o-Succinylbenzoate: Coenzyme A Ligase, an Enzyme Involved in Menaquinone (Vitamin K_2) Biosynthesis, Displays Broad Specificity

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o-Succinylbenzoate: coenzyme A ligase, an enzyme involved in menaquinone biosynthesis, was purified from *Mycobacterium phlei* and characterized with respect to isoelectric point, molecular weight, pH optimum, temperature optimum and kinetic data. The enzyme hydrolyses ATP to AMP. The substrate and cofactor specificity of the enzyme was tested with analogues of *o*-succinylbenzoic acid, different nucleotides, thiols and divalent cations. The enzyme appears to possess broad specificity for substrates and cofactors.

o-Succinylbenzoic acid (OSB, *i.e.* 4-(2′-carboxyphenyl)-4-oxobutyric acid) is an intermediate in the biosynthesis of menaquinones (vitamin K_2) in bacteria and of a series of quinonoid natural products in higher plants [1, 2]. Cell free protein extracts from different menaquinone producing bacterial strains including *Escherichia coli* and *Mycobacterium phlei* catalyze the coenzyme A, ATP and Mg^{2+} dependent conversion of OSB into 1,4-dihydroxy-2-naphthoic acid (DHNA) [3, 4]. The intermediate in this conversion is a mono coenzyme A ester [5] which has been synthesized, characterized [6] and converted enzymically to DHNA [7]. This coenzyme A ester is also formed in cell free preparations of anthraquinone producing cell suspension cultures of *Galium mollugo* [8].

Attempts to determine the site of activation of OSB led to the assumption that the "aromatic" carboxyl group (Fig. 1) is activated. This conclusion was erroneous [6, 7, 9, 10]. The question as to the site of activation was eventually solved in favour of the "aliphatic" carboxyl group of OSB (compare structure of OSB-1-CoA in Fig. 1) [6, 7].

Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; KP_i buffer, potassium phosphate buffer; MOPS, 3-(N-morpholino)propane sulfonic acid; OSB, *o*-succinylbenzoic acid; OSB-1-CoA, "aliphatic" coenzyme A ester of OSB or 4-(2′-carboxyphenyl)-4-oxobutyryl coenzyme A ester; OSB-2″-CoA, "aromatic" coenzyme A ester of OSB or 2-(3′-carboxypropionyl)-benzoyl coenzyme A ester; OSB-di-CoA, 4-(2′-carboxyphenyl)-4-oxobutyryl-di-coenzyme A ester.

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One of the reasons for the difficulty to determine the site of activation of OSB was that crude enzyme extracts catalyzing the activation of OSB contain a mixture of the "aliphatic" (OSB-1-CoA), the "aromatic" (OSB-2″-CoA) and the dicoenzyme A ester of OSB (OSB-di-CoA) [7]. In this mixture the "aliphatic" ester (OSB-1-CoA) prevails (80 to 85%) and only this isomer is enzymically converted to DHNA [7].

These observations suggest that the coenzyme A ligase that activates OSB may be an enzyme with low specificity. We have enriched this enzyme from protein extracts of *Mycobacterium phlei* and show in the present communication that the low specificity hypothesis appears correct.

Materials and Methods

Bacteria. Mycobacterium phlei was obtained from the Institut für Mikrobiologie of the Westfälische Wilhelms-Universität, 4000 Münster, West Germany.

Growth of Mycobacterium phlei. The organism was grown as described [11].

Assays for enzyme activity

When large amounts of enzyme fractions were checked for the presence of the ligase, activity was monitored after decomposition of the OSB coenzyme A ester to coenzyme A und OSB dilactone [6]: An incubation mixture (70 µl) contained ATP (5.89 nM), CoASH (860 µM), $MgCl_2$ (14 µM), [1-^{14}C] OSB (*i.e.* 4-(2′-carboxyphenyl)-[1^{14}C]-4-oxobutyric acid) (200 Bq, 300 pmol) and protein

(50 µl) in KP_i buffer (0.1 M, pH 7.2). K_M values were determined with 9.4 µg protein.

The mixture was incubated 12 min at 30 °C and the dilactone formed was extracted into toluene (1 ml). After separation of the aqueous and organic phase by centrifugation 0.9 ml of the toluene solution was withdrawn and its radioactivity (OSB dilactone) determined by liquid scintillation counting. A linear correlation was found between the amount of CoA ester formed (determined by tlc and radioscanning) and the level of radioactivity in the toluene phase. Under the conditions described, approximately 50% of the CoA ester synthesized was recovered as the dilactone (Fig. 1).

Alternatively, a coupled assay with myokinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) was employed to determine the product of hydrolysis of ATP (ADP or AMP) qualitatively and quantitatively [12, 13].

Nucleotides (AMP, retention time = t_r = 8.5 min; ADP, t_r = 11.9 min; ATP, t_r = 15.4 min), OSB (t_r = 22.9 min), CoASH (t_r = 32.1 min) and OSB-1-CoA (t_r = 36.9 min) were also qualitatively and quantitatively determined by HPLC following an LKB application note (LKB, Bromma, Sweden). This technique was employed when enzyme activity was determined as hydrolysis of ATP to AMP or when products of the enzyme reaction (AMP, OSB-1-CoA) were identified. A Lichrosorb RP-18 column (particle size 5 µm), equipped with a precolumn packed with Perisorb RP-18 (particle size 30–40 µm), was eluted with a gradient consisting of solvent A: Na_2HPO_4 (0.2 M) and tetrabutylammonium hydrogensulfate (0.025 M) in H_2O adjusted to pH 6 with NaOH and solvent B: Na_2HPO_4 (0.2 M) and tetrabutylammoniumhydrogensulfate (0.025 mol × liter^{-1}) in methanol/H_2O (50%, v/v) adjusted to pH 6 with NaOH. Solvent A contained 4% B during the first 5 min increasing to 16% (6–14 min), to 24% (15–31 min) and to 96% (32–45 min). Flow rate was 1 ml per min.

Identification of products formed during incubation

The identity of the coenzyme A ester was investigated by HPLC using two solvent systems [6 and *vide supra*] before and after hydrolysis (pH 11.5, 10 min, 30 °C). The coenzyme A formed upon hydrolysis was also determined by Ellman's reagent [6]. The identity of the coenzyme A ester was further confirmed by its enzymic conversion to 1,4-dihydroxy-2-naphthoic acid (DHNA) [6, 7].

Enzyme purification (Table I)

10 g of frozen cell paste was thawed and suspended in MOPS buffer (10 ml, pH 6.9, 20 mM, containing 0.2 mM DTT and DMSO, 20%). The cell suspension was cooled in an ice salt mixture and sonicated (Branson Sonifier) 10 times for 60 s with 60 watts with thirty-s intervals. The solution was centrifuged (10 min, 50000 × *g*), and the supernatant (10 ml) was cooled (0 °C) and treated with a protamine sulfate solution (9.5 ml, 2%) in MOPS buffer with stirring over 10 min. After stirring (20 min) at 0 °C the mixture was centrifuged (10 min, 12000 × *g*) and the supernatant desalted on a column of Sephadex G-25: Sephadex G25 fine was equilibrated in KP_i buffer (0.1 M, pH 8.0) overnight and packed into a short glass column (2 × 6 cm). The column was centrifuged (5 min, 1000 × *g*) at 4 °C to remove excess of buffer, the enzyme extract (3 ml) mentioned above was applied to the column and centrifugation repeated. The eluent contained the ligase in phosphate buffer.

Hydroxyapatite chromatography

A column of Biogel HT (Bio-Rad, München) (2.8 × 5.5 cm) was equilibrated with KP_i buffer (10 mM, pH 7.5). The desalted protein solution was applied to the column, and the column was washed with a gradient of KP_i buffer increasing to 0.6 M. The enzyme eluted at a concentration of 0.15 to 0.25 M. The enzyme was again desalted on a Sephadex G25 column as described above and further purified by CoA-Sepharose ligand chromatography.

CoA-Sepharose ligand chromatography

A column of agarose-hexane-coenzyme A Type 5 (Pharmacia, LKB, Freiburg) (0.6 × 3 cm) was equilibrated with degassed KP_i buffer (10 mM, pH 7.5). The desalted enzyme solution after hydroxyapatite chromatography was applied to the column and the column eluted with 0.6 M KP_i buffer, pH 7.5, at a flow rate of 10 ml per h.

Analyses of protein

Protein concentration was determined by the method of Bradford [14]. The isoelectric point of the ligase was determined as described by Harzer [15]. The molecular weight of the enzyme was determined by gel filtration chromatography using an LKB-HPLC system equipped with a precolumn (LKB 2135 Ultro Pac TSK-GSWP 7.5 × 75 mm) and a main column (LKB 2135 Ultro Pac TSK-G 3000 SW 7.5 × 600 mm). The column system was equilibrated with KP_i buffer (0.1 mM, pH 7.5, plus 0.2 mM DTT) at a flow rate of 0.8 ml per min. Protein was detected UV-spectrophotometrically at 280 nm. The molecular weight standards used included ribonuclease A (M_r 13700), chymotrypsinogen A (M_r 25000), ovalbumin (M_r 43000), bovine serum albumine (M_r 67000), aldolase (M_r 158000) and ferritin (M_r 440000).

Results and Discussion

The enzyme was purified as shown in Table I. The purification factor and the specific activity of the enzyme did not increase during chromatography on Sephadex G25 (step 3) and affinity chromatography on CoA sepharose (step 4) indicating that the ligase denatured as purification progressed.

Different methods were employed for the assay of the OSB coenzyme A ligase. The coenzyme A ester formed could be determined quantitatively by HPLC (Materials and Methods). Alternatively

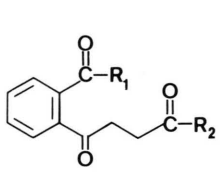
Table I. Purification of *o*-succinylbenzoate: CoA ligase from 10 g of bacterial cells of *Mycobacterium phlei*.

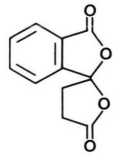
Purification step	Protein [mg]	Specific activity [nkat/kg]	Purification factor [-fold]	Yield [%]
Crude extract	427	111.7	1	100.0
Protaminsulfate precipitation (1)	90	2652	24	500.0
Hydroxyapatite (2)	8.0	24663	221	413.6
Sephadex G25 (3)	4.4	13826	124	127.9
CoA-Sepharose (4)	0.75	14933	134	23.6

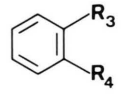
A procedure for the purification of the ligase had been published previously. Activity of the enzyme, however, was determined by an indirect method. The enzyme remained uncharacterized. Experimental details were not given [10].

it is possible to take advantage of the fact that the thiol ester is labile [6, 7] and undergoes rapid nonenzymatic lactonization with elimination of coenzyme A at neutral pH. The spirodilactone of OSB (Fig. 1) so formed can be quantitatively determined after extraction into toluene. When radioactively labelled OSB is employed radioactivity in the toluene is a measure of the enzyme activity. Product formation was linear with time (0–15 min) and showed a linear increase with increasing amounts of purified ligase preparation (0 to 12 µg per standard incubation).

Previous experiments on the cell free conversion of OSB to 1,4-dihydroxy-2-naphthoic acid (DHNA) indicated that during this reaction ATP







SPIRODILACTONE OF OSB

OSB: $R_1, R_2 = OH$

OSB–1–CoA: $R_1 = OH$, $R_2 = SCoA$

OSB–2″–CoA: $R_1 = SCoA$, $R_2 = OH$

OSB–di–CoA: $R_1, R_2 = SCoA$

OMB: $R_3 = COOH$, $R_4 = C(=O)-CH_2-COOH$

BPA: $R_3 = H$, $R_4 = C(=O)-CH_2-CH_2-COOH$

BEA: $R_3 = COOH$, $R_4 = H$

ABA: $R_3 = COOH$, $R_4 = C(=O)-CH_3$

PHA: $R_3, R_4 = COOH$

Fig. 1. Derivates and substrate analogues of *o*-succinylbenzoic acid (OSB). ABA, *o*-acetylbenzoic acid; BEA, benzoic acid; BPA, benzoylpropionic acid; OMB, *o*-malonylbenzoic acid; PHA, phthalic acid.

may be hydrolyzed to AMP [4]. We confirmed this using the purified enzyme preparation (Table I): AMP was detected by HPLC after incubation with OSB, coenzyme A, ATP and Mg^{2+}, and its formation depended on the presence of intact enzyme and OSB. ADP was not formed. The presence of AMP was also determined in a coupled assay using myokinase [12, 13]: 31.0 nmol AMP was formed in this assay system per minute and mg purified OSB coenzyme A ligase.

The partially purified enzyme was characterized with respect to isoelectric point, molecular weight, pH optimum, temperature optimum and kinetic data (Table II). Determination of pH- and temperature optimum gave bell shaped curves in both cases. Kinetic data of the ligase are also given in Table II. The half maximum velocity of the reaction was reached with approximately 800 μM ATP in a standard incubation mixture. In contrast to the results with OSB and coenzyme A, the double reciprocal plot according to Lineweaver and Burk gave a non-linear curve when ATP concentration was varied.

Table II. Properties of OSB coenzyme A ligase from *M. phlei*[a].

Isoelectric point[b] at pH	4.9
Molecular weight (gel filtration)	28,000
pH-Optimum	7.3
Temperature optimum	38 °C
Kinetic data	
K_M [μM]	
ATP	see text
CoASH	16.5
OSB	148.1
V_{max} [pmol/mg protein/min]	480

[a] The standard incubation mixture including invariant components for determination of K_M values is given under Materials and Methods.

[b] The IEP has been determined with a protein preparation obtained after the protamine sulfate step. All other determinations were carried out after purification step 4 (compare Table I).

In Tables III and IV results of experiments are listed in which the substrate specificity and cofactor requirement of the ligase were tested.

Six substrate analogues of OSB were investigated using hydrolysis of ATP to AMP as a measure of enzyme activity. The AMP formed was determined by HPLC. No AMP formation was observed with *p*-coumaric acid, phthalic acid (PHA), 2-acetyl-benzoic acid (ABA) and benzoic acid (BEA). Interesting, however, AMP was found in incubation mixtures with *o*-malonylbenzoic acid (OMB) and benzoylpropionic acid (BPA) (Fig. 1). The length of the aliphatic side chain seems to be important for enzyme activity because more hydrolysis of ATP is observed with benzoylpropionic acid (BPA) than with *o*-malonylbenzoic acid (OMB) (Table III). The fact that benzoylpropionic acid (BPA) is the most effective substrate analogue while benzoic acid (BEA) is completely inactive is consistent with the observation that the ligase activates the aliphatic rather than the aromatic carboxyl group [6, 7, 9].

Table III. Hydrolysis of ATP to AMP by *o*-succinylbenzoate: CoA ligase in the presence of various substrate analogues of OSB.

Substrate	Formation of AMP [nmol/min]	Relative activity [%]
o-Succinylbenzoic acid	138.5	100.0
o-Malonylbenzoic acid	16.1	11.6
Benzoylpropionic acid	29.7	21.6
Benzoic acid	n.d.	–
o-Acetylbenzoic acid	n.d.	–
Phthalic acid	n.d.	–
p-Coumaric acid	n.d.	–

The incubation mixture contained 1.6 μmol of the individual substrate analogues of OSB and 22.5 μg of protein. n.d., not detectable.

Table IV. Activity of *o*-succinylbenzoate: CoA ligase as influenced by different nucleoside triphosphates. The incubation mixture contained 500 nmol nucleoside triphosphates.

	OSB-1-CoA formed [pmol/12 min]	Relative activity [%]
ATP	153.2	100.0
GTP	20.6	13.4
ITP	16.7	10.9
CTP	20.7	13.5
UTP	18.9	12.3
TTP	18.5	12.1

Activation of OSB is best accomplished in the presence of ATP, but other nucleotides are also accepted by the enzyme. The activity of the enzyme is slightly more than 10% in these cases when compared to ATP (100%). In these experiments (Table

IV) enzyme activity was determined as the amount of OSB-1-CoA detected by HPLC. The same technique was employed when dephosphocoenzyme A was compared to coenzyme A. The former thiol was almost as effective (96%) as the latter (100%).

Finally, divalent cations were tested for their ability to replace Mg^{2+} (100%) in the activation reaction. Enzyme activity was observed in the presence of cobalt (63%), nickel (46%) and manganese (36%) ions. The activity of calcium (8%), copper (7%) and zinc (3%) ions was lowest.

Eventually formation of thiol esters of OSB (*viz.* OSB-1-CoA, OSB-2″-CoA and OSB-di-CoA) was tested in enzyme preparations after different purification steps (Table V). Formation of OSB-2″-CoA and OSB-di-CoA was again observed at pH 7.9 but not at pH 6.5 when crude extracts were employed (compare ref. [7]). The enzyme preparation obtained after purification step 4 did not yield any OSB-2″-CoA at either pH value but still considerable amounts of OSB-di-CoA. This may indicate that a CoA ligase activating the "aromatic" carboxyl group of OSB, or a hydrolase active on the "aliphatic" thiol ester group, was removed through purification step 1 to 4.

In our previous experiments we had proposed that the pH of the assay buffer influences the formation of OSB-2″-CoA and OSB-di-CoA [7]. We found additional evidence that this may be true for the OSB-CoA ligase since incubation of the purified enzyme preparation at different pH values gives a varying ratio of OSB-1-CoA to OSB-di-CoA. The maximum ratio is obtained at pH 7 as shown in Fig. 2. We do not assume that the occurrence of OSB-di-CoA is due to a chemical intra- or intermolecular transfer of coenzyme A [7] but rather that the ligase displays broad specificity. Our results do not constitute an isolated example of a coenzyme A ligase with low substrate specificity [13].

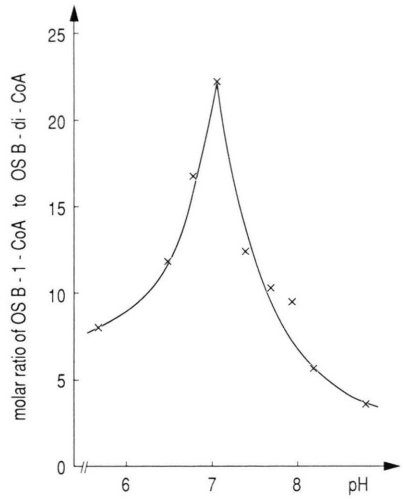


Fig. 2. Molar ratio of OSB-1-CoA to OSB-di-CoA formed at different pH values of the incubation mixture. The reaction was carried out with a purified ligase preparation.

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Table V. Activation of OSB catalyzed by *o*-succinylbenzoate: CoA ligase preparations after different purification steps. Incubations were carried out at pH 6.5 or pH 7.9 respectively.

pH of incubation mixture	Purification step (compare Table I)	OSB-1-CoA [%]	OSB-2″-CoA [%]	OSB-di-CoA [%]
6.5	crude extract	100	0	0
	step 1	95.3	1.35	3.32
	step 2	92.13	0	7.87
7.9	crude extract	91.5	4.4	4.1
	step 1	86.6	4.15	9.25
	step 2	90.45	0	9.55

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