Menaquinone (Vitamin K_2) Biosynthesis in *Escherichia coli*: Synthesis of *o*-Succinylbenzoate Does Not Require the Decarboxylase Activity of the Ketoglutarate Dehydrogenase Complex†

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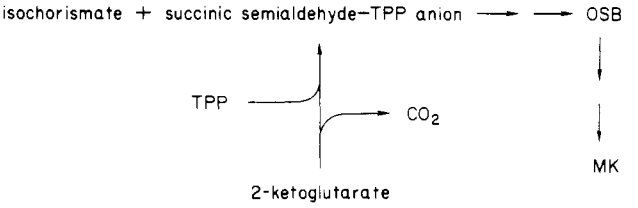
ABSTRACT: The committed step in menaquinone biosynthesis is the formation of *o*-succinylbenzoate (OSB). It is presumed to require the reaction of a seven-carbon intermediate of the shikimate pathway with a succinic semialdehyde–thiamin pyrophosphate (TPP) anion, derived by decarboxylation of 2-ketoglutarate. The following evidence indicates that the decarboxylation is not a function of the ketoglutarate dehydrogenase complex but is carried out by a separate activity. (A) Cell-free extracts of *Escherichia coli* K12 without added TPP lose OSB synthase activity but retain all of the ketoglutarate dehydrogenase complex activities. (B) OSB synthase activity is inhibited by addition of tetrahydro-TPP (th-TPP) to the incubations. The ketoglutarate dehydrogenase complex activities are only inhibited by this analogue after an initial preincubation period. (C) The high molecular weight ketoglutarate dehydrogenase complex can be separated from OSB synthase activity by gel-permeation chromatography on Sepharose CL-6B. Experiment series A and B also provide supporting evidence that TPP does play an important role in menaquinone biosynthesis.

Shikimic acid is a major precursor of the naphthoquinone ring system of the menaquinone (MK, vitamin K_2) components of bacteria (Bentley & Meganathan, 1982). The six carbon atoms of the cyclohexene ring of shikimate ultimately form the A ring of the naphthoquinone nucleus (see Figure 1). The carboxyl carbon is incorporated into ring B. The construction of the B ring also requires the introduction of the three central carbon atoms (C-2, C-3, and C-4) of 2-ketoglutarate (Campbell et al., 1971; Robins et al., 1970; Robins & Bentley, 1972). The mechanism for the utilization of the non-carboxyl carbon atoms of 2-ketoglutarate has remained obscure. However, the first aromatic intermediate in the menaquinone biosynthetic pathway is known to be *o*-succinylbenzoate (OSB,[1] see Figure 1), which contains four of the carbon atoms of 2-ketoglutarate (Dansette & Azerad, 1970; Campbell et al., 1971; Meganathan & Bentley, 1981). It is necessary, therefore, to account for the production of OSB from shikimate and a decarboxylated (i.e., C_4) unit derived from 2-ketoglutarate.

For some time, the evidence indicated that shikimate was first converted to chorismate. In fact, cell-free extracts have been obtained from *Escherichia coli* that produce OSB when supplied with chorismate, 2-ketoglutarate, and necessary cofactors (Meganathan, 1981). However, it now appears (Weische & Leistner, 1985) that isochorismate is the actual precursor as originally suggested (Dansette & Azerad, 1970). Although the chorismate preparations used in studying OSB synthase may contain small amounts of isochorismate (Weische & Leistner, 1985), the cell-free extracts used by us have the ability to convert chorismate to isochorismate. Thus, OSB synthesis has been demonstrated with cell extracts of *E. coli* AN 154 supplied with shikimate and appropriate cofactors (Meganathan & Bentley, 1983).

It was suggested by Campbell (1969) that 2-ketoglutarate was converted to the succinic semialdehyde–thiamin pyrophosphate (TPP) anion by the decarboxylation mechanism shown in Figure 2. Hence, the overall process of menaquinone biosynthesis could be represented as



Evidence has, in fact, been obtained for the involvement of TPP in OSB biosynthesis (Meganathan & Bentley, 1983), thus supporting the proposed role of the anionic intermediate.

The postulated decarboxylation mechanism for 2-ketoglutarate (see Figure 2) is the same as that believed to be involved in the initial reaction of the 2-ketoglutarate dehydrogenase (KGDH) complex. This reaction is catalyzed by the first enzyme of this complex, usually termed E1 [EC 1.2.4.2, oxoglutarate dehydrogenase (lipoamide)]. The question arises, therefore, as to whether MK biosynthesis utilizes this E1 activity of the KGDH complex or whether a separate decarboxylating activity is involved.

A direct approach to this question has now been undertaken. The results reported here show that a separation of the KGDH complex and the OSB synthase enzyme system is possible. Additional evidence supporting this conclusion and confirming a role for TPP is also reported.

Materials and Methods

All experiments were carried out with cell-free extracts of *E. coli* K12 grown in tryptic soy broth; extracts were obtained

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[1] Abbreviations: OSB, *o*-succinylbenzoic acid; KGDH, 2-ketoglutarate dehydrogenase; TPP, thiamin pyrophosphate; th-TPP, tetrahydrothiamin pyrophosphate; CoA, coenzyme A.

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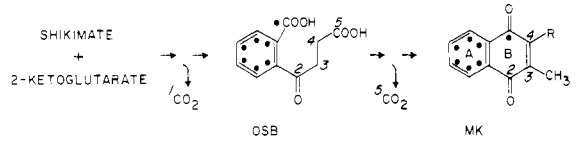

FIGURE 1: Incorporation of precursor carbon atoms into menaquinone (MK) and its biosynthetic precursor *o*-succinylbenzoate (OSB). The seven carbon atoms derived from the shikimate pathway via isochorismate are indicated with (●). The numbered atoms refer to carbon atoms derived from the specified atoms of 2-ketoglutarate. Although intermediates beyond OSB are known (Bentley & Meganathan, 1982), they are not indicated here. In the MK structure, R = a "prenyl" residue, known to be derived biosynthetically from mevalonate. The methyl substituent is known to be derived from *S*-adenosylmethionine.

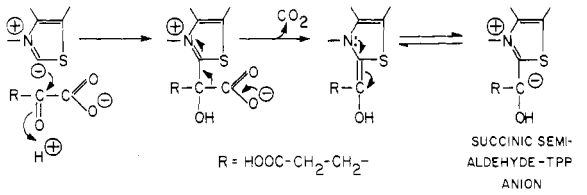

FIGURE 2: Postulated mechanism for the conversion of 2-ketoglutarate to the succinic semialdehyde–TPP anion. Only the thiazole portion of the TPP structure is shown since this is the active site of the reaction.

by use of a French press as previously described (Meganathan & Bentley, 1983). The extracts were treated with DNase (100 μg, 30 °C, 5 min) to reduce viscosity and were then centrifuged (Sorvall RC-5B, 30 min, 10000 rpm). Before use, the extracts were dialyzed against potassium phosphate buffer (0.02 M, pH 7.0, 6 h).

Assays for enzyme activities of the KGDH complex were as follows. The ferricyanide reduction assay (Reed & Mukherjee, 1969) was carried out with cell-free extract containing between 10 and 15 mg of protein. Release of $^{14}CO_2$ from [1-^{14}C]-2-ketoglutarate was followed in sealed Erlenmeyer flasks equipped with plastic center wells (Dennis et al., 1978). The assay mixture contained 4 mM NAD^+, 0.4 mM CoA-SH, 0.8 mM TPP, 2 mM dithiothreitol, and potassium phosphate buffer (0.1 M, pH 7.0) to a final volume of 2.0 mL. The substrate was 5 mM [1-^{14}C]-2-ketoglutarate. After incubation at 30 °C for 5 min, the reaction was terminated by addition of 1 mL of 2 N HCl. The released radioactive CO_2 was absorbed by 0.2 mL of hyamine hydroxide placed in the center wells; radioactivity was determined by transferring the hyamine hydroxide to a scintillation counting vial containing 10 mL of "formula 963" (New England Nuclear). Reduction of 3-acetylpyridine adenine dinucleotide ($APAD^+$) was followed spectrophotometrically at 365 nm by the method described earlier for NAD^+ reduction (Hager & Kornberg, 1961).

Assay for OSB synthase activity used the radio–gas chromatographic method described previously (Meganathan & Bentley, 1983); either [U-^{14}C]-2-ketoglutarate or [U-^{14}C]-glutamate was used as the radioactive substrate.

Tetrahydrothiamin pyrophosphate was prepared by borohydride reduction of TPP itself (Lowe et al., 1983). The analogue was separated from unreacted TPP by paper electrophoresis at pH 2.0. The nonfluorescent UV-absorbing material near the origin was eluted and lyophilized. This material was used without further separation of the active diastereoisomer.

Barium chorismate, TPP-Cl, NAD^+, $APAD^+$, and CoA-SH were obtained from Sigma Chemical Co., St. Louis, MO, [U-^{14}C]-2-ketoglutarate was from ICN, Irvine, CA, and [1-^{14}C]-2-ketoglutarate was from Amersham Corp., Arlington Heights, IL. Other chemicals were of highest quality, and organic solvents were redistilled before use. Protein assays were carried out by the method of Bradford (1976).

Table I: Enzyme Activities of the KGDH Complex of *E. coli* with and without Thiamin Pyrophosphate in the Incubation Mixtures

TPP	ferricyanide reduction[a,b] [nmol of $Fe(CN)_6^{4-}$ min^{-1} (mg of protein)$^{-1}$][c] expt A	expt B	$APAD^+$ reduction[a] [nmol min^{-1} (mg of protein)$^{-1}$][d]
+[e]	0.58	0.55	3.3
−	0.68	0.55	4.1

[a] Different batches of extract were used for measurement of the two activities, and the experimental conditions are different in the two cases. Hence, the values for one activity against the other are not directly comparable. [b] Each incubation mixture contained the following (in μmol) in a total volume of 1.4 mL: TPP, 0.20; $MgCl_2$, 0.30; potassium ketoglutarate, 50; potassium ferricyanide, 25. Potassium phosphate buffer was also present (1.0 M, pH 6.0, 0.25 mL). Cell-free extract was added so that from 10 to 15 mg of protein was present. After incubation for 30 min at 30 °C, the reaction was terminated with 10% trichloroacetic acid (1.0 mL); the denatured protein was removed by centrifugation. The ferrocyanide present in a 0.5-mL aliquot of the supernatant was determined by the method of Reed and Mukherjee (1969). [c] The actual experimental values were divided by 2, since 2 mol of $Fe(CN)_6^{3-}$ is reduced for a two-electron transfer. [d] The assay mixture contained the following (in μmol) in a final volume of 3.0 mL: $MgCl_2$, 67; cysteine HCl (neutralized), 33; thiamin pyrophosphate, 0.17; CoA-SH, 0.33; potassium ketoglutarate, 33; potassium phosphate, pH 7.0, 61; $APAD^+$, 10. The reaction was initiated by addition of the $APAD^+$ and was followed in a spectrophotometer at 365 nm. [e] In this case, the amount of TPP added was 0.8 μmol.

RESULTS

In preliminary experiments using cell-free extracts from *E. coli* K12, it was confirmed that the absence of TPP from the incubation mixtures resulted in almost complete loss of OSB synthase activity (95%) as previously reported (Meganathan & Bentley, 1983). Under these conditions, however, there was little or no effect on the KGDH complex activities (see Table I). These activities were measured in several ways: (a) The first is by following the reduction of ferricyanide in the reaction 2-ketoglutarate + $2Fe(CN)_6^{3-}$ + H_2O → succinate + CO_2 + $2Fe(CN)_6^{4-}$ + $2H^+$. This reaction is stated (Reed & Mukherjee, 1969) to provide a measure of the enzymatic activity of the E1 component of the KGDH complex (i.e., the decarboxylase activity). (b) The second is by following the decarboxylase activity directly by measuring the release of $^{14}CO_2$ from [1-^{14}C]-2-ketoglutarate. This technique was used previously with [1-^{14}C]pyruvate to study the corresponding pyruvate dehydrogenase complex (Dennis et al., 1978). (c) The third is by following the reduction of 3-acetylpyridine adenine dinucleotide ($APAD^+$); this substrate was preferred to the usual NAD^+ since *E. coli* extracts have considerable NADH oxidase activity (Amarasingham & Davis, 1965). This method provides a measure of the overall activity of the complex: 2-ketoglutarate + $APAD^+$ + CoA-SH → succinyl-CoA + CO_2 + APADH + H^+. As shown in Table I, none of these activities were reduced in the absence of TPP from the incubation mixtures.

That the absence of TPP has essentially no effect on the KGDH complex is presumably to be attributed to the presence of tightly bound TPP in the complex. Since the absence of TPP reduced the OSB synthase activity almost to zero, it immediately appeared that the OSB synthase activity was independent of the KGDH activity.

This preliminary conclusion was further supported by experiments with tetrahydro-TPP (th-TPP). This TPP analogue was previously shown (Lowe et al., 1983) to be an inhibitor

Table II: Effect of Tetrahydrothiamin Pyrophosphate (th-TPP) on 2-Ketoglutarate Decarboxylation by *E. coli* Extracts

th-TPP (μmol)[a]	$^{14}CO_2$ production from [1-^{14}]-2-ketoglutarate [nmol min^{-1} (mg of protein)$^{-1}$][b]	inhibition (%)[c]
0	30.3	0
0.4	14.0	54
0.8	7.0	77
1.6	1.8	94

[a] In these experiments, the *E. coli* extracts were preincubated with the th-TPP for a 2-h period at 4 °C. Each incubation mixture contained 0.5 mg of protein, [1-^{14}C]-2-ketoglutarate (400 dpm/nmol, 0.4 μmol), TPP (0.8 μmol), and the other materials listed under Materials and Methods. [b] These values have been corrected for the blank observed in the absence of enzyme extract. [c] In another experiment, $APAD^+$ reduction was measured; a 40% inhibition was observed with 0.25 μmol of th-TPP and with the same preincubation conditions.

Table III: Effect of Tetrahydrothiamin Pyrophosphate (th-TPP) on OSB Synthase Activity

th-TPP (μmol)[a]	OSB production [pmol min^{-1} (mg of protein)$^{-1}$][b]	inhibition (%)
0	17.0	0
0.02	4.4	74
0.07	3.3	81
0.15	2.4	86

[a] Note that, in contrast to the experiments described in Table II, th-TPP was added to the incubation mixtrues at the same time as [U-^{14}C]-2-ketoglutarate. [b] Each assay mixture contained the following components (in μmol) in 0.1 M glycylglycine buffer, pH 8.5, with 5 mM mercaptoethanol: chorismate, 1.4; TPP, 0.11; [U-^{14}C]-ketoglutarate, 0.4 (5 μCi). After incubation for 30 min at 30 °C, the reaction was terminated with 4 drops of concentrated HCl. The OSB was extracted, purified, and converted to the dimethyl derivative as previously described, prior to radio–gas chromatography (Meganathan & Bentley, 1983). The amount of OSB produced in each assay was determined from the mass and radioactivity peaks observed.

of the pyruvate dehydrogenase complex activity of *E. coli*. In our work, it was also found to inhibit the KGDH complex; as was the case with the pyruvate dehydrogenase system, it was necessary to preincubate the cell-free extracts with th-TPP in order to demonstrate this inhibition (see Table II). On the other hand, OSB synthase activity was strongly inhibited without the need for any preincubation (see Table III). It was also the case that the OSB synthase activity appeared to be more sensitive to the action of the inhibitor than did the KGDH complex. Thus, 0.02 μmol of th-TPP gave a 74% inhibition of OSB synthase activity while 0.8 μmol was required for a 77% inhibition of the KGDH complex (see Tables II and III). This different behavior of the two systems was also consistent with the presence of a separate decarboxylating activity for the OSB synthase. All of the experiments so far described also provide supporting evidence for a role of TPP in OSB biosynthesis.

In further work, gel-permeation chromatography using a Sepharose CL-6B column (10K–400K exclusion limit) was found to separate the very high molecular weight KGDH complex (as measured by reduction of $APAD^+$) from the OSB synthesizing component (see Figure 3). When release of $^{14}CO_2$ from [1-^{14}C]-2-ketoglutarate was determined in the column fractions, a definite peak of decarboxylating activity was observed to coincide, as expected, with the fractions containing the high molecular weight KGDH complex. No defined peak of decarboxylating activity, however, was seen in the fractions containing OSB synthase activity (see Figure 3). This apparently is the result of the rather high background observed with this assay; the background is in the nanomolar range while the maximum measured OSB synthase activity

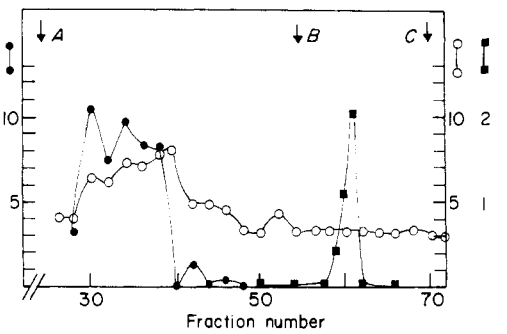

FIGURE 3: Fractionation of *E. coli* K12 extract on a Sepharose CL-6B column. The column (diameter 4.6 cm, height 55 cm) was equilibrated with 0.1 M potassium phosphate buffer, pH 7, containing 10 mM mercaptoethanol. The cell-free extract contained 1.95 g of protein in 0.02 M potassium phosphate buffer, pH 7.0 (50 mL). The flow rate was 2.5 mL/min, and the fraction size was 10 mL. Molecular weight markers (indicated by arrows) were A = Blue Dextran (M_r 2×10^6), B = bovine serum albumin (M_r 66 200), and C = K_2CrO_4. All fractions were assayed for three enzyme activities. (●) KGDH complex determined by measuring $APAD^+$ reduction, with the ordinate representing nmol of 2-ketoglutarate min^{-1} (mg of protein)$^{-1}$. (○) Release of $^{14}CO_2$ from [1-^{14}C]-2-ketoglutarate, with the ordinate representing nmol of $^{14}CO_2$ min^{-1} (mg of protein)$^{-1}$. These values have not been corrected for the background level of $^{14}CO_2$ production in the absence of enzyme extract. (■) OSB production with the ordinate representing pmol of OSB min^{-1} (mg of protein)$^{-1}$. All fractions were assayed, but no OSB synthase activity was detected below fraction 50 or above fraction 65, and no KGDH complex activity (of either kind) was observed beyond fraction 50 (these zero values are not plotted).

in the most active column fraction was 2.03 pmol min^{-1} (mg of protein)$^{-1}$.

Discussion

The number of enzymes, or of enzyme activities, needed for OSB biosynthesis is an intriguing question. It has been suggested that the maximum would be four, in addition to a ketoglutarate decarboxylase (Bentley & Meganathan, 1982). So far, however, only two groups of *E. coli* mutants (*menD* and *menC*) have been identified that are deficient in OSB biosynthesis (Guest, 1979). Provisionally, we have referred to two enzyme activities as OSB synthase I and II. OSB synthase I (the product of the *menD* gene) is known to form an intermediate, probably 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (Emmons et al., 1985); this intermediate is converted to OSB by OSB synthase II (the product of the *menC* gene). If OSB synthase I contains an integral decarboxylase, the minimum number of enzymes would be two; if there is a completely separate decarboxylase in addition to the two OSB synthases, at least three enzyme activities are required.

Previous work showed that the *E. coli sucA* mutant JRG72, which does not show significant KGDH activity due to a deficiency of the E1 component, had as much total OSB synthase activity as did the wild-type strain PL 2024 (Meganathan & Bentley, 1983). This *sucA* mutant requires both lysine and methionine for growth on glucose-minimal salts medium; alternatively, the requirement for both of these amino acids can be replaced by succinate (Herbert & Guest, 1968). Succinate is needed only in catalytic amounts, since it is recycled (Creaghan & Guest, 1978). This property is consistent with the established biosynthetic pathways to lysine and methionine; in both cases, a succinylation with succinyl-CoA is required, and succinate is regenerated subsequently. In the absence of the KGDH complex, succinyl-CoA cannot be obtained from glucose. Our finding of a normal level of OSB synthase activity in the *sucA* mutant indicated that either there

was a decarboxylase activity separate from the KGDH complex or, alternatively, there was sufficient leakiness in this mutant to supply the succinic semialdehyde–TPP anion in the very small quantities needed for OSB synthase I activity (although not sufficient to supply succinyl-CoA for lysine and methionine synthesis). In the first case, the decarboxylase activity could be a separate enzyme, or an activity associated with OSB synthase I; in the second case, a separate enzyme for the anion addition step would still be required.

The clear-cut separation of the KGDH complex from the OSB synthase now confirms these preliminary suggestions that the OSB synthase does not rely on the decarboxylating activity of the complex. This conclusion requires that no separation of the E1 component of the KGDH complex has occurred on the column. This seems improbable since the proteins of this complex are known to be tightly bound together and the decarboxylase activity is only removed by treatment with 1 M NaCl at pH 9.0 (Pettit et al., 1973). The possible occurrence of the E1 enzyme activity with the OSB synthase also seems unlikely in terms of the following molecular weight considerations. The E1 decarboxylase component occurs as a dimer of subunits with individual molecular weights of 95 000. Since comparison with known molecular weight markers (bovine serum albumin, RNase A) indicated an approximate molecular weight of 35 000 for the OSB synthase enzyme, it is apparent that any protein of the OSB synthase system has a much smaller molecular weight than does the E1 decarboxylase. After completion of our work, Weische and Leistner (1985) also reported a separation of the KGDH complex activity from OSB synthase activity by precipitation with protamine sulfate; experimental details have not been provided.

The structure of the OSB synthase I thus becomes of considerable interest. As already indicated, the necessary enzyme protein(s) that form(s) the 2,4-cyclohexadiene intermediate is (are) controlled by the *menD* gene. Unless another gene remains to be identified, the product(s) of this gene is (are), therefore, responsible for the formation of the succinic semialdehyde–TPP anion (by decarboxylation of 2-ketoglutarate) as well as for its addition to isochorismate. Since the ketoglutarate decarboxylase and anion adding activity coelute from the Sepharose column, the properties of the *menD* mutants are of importance. These mutants completely lack the ability to synthesize OSB. However, they are not deficient in the formation of the KGDH complex, and they are lysine- and methionine-independent. Their properties are, in fact, the inverse of the *sucA* mutants:

	KGDH	Lys and Met dependent	OSB synthase
sucA	absent	yes	yes
menD	present	no	no

These properties provide further support for the postulate that the KGDH complex is a separate and independent entity from the decarboxylase activity associated with OSB synthase I.

The postulate is also consistent with the known *absence* of the KGDH complex activity in anaerobically grown *E. coli* (Amarasingham & Davis, 1965); under anaerobic conditions, however, there is a 2–3-fold *increase* in the level of overall OSB synthase activity, both in wild-type *E. coli* (PL 2024) and in the *sucA* mutant (Meganathan & Bentley, 1983). This increase in OSB synthase activity is, of course, related to the well-known increase in MK content of *E. coli* when grown anaerobically (Bentley & Meganathan, 1982).

Acknowledgments

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Registry No. OSB, 27415-09-4; succinic semialdehyde–TPP, 100046-06-8; OSB synthase, 97089-83-3; tetrahydro-TPP, 35728-83-7; menaquinone, 1182-68-9; 2-ketoglutarate, 328-50-7; ketoglutarate dehydrogenase, 9031-02-1.

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