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Menaquinone (Vitamin K_2) Biosynthesis: Overexpression, Purification, and Properties of *o*-Succinylbenzoyl-Coenzyme A Synthetase from *Escherichia coli*

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The coenzyme A (CoA)- and ATP-dependent conversion of *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyric acid], to *o*-succinylbenzoyl-CoA is carried out by the enzyme *o*-succinylbenzoyl-CoA synthetase. *o*-Succinylbenzoyl-CoA is a key intermediate in the biosynthesis of menaquinone (vitamin K_2) in both gram-negative and gram-positive bacteria. The enzyme has been overexpressed and purified to homogeneity. The purified enzyme was found to have a native molecular mass of 185 kDa as determined by gel filtration column chromatography on Sephacryl S-200. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis established a subunit molecular mass of 49 kDa. Thus, the enzyme is a homotetramer. The enzyme showed a pH optimum of 7.5 to 8.0 and a temperature optimum of 30 to 40°C. The K_m values for OSB, ATP, and CoA were 16, 73.5, and 360 μM, respectively. Of the various metal ions tested, Mg^{2+} was found to be the most effective in stimulating the enzyme activity. Studies with substrate analogs showed that neither benzoic acid nor benzoylpropionic acid (succinylbenzene) is a substrate for the enzyme. Thus, it appears that both the benzoyl carboxyl group and the succinyl side chain are required for activation of the aliphatic carboxyl group.

Menaquinone (vitamin K_2) functions in several anaerobic electron transport systems of *Escherichia coli* (7). The pathway for the biosynthesis of menaquinone has been reviewed (8). To date, seven genes, designated *menA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G*, encoding the various enzymes of the pathway have been identified (8). An important step in the biosynthesis is the enzymatic conversion of the benzenoid aromatic compound *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyric acid] to the naphthalenoid aromatic compound 1,4-dihydroxy-2-naphthoic acid (DHNA). During this conversion, the bicyclic ring system makes its first appearance. The enzymatic cyclization of OSB to DHNA was shown to be dependent on the presence of ATP and coenzyme A (CoA), suggesting the formation of an OSB-CoA derivative as an intermediate (3, 9). Subsequently, by using cell extracts of *Mycobacterium phlei*, it was shown that two enzymatic activities, OSB-CoA synthetase and DHNA synthase, are required for this conversion. It was further demonstrated that the OSB-CoA synthetase forms a highly unstable intermediate, OSB-CoA, which was subsequently cyclized into DHNA by DHNA synthase (9). During the formation of OSB-CoA, the ATP was converted to AMP and PP_i. Consistent with these observations, two groups of mutants, designated *menE* and *menB*, lacking OSB-CoA synthetase and DHNA synthase, respectively, were identified (10, 14). These reactions, enzymes, and genes are summarized in Fig. 1. The gene encoding OSB-CoA synthetase has been cloned from *E. coli* (15), and its complete nucleotide sequence has been determined (12).

In this report, we describe the overexpression, purification, and properties of OSB-CoA synthetase from *E. coli*.

(A preliminary report of some of these findings has appeared [5]).

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MATERIALS AND METHODS

Materials. ATP, CoA, *E. coli* pyrophosphatase, DEAE Sephadex A-50, Reactive Blue 2 Sepharose CL-6B, protamine sulfate, trichloroacetic acid, ammonium molybdate, β-mercaptoethanol, *N,N,N′,N′*-tetramethylethylendiamine (TEMED), rifampin, and isopropyl-β-D-thiogalactopyranoside (IPTG) were products of Sigma Chemical Co., St. Louis, Mo. Low-molecular-weight protein standards were purchased from Bio-Rad Laboratories, Hercules, Calif. Centricon concentrators were from Amicon, Beverly, Mass. Ascorbic acid, sodium dodecyl sulfate (SDS), polyacrylamide, benzoic acid, phthalic acid, benzoylpropionic acid, *p*-coumaric acid, and *o*-acetylbenzoic acid were from Fisher Scientific Co., Pittsburgh, Pa. Restriction enzymes and the Altered Sites II in vitro mutagenesis kit were purchased from Promega, Madison, Wis. OSB was synthesized as described before (3).

Media and growth conditions. Cultures were routinely stored at −80°C in glycerated Luria-Bertani (LB) broth and grown on LB agar. Ampicillin, kanamycin, or tetracycline, when required, was added to the medium at a concentration of 50, 40, or 12.5 μg/ml, respectively, unless specified otherwise.

Construction of the overexpression plasmid. Plasmid pMS73 (12), containing the entire *menE* gene, was digested with *Eco*RI and *Hin*dIII. The resulting 2.4-kb fragment containing *menE* was subcloned into plasmid pAlter-1 (Promega) after restriction with the same enzymes, and the plasmid so formed was designated pME73. An *Nde*I restriction site was created near the ATG initiation codon of *menE* by site-directed mutagenesis with the Altered Sites II in vitro mutagenesis kit. An oligonucleotide primer spanning the region of the *menE* initiation codon was used for mutagenesis. The following sequence of the *menE* gene near the ATG codon, 5′TGGAGCGGTTGTTATGATCTTCTCTG3′, was altered by changing the two underlined bases to CA, and an *Nde*I site (CATATG) was introduced. The presence of the desired mutation was verified by restriction mapping, and the plasmid was designated pAE73. The expression vector pT7-7 (which contains the T7 promoter and ribosomal binding site [18]), was linearized with *Nde*I and *Hin*dIII and ligated with the 2.2-kb *Nde*I-*Hin*dIII fragment of pAE73, resulting in pME737. This overexpression plasmid was transformed into *E. coli* BL21 (DE3), which carries the T7 RNA polymerase gene under *lac* UV5 control (17).

Overexpression and preparation of cell extracts. A 5-ml overnight LB broth culture of *E. coli* BL21 (DE3) containing plasmid pME737 was inoculated into 500 ml of LB broth containing 0.2% glucose and 200 μg of ampicillin per ml and grown with shaking until the A_{600} reached 1.0. At this time, the T7 RNA polymerase was induced by addition of 0.4 mM IPTG, and after 30 min, rifampin was added at 200 μg/ml and growth continued for a further 3 h. The cells were harvested by centrifugation at 4,000 × *g* for 10 min. The pellet was washed by resuspension in 20 mM Tris-HCl buffer (pH 7.2), centrifuged, and stored frozen at −20°C. The frozen cells were suspended in a buffer of the same composition containing 10 mM β-mercaptoethanol (buffer A) at a ratio of 1 g/1.5 ml and passed through a French pressure cell at 12,000 lb/in^2. The extract was treated with DNase for 5 min at 37°C to reduce viscosity and centrifuged at 30,000 × *g* for 30 min, and the resulting supernatant was used for further purification.

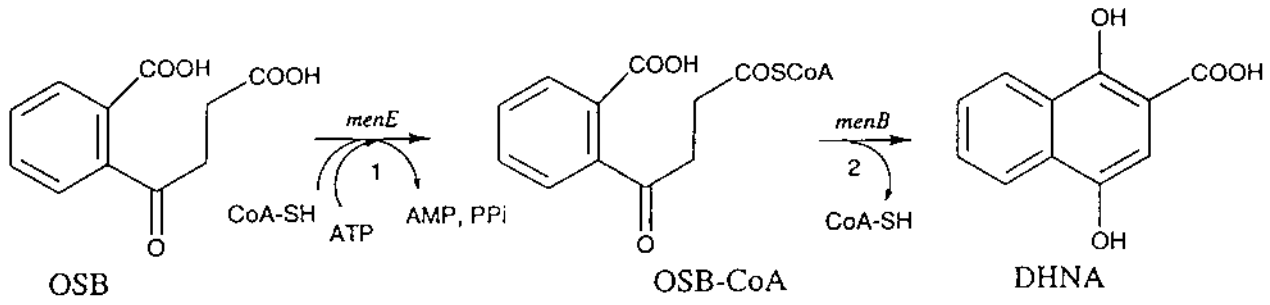

FIG. 1. The formation of OSB-CoA and its conversion to DHNA. 1, OSB-CoA synthetase; 2, DHNA synthase.

Protein determination. The protein concentrations were determined by the method of Bradford (2) with the Bio-Rad Laboratories protein assay kit. Bovine serum albumin was used as the standard.

Determination of pH optimum. For pHs 6.5 and 7.0, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*-′2-ethanesulfonic acid) buffer was used. From pH 7.0 to pH 8.5, 0.1 M Tris-HCl [Tris(hydroxymethyl)aminomethane] buffer was used. Enzyme assays were routinely performed in 0.1 M Tris-HCl buffer, pH 8.0.

Assay of enzyme activity. (i) By coupling with DHNA synthase. OSB-CoA, the product of the OSB-CoA synthetase reaction, is a highly unstable compound which spontaneously converts to OSB-spirodilactone in the absence of DHNA synthase (9, 12). Thus, it was assayed as accumulated DHNA following its conversion in the presence of excess DHNA synthase. The DHNA synthase was supplied in the form of an extract from *menE* mutant strain AN213 carrying plasmid pMS9 (13). The enzymatic incubation mixture, the assay conditions used, and the spectrophotofluorometric determination of the DHNA formed were as previously described (12, 13).

(ii) By coupling with pyrophosphatase. Based on the previous report that ATP is hydrolyzed to AMP and PP_i during the formation of OSB-CoA (9) (Fig. 1), a new, simple assay method for OSB-CoA synthetase was developed. The PP_i formed was converted to P_i by using *E. coli* pyrophosphatase, and the P_i was determined. The incubation mixture and the conditions were the same as those of the DHNA synthase coupled assay without the addition of AN213(pMS9) extract. The reaction was terminated by heating at 70°C. After termination, 1 U of *E. coli* pyrophosphatase was added to the reaction mixture and incubation was continued for 10 min at room temperature. The reaction was terminated by addition of 1 ml of 10% (wt/vol) trichloroacetic acid, and the precipitated protein was removed by centrifugation at 2,000 × *g*. The P_i was estimated as described by Chen et al. (4).

With the DHNA determination and phosphate determination methods described above, identical results were obtained. However, DHNA synthase has not been purified, necessitating the use of crude enzyme preparations in conjunction with homogeneous preparations of OSB-CoA synthetase. Hence, for all of the results reported here, the reaction was coupled with pyrophosphatase. Since the OSB-CoA synthetase in the strain has been amplified more than 3,000-fold, even in crude cell extracts, the phosphatase activity was very low. During the assay, a control containing all of the components except the substrate, OSB, was always included and the amount of phosphate formed was used to correct the experimental results. All of the assays were repeated at least three or four times, and the variation was less than 5%.

Protamine sulfate precipitation. The crude cell extract was acidified to pH 6.2 with 1% acetic acid. A 2% solution of protamine sulfate (adjusted to pH 6.2) was added dropwise with constant stirring until the volume increased by 40%, and the stirring was continued for another 30 min. The precipitate formed was removed by centrifugation at 30,000 × *g* for 30 min, and the clear supernatant was used for further purification.

DEAE Sephadex A-50 anion-exchange chromatography. A column (18 by 0.8 cm) of DEAE Sephadex A-50 was equilibrated with buffer A. Protamine sulfate supernatant (6.0 ml) was applied to the column, and the column was washed with 10 column volumes of buffer A until all of the unbound protein was removed. The bound enzyme was eluted with 160 ml of a linear gradient of 0 to 600 mM KCl in buffer A, and the A_{280} of the eluent was monitored. Fractions (2.0 ml) were collected, and the active fractions (no. 29 to 39) were pooled and concentrated with Centricon 30 concentrators (Amicon).

Blue Sepharose CL-6B affinity chromatography. The concentrated protein from the DEAE Sephadex A-50 column was applied to a Blue Sepharose CL-6B column (6 by 0.5 cm) equilibrated with buffer A and allowed to bind for 30 min. The column was washed with 10 volumes of buffer A to remove unbound protein. The enzyme was eluted with 160 ml of a linear gradient of 0 to 500 mM KCl in buffer A, and 2.2-ml fractions were collected. Fractions (no. 27 to 38) containing the enzyme activity were pooled and concentrated with Centricon 30 concentrators.

Spectroscopic methods. UV absorption spectra were recorded with a Perkin-Elmer lambda 4A spectrophotometer at 25°C.

SDS-PAGE. The purity of the enzyme preparation was monitored on SDS–12% polyacrylamide gel electrophoresis (PAGE) gels as described by Laemmli (6). The mini PROTEAN II cell apparatus of Bio-Rad Laboratories was used in accordance with the instructions of the manufacturer. The following low-molecular-weight Bio-Rad protein standards were used: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Native molecular mass determination. The native molecular mass of the enzyme was determined by Sephacryl S-200 gel filtration column chromatography as described in Sigma Chemical Co. technical bulletin GF-3. A column (1.5 by 100 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.2) and calibrated with the gel filtration kit of Sigma Chemical Co. The kit contained cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2,000 kDa). The molecular mass of OSB-CoA synthetase was determined from the calibration curve.

RESULTS

Purification of OSB-CoA synthetase. Overexpression strain BL21 (DE3) containing plasmid pME737 was grown and the enzyme was induced as described in Materials and Methods. Cell extracts of this strain produced 0.37 μmol of OSB-CoA min^{-1} mg of $protein^{-1}$ (Table 1). By using extracts of this overexpression strain as the starting point, the enzyme was purified by various methods (Table 1). Treatment of the clarified cell extract with protamine sulfate and removal of the precipitate by centrifugation resulted in approximately 1.4-fold purification of the enzyme in the supernatant, with greater than 60% recovery. The protamine sulfate supernatant was chromatographed on DEAE Sephadex A-50 anion-exchange resin, resulting in 3.5-fold purification with 53% recovery of the enzyme. The active fractions were combined and applied to a Blue Sepharose CL-6B affinity chromatography column, re-

TABLE 1. Purification of OSB-CoA synthetase of *E. coli*

Purification step	Total protein (mg)	Sp act (μmol min^{-1} mg of $protein^{-1}$)	Total activity (μmol min^{-1})	Yield (%)	Purification (fold)
Crude extract	390	0.37	144.3	100	1.0
Protamine sulfate	165	0.53	87.5	60.6	1.4
DEAE Sephadex A-50	58.5	1.31	76.7	53.2	3.5
Blue Sepharose CL-6B	22	3.20	70.4	48.8	8.6

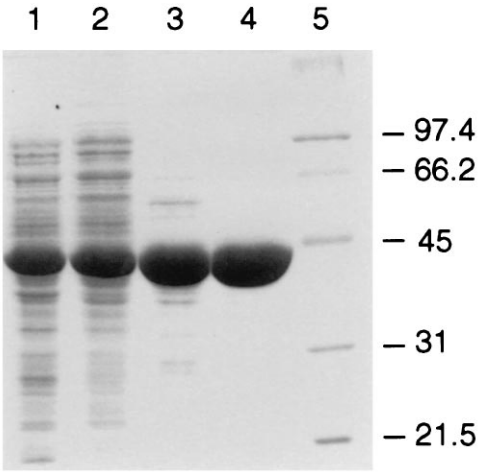

FIG. 2. SDS-PAGE analysis of protein fractions from various stages of purification. Proteins were separated on an SDS–12% PAGE gel and stained with Coomassie blue. Each lane contained 20 μg of protein. Lanes: 1, crude extract; 2, protamine sulfate fraction; 3, DEAE Sephadex A-50 fraction; 4, Blue Sepharose CL-6B fraction; 5, low-molecular-weight markers from Bio-Rad Laboratories. The values on the right are molecular masses in kilodaltons.

sulting in a ninefold-purified homogeneous preparation with a recovery of 49%. The purity of the enzyme during various stages of purification as determined by SDS-PAGE is shown in Fig. 2.

Molecular mass of the enzyme. The native molecular mass of the enzyme, as determined on a calibrated Sephacryl S-200 gel filtration column, was found to be 185 kDa. SDS-PAGE of the same sample gave a single band of 49 kDa.

Determination of pH and temperature optima. The pH optimum of the enzyme was determined in the range of 6.5 to 8.5. The enzyme exhibited optimum activity at pHs 7.5 to 8.0. At a pH of 7.0, the activity decreased only slightly. However, the activity decreased sharply at pHs of 6.5 and 8.5 to about 55 and 80%, respectively, of that obtained at the optimum pH (data not shown). The enzyme showed maximum activity in the temperature range of 30 to 40°C (data not shown).

Determination of kinetic constants. The OSB-CoA synthetase activity increased with increasing concentrations of the substrates OSB, ATP, and CoA, and typical hyperbolic curves were obtained (data not shown). The apparent K_m and V_{max} were determined for each of the substrates by using double-reciprocal plots. The K_m values for OSB, ATP, and CoA were 16, 73.5, and 360 μM, respectively, and the corresponding V_{max} values were 2, 2.4, and 4.3 μmol min^{-1} mg of $protein^{-1}$.

Activity with substrate analogs. Various benzenoid substrate analogs, *o*-malonylbenzoic acid, benzoylpropionic acid, benzoic acid, *o*-acetylbenzoic acid, phthalic acid, and *p*-coumaric acid, were tested for enzyme activity and were found to be inactive (data not shown).

Absorption spectra of the enzyme. The absorption spectrum of the purified enzyme was recorded at wavelengths of 250 to 700 nm. It showed a single absorption peak at 278 nm (data not shown).

Metal ion requirement for enzyme activity. The metal ion requirement for the enzyme activity was determined at 0.5, 1.0, and 5.0 mM concentrations. As seen in Table 2, the presence of Na^+, K^+, Ca^{2+}, or Zn^{2+} resulted in approximately twofold higher activity than the control at all of the concentrations tested. Addition of Co^{2+} or Mn^{2+} increased the activity approximately fourfold at 0.5 and 1.0 mM, while at 5 mM, the increase was only twofold. Maximum stimulation of sixfold was observed at 1 mM Mg^{2+}, while at 0.5 and 5.0 mM, the increases were only five- and fourfold, respectively. In the presence of Fe^{2+} and Hg^{2+}, the enzyme was inhibited.

TABLE 2. Effects of various metal ions on OSB-CoA synthetase activity[a]

Metal ion	Sp act (μmol min^{-1} mg of $protein^{-1}$) at ion concn of:			
	0 mM	0.5 mM	1 mM	5 mM
None	0.53			
Na^+		0.83	0.83	0.83
K^+		0.83	0.90	1.0
Mg^{2+}		2.65	2.92	2.2
Ca^{2+}		1.0	1.0	ND[b]
Zn^{2+}		0.92	1.26	ND
Co^{2+}		1.75	2.25	1.07
Mn^{2+}		1.87	2.02	1.14
Fe^{2+}		0.29	0.43	ND
Hg^{2+}		0.18	0.19	ND

[a] For each assay, 1.0 μg of the enzyme was used. Each value is the average of at least four determinations.
[b] ND, not determined due to precipitation.

Since the enzyme showed maximum activity with Mg^{2+}, the effect of various concentrations of this metal on the enzyme activity was determined (Fig. 3). Very little activity was observed at concentrations of 0.1 to 10 μM, and the activity increased sharply from 25 μM, reaching a maximum at 1 mM. At concentrations above 1 mM, the enzyme was inhibited.

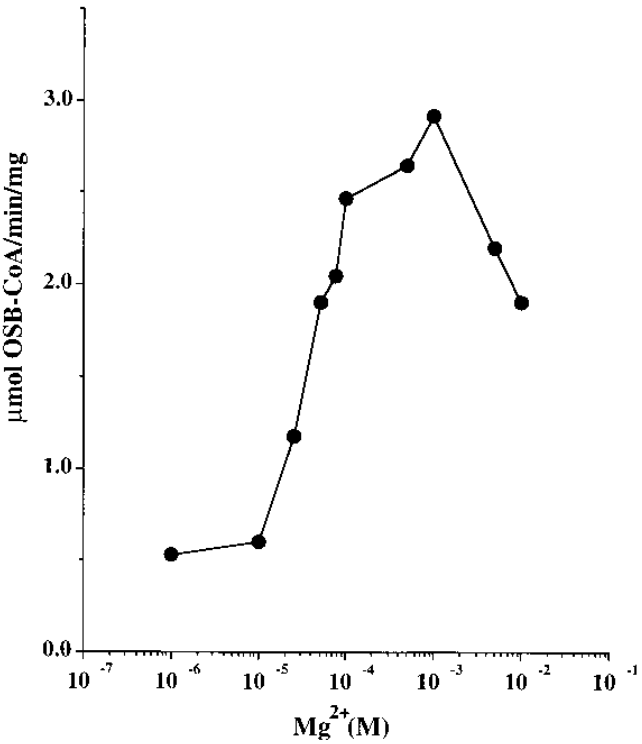

FIG. 3. Effect of various concentrations of Mg^{2+} on OSB-CoA synthetase activity. Enzyme activity was measured as described in Materials and Methods, and the Mg^{2+} concentration was varied from 10^{-7} to 10^{-2} M as indicated.

DISCUSSION

Conversion of the benzenoid OSB to the naphthalenoid DHNA requires the elimination of water and cyclization. This process is initiated by the enzyme OSB-CoA synthetase, resulting in the formation of OSB-CoA as an intermediate. The presence of OSB-CoA synthetase and DHNA synthase has been reported in the gram-negative bacterium *E. coli* (14) and a number of gram-positive organisms, such as *Bacillus subtilis* (10), *Micrococcus luteus* (11), and *M. phlei* (9). Since the enzymes involved in menaquinone biosynthesis are present at extremely low levels, early attempts at purification and characterization of the enzyme met with very limited success. Partial purification of OSB-CoA synthetase to near homogeneity from *M. phlei*, an organism which shows, in crude extracts, a specific activity 1.5 times that of *E. coli*, was achieved (1). However, the enzyme was not characterized further due to the small quantities available. Subsequently, a 200-fold partial purification and characterization of the enzyme from *M. phlei* were reported (16).

The activity of OSB-CoA synthetase in the wild-type strain of *E. coli* is about 0.12 nmol min^{-1} mg of $protein^{-1}$, which is extremely low for meaningful purification and characterization of the enzyme. Recently, we reported the complete nucleotide sequence of the *menE* gene encoding the OSB-CoA synthetase in *E. coli* (12). This information enabled us to overexpress this protein. The overexpressed strain had an activity of 370 nmol min^{-1} mg of $protein^{-1}$, which is about 3,000-fold higher than that of the wild-type strain and constituted about 11% of the total cellular protein. Extracts from this strain were used to purify the protein ninefold, resulting in a homogeneous preparation with a specific activity of 3.2 μmol min^{-1} mg of $protein^{-1}$. It was calculated that this level of activity is over 25,000-fold higher than that of the wild-type strain and allowed further characterization of the enzyme. The product of the reaction, OSB-CoA, is a highly unstable intermediate which cannot be assayed directly (9). We developed a simple colorimetric assay by determining PP_i after its hydrolysis to P_i by pyrophosphatase. This assay is based on previous studies establishing the hydrolysis of ATP to AMP and PP_i during the formation of OSB-CoA (9).

The purified enzyme had a molecular mass of 49 kDa as determined by SDS-PAGE, which is in agreement with the calculated molecular mass of 50.2 kDa as determined by sequence analysis (12). Since the native molecular mass as determined by gel filtration was 185 kDa, it appears that the enzyme is a homotetramer. The *M. phlei* enzyme, in contrast, has been reported to have a native molecular mass of 28 kDa (16). The K_m values for OSB, ATP, and CoA were found to be 16, 73.5, and 360 μM, respectively, while the *M. phlei* enzyme had K_m values of 148.1 and 16.5 μM for OSB and CoA, respectively. Sieweke and Leistner were unable to determine the K_m for ATP (16).

Further, it was reported for the *M. phlei* enzyme that the substrate analogs *o*-malonylbenzoic acid and benzoylpropionic acid had 11.6 and 21.6% of the activity, respectively, of that obtained with OSB, while benzoic acid, *o*-acetylbenzoic acid, phthalic acid, and *p*-coumaric acid were inactive (16). All of the above substrate analogs, except for *o*-malonylbenzoic acid, were tested and found to be inactive for the *E. coli* enzyme. Since neither benzoic acid nor benzoylpropionic acid (succinylbenzene) served as a substrate for the enzyme, it appears that both the benzoyl carboxyl group and the succinyl side chain are required for activation of the aliphatic carboxyl group.

While the partial purification and properties of OSB-CoA synthetase from *M. phlei* have been reported previously (1, 16), this is the first report of the purification of the enzyme to homogeneity. To further clarify the catalytic and regulatory properties of the enzyme, chemical modification and site-directed mutagenesis studies are in progress.

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