-[4'-^{14}C]-succinylbenzoic acid was synthesized according to [5]. [G-^{3}H] Coenzyme A (spec. act. 30.0 μCi/μmol) was purchased from New England Nuclear, (Dreieich).

2.2. *Growth of organism and enzyme extraction*

Mycobacterium phlei was grown in 300 ml Erlenmeyer flasks containing 50 ml medium [6] on a gyratory shaker (150 rev./min) at 37°C for 48 h. The cells were harvested by centrifugation (3000 × *g*, 10 min) and stored at −20°C.

Enzyme preparations were obtained according to [7] except that sonication (10 min) was applied to rupture cells and β-mercaptoethanol was replaced by dithiothreitol (0.2 mM). The crude enzyme preparation was treated with protamine sulfate [7] and after centrifugation (12 000 × *g*, 10 min) the supernatant was centrifuged through Sephadex G-25 [8] equilibrated with phosphate buffer (0.1 M, pH 8.0) containing dithiothreitol (0.2 mM). Protein was determined according to [9].

2.3. *Enzymic synthesis of II*

The incubation mixture contained protein (5 mg), potassium phosphate (100 μmol, pH 6.5), dithiothreitol (0.2 μmol), $MgCl_2$ (20 μmol), ATP (10 μmol), CoASH (2.5 μmol), *o*-[4'-^{14}C]succinylbenzoic acid (I) (0.5 μCi, 0.07 μmol) in a final volume of 1 ml. Incubation was carried out for 20 min at 30°C and terminated by addition of 100 μl formic acid.

2.4. *Isolation of II*

The incubation mixture (see 2.3) was evaporated to dryness at 10^{-1} Torr and room temperature, the residue applied to Whatmann 3 MM chromatography paper (washed with water) and developed in *n*-butanol–acetic acid–water (5:2:3, by vol.). The CoA-derivative (R_F 0.48), hereafter named OSB-Co A ester, was detected as shown in fig.2 and eluted with formic acid (3 M). When [^{3}H]CoASH in addition to [^{14}C]OSB was used, the eluate was evaporated to dryness, the residue dissolved in formic acid (0.1 M) containing EDTA (1 mM) and applied to a Hg–Sepharose column (0.55 × 6 cm; Affi-Gel 501, BioRad). While residual CoASH was retained on the column the OSB-CoA ester eluted (0.1 M formic acid, 1 mM EDTA) with the void volume. Subsequently the paper chromatographic step was repeated, and the ^{3}H/^{14}C ratio of the eluate determined.

2.5. *Enzymic synthesis of III from II*

An enzyme preparation was obtained as in section 2.2 except that the protamine sulfate step was omitted and the pH adjusted to 8.0. The incubation mixture (final vol. 190 μl) containing protein (1 mg) and [^{14}C]OSB-CoA ester (5645 dpm, 0.352 nmol) was kept at 30°C for 30 min. The reaction was terminated by addition of HCl (40 μl, 6 N).

2.6. *Identification of III*

The product of the reaction (see 2.5) was extracted into ethyl ether and 0.3 mg authentic DHNA (III) was added. Chromatography (TLC; silica gel; chloroform–ethylactetate–formic acid (45:6.6:0.5, by vol.)) showed that radioactivity coincided with authentic DHNA (III) (R_F 0.4). Treatment of the ethereal extract with diazomethane (1 min at 0°C) gave a radioactive derivative of II which on radiogas chromatography was identified as the methyl ester of III. Prolonged treatment (up to 1 h, at room temperature) with diazomethane gave two additional derivatives of III one of which was identified by radiogas chromatography as the methyl ester of 1-hydroxy-4-methoxy-2-naphthoic acid.

3. Results and discussion

Cell-free extracts obtained from *M. phlei* have been reported to convert *o*-succinylbenzoic acid (OSB I) to 1,4-dihydroxy-2-naphthoic acid (III), ATP and CoASH being essential cofactors in the reaction [3,4,7]. In our hands the yield in 1,4-dihydroxy-2-naphthoic acid (III) was up to 30% of the OSB (I) employed. When the naphthoate synthase was removed from the enzyme extract with the aid of protamine sulfate (see 2.3) prior to incubation, paper chromatography of the reaction mixture revealed a radioactive peak with an R_F of 0.48 (fig.2). This chromatographic behaviour is typical of CoASH-esters [10]. The formation of this radioactive material was only observed when incubations were carried out in the presence of ATP and CoASH (table 1). In the presence of heat-denatured

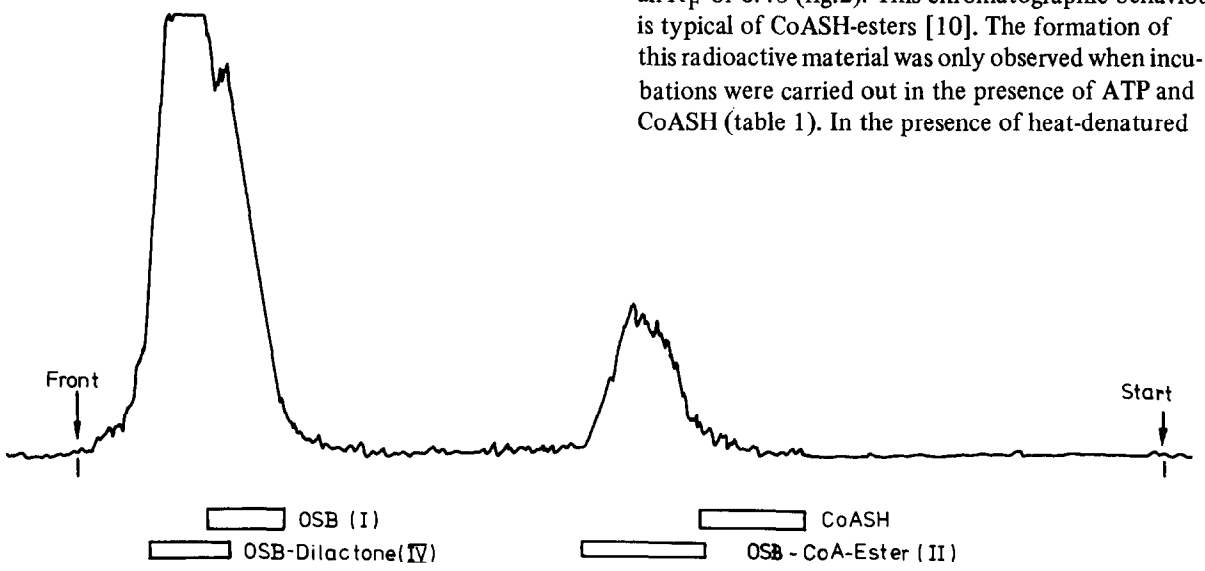

Fig.2. Detection of the thioester of *o*-succinylbenzoic acid on a paper chromatogram (section 2.4) of a complete incubation mixture (table 1). Radioactivity was recorded with the thin-layer scanner LB 2722 (Berthold, Wildbad).

Table 1
Activation of *o*-[4'-^{14}C]succinylbenzoic acid (I) with an enzyme preparation from *M. phlei*

Incubation mixture	OSB-CoA ester (II) (dpm)	Relative activity (%)
Complete plus crude extract	22 400	100
Complete plus heat-inactivated extract	<100	<0.4
Complete plus crude extract		
–ATP	289	1.2
–CoASH	<100	<0.4
–Mg^{2+}	4650	20.7

enzyme no such peak appeared. This compound was radioactively labelled when either [^{14}C]OSB (table 1) or [^{3}H]CoASH (not shown) were employed. At alkaline pH the product decomposed to *o*-succinylbenzoic acid (I) and coenzyme A. At neutral pH, the dilactone (IV) of I was formed. At acid pH the compound (II) was more stable, and formation of the dilactone was considerably reduced. This behaviour suggested that the product is a CoA ester of *o*-succinylbenzoic acid. We therefore attempted to determine whether one or both carboxyl groups of I are activated. Simultaneous incubation of [G-^{3}H]coenzyme A and *o*-[4'-^{14}C]succinylbenzoic acid (table 2) gave doubly-labelled II which after complete removal of residual [G-^{3}H]coenzyme A with the aid of a Hg–Sepharose column (section 2.4) showed a ^{3}H/^{14}C ration (table 2) that allowed

Table 3
Conversion of the OSB-CoA ester (II) to 1,4-dihydroxy-2-naphthoic acid (III) with an enzyme preparation from *M. phlei*

Incubation mixture	1,4-Dihydroxy-2-naphthoic acid (III) (dpm)	Relative conversion (%)
OSB-CoA ester plus crude extract	3235	100
OSB-CoA ester plus heat-inactivated extract	<100	<3.1

the conclusion that the compound in question is a mono-CoA ester rather than a di-CoA ester.

It remained to be established that this CoA ester is an intermediate in the formation of DHNA (III) from OSB (I) and CoASH. Incubation of [^{14}C]OSB-CoA ester with an enzymic preparation (section 2.5) gave ^{14}C-labelled 1,4-dihydroxy-2-naphthoic acid (III) with 57% yield (table 3). The product (III) was identified as in section 2.6. Neither ATP, CoASH or Mg^{2+}, or a mixture of these increased the yield of III.

We conclude that the activated intermediate is a CoA ester of *o*-succinylbenzoic acid (I) and that only 1 of the 2 carboxyl groups is activated. The CoA-ester decomposes non-enzymically to the dilactone (IV) of I (not shown). Formation of this dilactone had been observed [3,4,7,11] and was assumed to result from the decomposition of an activated intermediate of the reaction. We now know that this assumption is correct. Our observations agree with suggestions in [3,4,7] on the role of a thioester of *o*-succinylbenzoic acid in menaquinone biosynthesis.

Table 2
Simultaneous incubation of [G-^{3}H]coenzyme A and *o*-[4'-^{14}C]succinylbenzoic acid (I) and determination of the ^{3}H/^{14}C ratio of the resulting OSB-CoA ester (II)

	Amount (μmol)	Radioact- (μCi)	Spec. act. (μCi/μmol)	Ratio of spec. act.
[G-^{3}H]Coenzyme A	0.05	1.50	30.0	4.16
(I)	0.02	0.15	7.22	
	Expected for a diester		^{3}H/^{14}C expected for a monoester	Found
[G-^{3}H]Coenzyme A thioester of (II)	8.31		4.16	4.76

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