JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 8111–8118
0021-9193/92/248111-08$02.00/0

Vol. 174, No. 24

Menaquinone (Vitamin K_2) Biosynthesis: Evidence that the *Escherichia coli menD* Gene Encodes Both 2-Succinyl-6-Hydroxy-2,4-Cyclohexadiene-1-Carboxylic Acid Synthase and α-Ketoglutarate Decarboxylase Activities

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The formation of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC), the first identified intermediate in the menaquinone biosynthetic pathway, requires two reactions. They are the decarboxylation of α-ketoglutarate by an α-ketoglutarate decarboxylase, which results in the formation of succinic semialdehyde-thiamine PP_i (TPP) anion, and the addition of the succinic semialdehyde-TPP anion to isochorismate carried out by the enzyme SHCHC synthase. Evidence is provided to support the conclusion that both enzymatic activities are encoded by an extended *menD* gene which is capable of generating a bifunctional 69-kDa protein. Consistent with the requirement for TPP in the decarboxylation of α-ketoglutarate, the translated amino acid sequence contains the characteristic TPP-binding motif present in all well-characterized TPP-requiring enzymes.

The pathway for the biosynthesis of menaquinone (vitamin K_2) has been recently reviewed (4). That menaquinones are shikimate-derived compounds was demonstrated in the early experiments of Cox and Gibson (10). Subsequently, isotopic tracer experiments established that all seven shikimate carbon atoms are incorporated into the naphthoquinone nucleus of menaquinone (9) and that the remaining three carbons are derived from α-ketoglutarate (or glutamate) with the loss of both carboxyl groups (29). The first aromatic intermediate in the menaquinone biosynthetic pathway is *o*-succinylbenzoate (OSB) (11), which contains four carbon atoms derived from α-ketoglutarate. Although for some time evidence indicated that shikimate was first converted to chorismate (3), it now appears that isochorismate is the actual precursor (13, 36, 37), as originally suggested (11). Campbell (8) postulated that α-ketoglutarate was converted to succinic semialdehyde-thiamine PP_i (TPP) anion by the decarboxylation reaction shown in Fig. 1. The enzymatic biosynthesis of OSB from chorismic acid and α-ketoglutarate was demonstrated by Meganathan (20). Additional experiments revealed a TPP requirement for the reaction and the presence of an intermediate prior to OSB formation (23). The structure of this intermediate was established on the basis of nuclear magnetic resonance data as that of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC) (13).

It was further established that the decarboxylase required for the formation of succinic semialdehyde-TPP is distinct from the decarboxylase activity of the α-ketoglutarate dehydrogenase complex (KGDH complex) (19). Hence, for the formation of OSB from isochorismate and α-ketoglutarate, the activities of three enzymes, namely, SHCHC synthase, α-ketoglutarate decarboxylase (KDC), and OSB synthase, are required (19, 26) (Fig. 1).

Weische et al. (35, 36) have reported the presence of a single enzyme complex, OSB synthase, for the conversion of isochorismate and α-ketoglutarate to OSB in the presence of TPP. Those authors isolated a subunit from OSB synthase and designated it as the decarboxylating subunit of OSB synthase (35, 36). However, the involvement of SHCHC or SHCHC synthase was not addressed in those reports.

Moreover, only two groups of mutants blocked in the formation of OSB, *menC* and *-D*, have been reported (14, 23) (Fig. 1). One group of mutants (*menD*) was found to be blocked in the formation of SHCHC because of the lack of SHCHC synthase activity, and the other group (*menC*) lacked OSB synthase activity (23, 26, 27). Both *menC* and *menD* are part of the *menBCDE* cluster located at 48.5 min on the *Escherichia coli* chromosome (4). This cluster has been cloned (33), and the complete nucleotide sequences of the *menB* and *menD* genes have been reported (26, 32). The gene coding for KDC activity has never been identified. In this study, we have reexamined and extended the previously published sequence (26) of the *menD* gene and show that it encodes KDC activity, in addition to SHCHC synthase activity, as part of a bifunctional enzyme.

(A preliminary report of these findings has appeared previously [31]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains and plasmids used are listed in Table 1. Aerobic growth of cells in Trypticase soy broth was as described previously (23, 32). For large-scale anaerobic growth of cells, the strains were inoculated into 50 ml of medium contained in two 25-ml screw-cap tubes filled to the top with Trypticase soy broth containing 1% glucose and incubated overnight at 37°C. This culture was used to inoculate 2 liters of medium of the same composition contained in a 2-liter Erlenmeyer flask and incubated at 37°C until growth reached 80 Klett units at 600 nm. For plasmid-transformed strains, the medium was supplemented with 100 μg of ampicillin per ml.

Harvesting of cells and preparation of cell extracts. Cells were harvested by centrifugation at 4,000 × *g* for 10 min.

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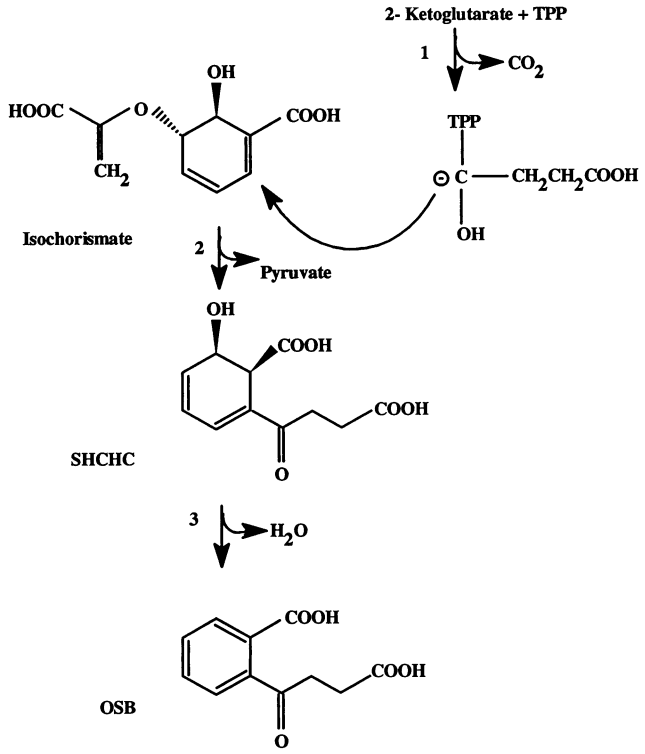

FIG. 1. Mechanism of formation of SHCHC and its conversion to OSB. Enzymes are numbered as follows: 1, α-ketoglutarate decarboxylase; 2, SHCHC synthase; 3, OSB synthase. Enzymes 1 and 2 are encoded by the *menD* gene, and enzyme 3 is encoded by the *menC* gene.

The pellet was washed by resuspension in 500 ml of 0.02 M potassium phosphate buffer (pH 7.0) and recentrifuged. The cells were then resuspended in buffer containing 10 mM 2-mercaptoethanol, at a ratio of 1 g/1.5 ml of buffer, and passed through a French pressure cell at 12,000 lb/in^2. The cell extract was incubated with DNase for 5 min at 37°C to reduce viscosity and then centrifuged at 30,000 × *g* for 30 min. The resulting supernatant was dialyzed in 1 liter of buffer containing 10 mM 2-mercaptoethanol for 3 h, and the buffer was changed and dialysis was continued for a further 3 h. Glycerol was added to the dialyzed extract at a concentration of 20%, and the extract was stored at −20°C.

TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
E. coli strains		
PL2024	*gal trpA trpR iclR rpsL*	23
JRG862	*menC1* (OSB-requiring mutant of PL2024)	23
JRG918	*menD7* (OSB-requiring mutant of PL2024)	23
JLP200	JRG862(pJP101)	26
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Laboratory stock
JM83	F$^-$ *ara* (*lac-proAB*) *rpsL* (Str^r) [φ80d (*lacZ*)M15]	Laboratory stock
TK3D18	Δ(*kdp-suc*)D18 Δ(*gal-bio*)	W. Epstein
Plasmids		
pJP101		26
pMS3		32
pKK223-3		Pharmacia, 26
pUC18		IBI
pUC19		IBI

Protamine sulfate precipitation. The dialyzed supernatant was fractionated by using protamine sulfate (28), with modifications. The extract was acidified to a pH of 6.2 with 1% acetic acid. To this extract, a 2% solution of protamine sulfate (pH 6.2) was added until the volume increased by 30%. The precipitate formed was removed by centrifugation at 17,000 × *g* for 15 min, and the clear supernatant was used in enzyme assays.

Assay of enzyme activity. The reaction mixture contained Tris-HCl buffer (pH 7.9) (50 mM), TPP (0.85 mM), 2-[U-^{14}C] ketoglutarate (0.5 μCi), and enzyme in a total volume of 2.0 ml. The tubes were incubated for 1 h at 37°C, and, after the addition of 2 μmol of succinic semialdehyde and 2 μmol of α-ketoglutarate as carrier, the reaction was terminated by boiling for 5 min. The contents of the tubes were cooled and then acidified by the addition of 200 μl of 10 N HCl. The precipitated protein was removed by centrifugation at 12,500 × *g* for 10 min. The supernatant fraction was treated with 1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl for 30 min at room temperature. The mixture was extracted three times with 3 ml of ethyl acetate. The combined ethyl acetate fractions were dried in a rotary evaporator. The dried material was dissolved in a small quantity of ethyl acetate and streaked onto silica gel thin-layer chromatography plates (Baker Si250) and developed with chloroform-methanol (9:1) as the solvent system. The plates were dried and scanned for radioactivity with a Packard model 7201 radiochromatogram scanner.

DNA isolation. Plasmid DNAs were isolated by the alkaline lysis procedure of Birnboim and Doly (5) and purified in ethidium bromide CsCl gradients.

Construction of plasmids. Plasmids used in this study consisted of fragments derived from pJP101 (26) and cloned into pUC18 or pKK223-3 as shown in Fig. 2. For some constructs, individual fragments were electroeluted from 0.8 or 1.0% agarose gel slices by using an IBI (New Haven, Conn.) unidirectional electroeluter. Ligation and transformation procedures were as described previously (18). Hosts for initial transformation were strains HB101 and JM83.

DNA sequencing and sequence analysis. DNA sequences were determined by the dideoxynucleotide chain termination method (30) by using the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{32}P]dATP (ICN, Costa Mesa, Calif.). The initial DNA sequence was determined from alkaline-denatured double-stranded plasmid DNAs. Compressed regions were resolved by single-stranded sequencing from equivalent M13 clones by using either dITP or 7-deaza-dGTP. Sequencing reactions were primed with universal, reverse, or sequence-generated synthetic oligonucleotide primers synthesized on an Applied Biosystems (Foster City, Calif.) model 391 DNA synthesizer. All nucleotide positions were confirmed by sequencing of the complementary strands. Nucleic acid and deduced protein sequences were analyzed by using the Pustell DNA sequence analysis program (IBI Inc.) and the Genetics Computer Group program (University of Wisconsin Biotechnology Center, Madison, Wis.).

Chemicals. Succinic semialdehyde, α-ketoglutarate, TPP hydrochloride, dinitrophenylhydrazine, and ampicillin were from Sigma Chemical Co., St. Louis, Mo. α-[U^{14}C]-ketogu-

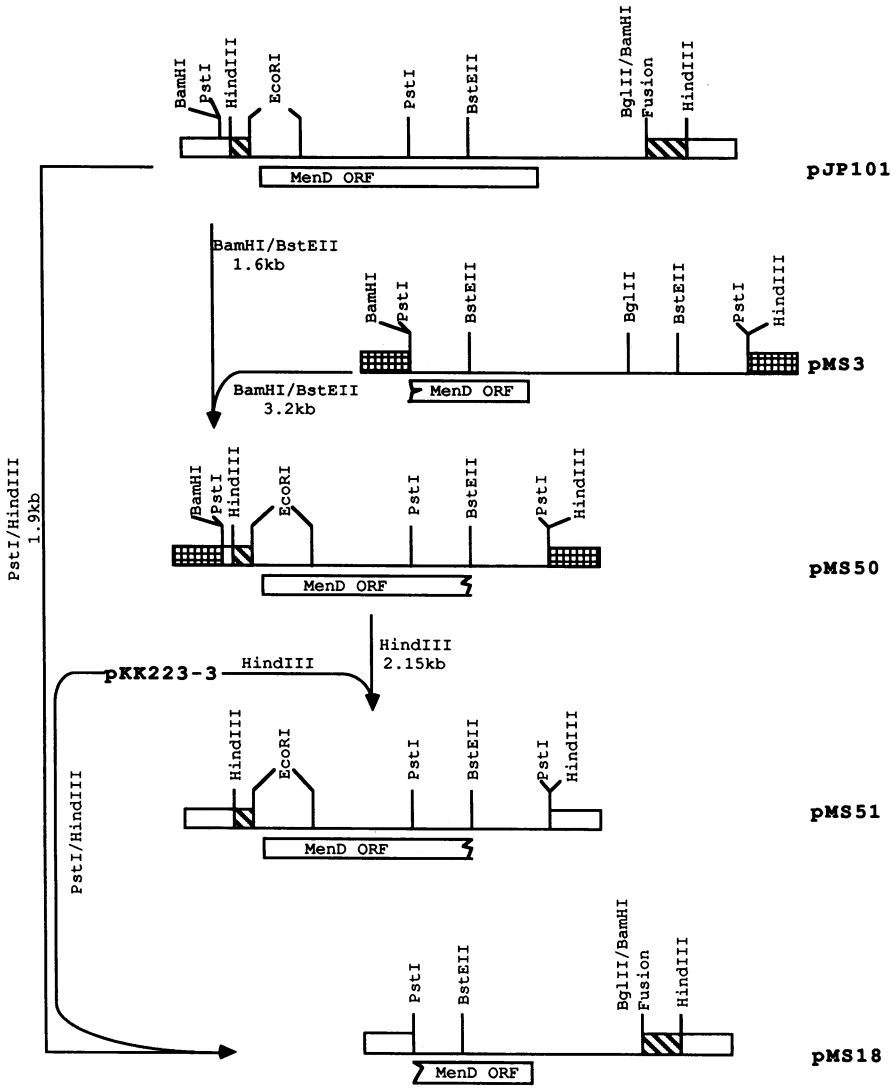

FIG. 2. Construction of the pKK223-3 *menD* deletion plasmids pMS18 and pMS51. For 5′ deletion (pMS18), a *Pst*I-*Hin*dIII fragment from pJP101 (26) was directly inserted into pKK223-3. For 3′ deletion (pMS51), a *Bam*HI-*Bst*EII fragment from pJP101 was first fused with a downstream *Bst*EII site of pMS3 (32) and then transferred as a *Hin*dIII fragment to pKK223-3. Only restriction sites relevant to the constructs are shown. Symbols: ▭, pKK223-3; ▦, pUC18; ▨, pBR322.

tarate was a product of New England Nuclear, Boston, Mass. Baker Si250 thin-layer chromatography plates were from J. T. Baker Chemical Co., Phillipsburg, N.J. Trypticase soy broth was from Difco Laboratories, Detroit, Mich. Solvents used were of high-performance liquid chromatography grade and were obtained from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals were of the highest quality commercially available. Protein was determined by the method of Bradford (6) by using chemicals supplied by Bio-Rad Laboratories, Richmond, Calif.

Nucleotide sequence accession number. The 1,800-bp sequence reported in this article appears in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number LO4464.

RESULTS

Presence of KDC in various strains after removal of KGDH complex. Two reactions have been identified in the formation of SHCHC. These are the decarboxylation of α-ketoglutarate, which results in the formation of succinic semialdehyde-TPP anion, and the addition of succinic semialdehyde-TPP anion to isochorismate with the elimination of the pyruvoyl moiety (Fig. 1). However, only a single group of mutants blocked in these activities, designated as *menD*, has been identified. The *menD* gene has been cloned and sequenced, and the enzyme encoded by this gene has been designated as SHCHC synthase (26). It has also been established that the enzyme responsible for the decarboxylation of α-ketoglutarate is distinct from the first enzyme of the KGDH complex, usually termed E1 (EC 1.2.4.2, oxoglutarate dehydrogenase [lipoamide]) (19). However, the possibility of both enzymatic activities (KDC and SHCHC synthase) being under the control of a single gene has never been ruled out. In order to establish whether a single gene or two discrete genes are involved in the formation of SHCHC, we assayed for KDC activity in wild-type and mutant

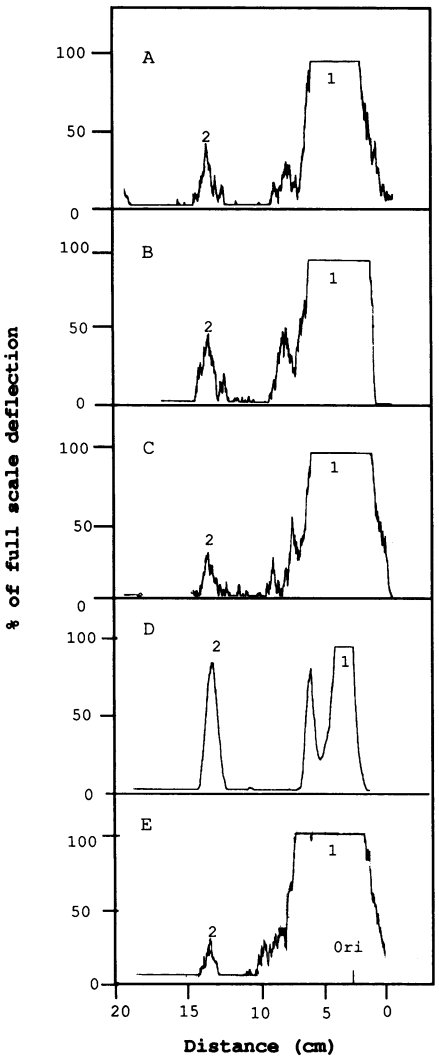

FIG. 3. Formation of succinic semialdehyde by various strains. The succinic semialdehyde formed was isolated as its dinitrophenylhydrozone (SS-DNP) and separated from α-ketoglutarate dinitrophenylhydrozone (KG-DNP) by thin-layer chromatography as described in Materials and Methods. Radioactivity tracings from five different experiments are shown. The ordinate shows the percentage of full-scale deflection of the radioactivity scanner. The instrument settings were as follows: range, 3×10^2 for panels A, B, C, and E and 3×10^3 for panel D; speed, 1 cm/min; time constant, 10 s; slit width, 2 mm. Ori indicates the origin of the thin-layer chromatogram. Peak 1 is KG-DNP, and peak 2 is SS-DNP. (A) Sample derived from wild-type strain PL2024 (*men*$^+$) grown aerobically; (B) PL2024 grown anaerobically; (C) the KGDH deletion mutant TK3D18 [Δ(*kdp-suc*)] grown aerobically; (D) JLP200 (JRG862 *menC* [pJP101]) grown aerobically; (E) JRG862 (*menC*) grown aerobically.

strains. To obtain unequivocal results in assaying for KDC activity, it was essential that the competing decarboxylase activity of the KGDH complex be eliminated. This was accomplished by precipitation of the KGDH complex with protamine sulfate as described in Materials and Methods. The KDC activity of the wild-type strain is shown in Fig. 3A. When the *menC* and *menD* mutants were assayed for KDC, they showed the same level of activity as the parent strain. The presence of KDC in the *menD* mutant and its reported inability to form SHCHC verified that it lacks SHCHC synthase but not KDC.

Presence of KDC activity in crude cell extracts from various strains grown anaerobically. Although it has been reported (28) that protamine sulfate precipitates the KGDH complex, it was essential to establish that it was completely precipitated and that KDC activity remained in the supernatant. Since it is known that the KGDH complex is repressed in anaerobically grown cells (1), we assayed for KDC activity in crude cell extracts of various strains grown anaerobically. Figure 3B demonstrates that the KDC activity of anaerobically grown wild-type cells is similar to that obtained after protamine sulfate precipitation of aerobically grown cell extracts. The *menC* and *menD* strains showed similar activities (data not shown).

Presence of KDC activity in a deletion mutant lacking KGDH complex. The level of the E1o component of the KGDH complex in anaerobically grown cells was reported to be 310 nmol/h/mg of protein (16), and since previous studies have established that the KDC involved in menaquinone biosynthesis, like the other enzymes of the pathway, is present in extremely low activities (19, 23), it was essential to demonstrate that the repressed level of KGDH was not contributing to the apparent KDC activity. Therefore, KDC activity was assayed in crude cell extracts from an aerobically grown mutant strain carrying a deletion in the KGDH complex (7). As seen in Fig. 3C, the deletion mutant showed nearly the same level of KDC activity as the wild type and the *menC* and *menD* mutants whether grown aerobically and subjected to protamine sulfate treatment or grown anaerobically. Similar results were obtained when the KGDH deletion mutant was grown anaerobically (data not shown). Furthermore, when the extract from the mutant strain was subjected to protamine sulfate treatment at the same concentration as the parent strain, KDC activity remained unchanged (data not shown).

Identification of the gene encoding KDC activity. A plasmid derivative containing a 3.14-kb *Sal*I-*Bgl*II insert in pBR322, designated as pGS51, has previously been shown to complement *menD* mutants (33). Subcloning of a 2.648-kb *Eco*RI-*Bgl*II fragment of pGS51 into pKK223-3 as pJP101 resulted in the overproduction of SHCHC synthase, and DNA sequence analysis identified a 1.4-kb open reading frame encoding SHCHC synthase (26). Since this enzyme, the product of *menD*, is required in conjunction with KDC for the formation of SHCHC, we assayed JLP200, which is a *menC* mutant (blocked in the conversion of SHCHC to OSB) carrying the plasmid pJP101, for increased KDC activity. This strain showed greatly amplified levels of KDC activity (Fig. 3D) compared with those of the parent strain JRG862 (*menC*) (Fig. 3E), even in the absence of induction by isopropyl-β-D-thiogalactopyranoside, and thus provided evidence that KDC is encoded by pJP101.

To identify the DNA that encodes KDC activity, we constructed two plasmids from pJP101 for complementation analyses. The first of these, pMS18 (Fig. 2), was deleted (*Eco*RI-*Pst*I) for all but the last 118 codons of *menD* (as previously defined). Unexpectedly, this plasmid failed to show increased KDC activity in the *menC* background, as if the *menD* gene were necessary for KDC activity. The second construct, pMS51, retained the apparent *menD* reading frame but was deleted (*Bst*EII-*Bgl*II) for downstream DNA. This construct surprisingly failed to complement and restore anaerobic growth of the *menD* mutant on glycerol-trimethylamine *N*-oxide medium, suggesting the absence of an intact SHCHC synthase.

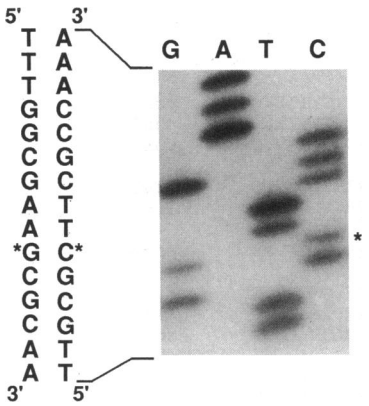

FIG. 4. Evidence for an additional nucleotide in the *menD* sequence. Shown is a section of the sequencing gel encompassing nucleotides 1834 to 1849 (Fig. 5) of the noncoding strand. The coding strand is shown with the additional nucleotide identified by an asterisk.

Thus, while pJP101 encoded both SHCHC synthase and KDC activities, deletion of either the 5′ or 3′ region (pMS18 or pMS51) resulted in the simultaneous loss of both enzymatic activities.

These results suggested that previous sequence analysis of the 3′ end of *menD* might be in error. DNA sequence analysis with dITP and 7-deaza-dGTP confirmed the reported sequence of this region except for the presence of an additional G residue in a region subject to strong compressions (Fig. 4). Insertion of this nucleotide after base 1842 of the reported *menD* sequence (26) extends the SHCHC synthase reading frame (Fig. 5). This single extended 619-codon reading frame, which encodes a 69-kDa protein, resolves the inconsistencies of the complementation assays with pJP101, pMS18, and pMS51 and identifies the *menD* gene product as a bifunctional protein containing both SHCHC synthase and KDC enzymatic activities.

Presence of a TPP-binding motif in the KDC region of the *menD* gene product. Despite the identical natures of reactions catalyzed by TPP-binding enzymes, little amino acid similarity was evident from initial comparison of a variety of such enzymes (12). Recently, however, Hawkins et al. (15), in an analysis of an expanded pool of TPP-requiring proteins, identified a putative TPP-binding motif. Given the TPP requirement of KDC, we analyzed the deduced bifunctional polypeptide encoded by the extended *menD* open reading frame for this motif. As shown in Fig. 6, the putative TPP-binding motif is evident in the C-terminal region of the polypeptide.

DISCUSSION

There is considerable evidence to show that bacterial menaquinones are formed by the shikimate pathway (for reviews, see references 3 and 4). The first aromatic intermediate identified in the pathway is the benzenoid compound OSB. Of the 11 carbon atoms of OSB, 7 are derived ultimately from shikimic acid and the remaining 4 are from α-ketoglutarate (22). To account for the role of α-ketoglutarate, the involvement of the anion form of the TPP complex of succinic semialdehyde formed by a reaction analogous to that of the first enzyme of the KGDH complex, usually

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    L Q Q A Q I V V Q L G S S L T G K R L L Q W Q A S C E P E E Y W I V
1561 CTGCAGCAGGCGCAAATTGTGGTGCAACTGGGAAGCAGCCTGACGGGGAAACGGCTCCTGCAATGGCAGGCAAGCTGTGAACCAGAAGAGTACTGGATTG
     PstI
     D D I E G R L D P A H H R G R R L I A N I A D W L E L H P A E K R
1661 TTGATGACATTGAAGGGCGACTTGATCCGGCACACCATCGCGGACGTCGCTTAATTGCCAATATTGCCGACTGGCTGGAGCTGCATCCGGCAGAAAAACG

     Q P W C V E I P R L A E Q A M Q A V I A R R D A F G E A Q L A H R
1761 CCAGCCCTGGTGCGTTGAAATCCCGCGCCTGGCGGAACAGGCAATGCAGGCGGTTATTGCCCGCCGTGATGCGTTTGGCGAAGCGCAACTGGCGCATCGC

     I C D Y L P E Q G Q L F V G N S L V V R L I D A L S Q L P A G Y P V
1861 ATCTGCGACTATCTGCCTGAACAGGGGCAATTGTTTGTTGGTAACAGCCTGGTGGTACGTCTGATTGATGCGCTTTCGCAACTTCCGGCAGGTTACCCGG

     Y S N R G A S G I D G L L S T A A G V Q R A S G K P T L A I V G D
1961 TGTACAGCAACCGTGGGGCCAGCGGTATCGACGGGCTGCTTTCGACCGCCGCCGGCGTTCAGCGGGCAAGCGGCAAACCGACGCTGGCGATTGTGGGCGA

     L S A L Y D L N A L A L L R Q V S A P L V L I V V N N N G G Q I F
2061 TCTCTCCGCACTTTACGATCTCAACGCGCTGGCGTTATTGCGTCAGGTTTCTGCGCCGCTGGTATTAATTGTGGTGAACAACAACGGCGGGCAAATTTTC

     S L L P T P Q S E R E R F Y L M P Q N V H F E H A A A M F E L K Y H
2161 TCGCTGTTGCCAACGCCGCAAAGCGAGCGTGAGCGTTTCTATCTGATGCCGCAAAACGTCCATTTTGAGCACGCCGCCGCGATGTTCGAGCTGAAATATC

     R P Q N W Q E L E T A F A D A W R T P T T T V I E M V V N D T D G
2261 ATCGTCCGCAAAACTGGCAGGAACTTGAAACGGCATTTGCCGACGCCTGGCGCACGCCAACCACCACGGTGATTGAAATGGTGGTTAACGACACCGATGG
                                                                 HpaI

     R K R S S N F W R R *
2361 TCGCAAACGCTCCAGCAACTTCTGGCGCAGGTAA
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FIG. 5. Nucleotide and deduced amino acid sequences of the 3′ region of *menD*. The noncoding strand is shown and numbered according to the method of Popp (26). Single-letter amino acid abbreviations are indicated above the codons. Amino acids implicated in the TPP-binding region (Fig. 6) are underlined and identify the KDC domain of *menD* as 3′ to the SHCHC synthase domain. With the exception of the additional underlined nucleotide at position 1843, the *Pst*I-*Hpa*I sequence is identical to that reported previously (26). The site for the new termination codon, 42 nucleotides downstream of the *Hpa*I, is shown by an asterisk.

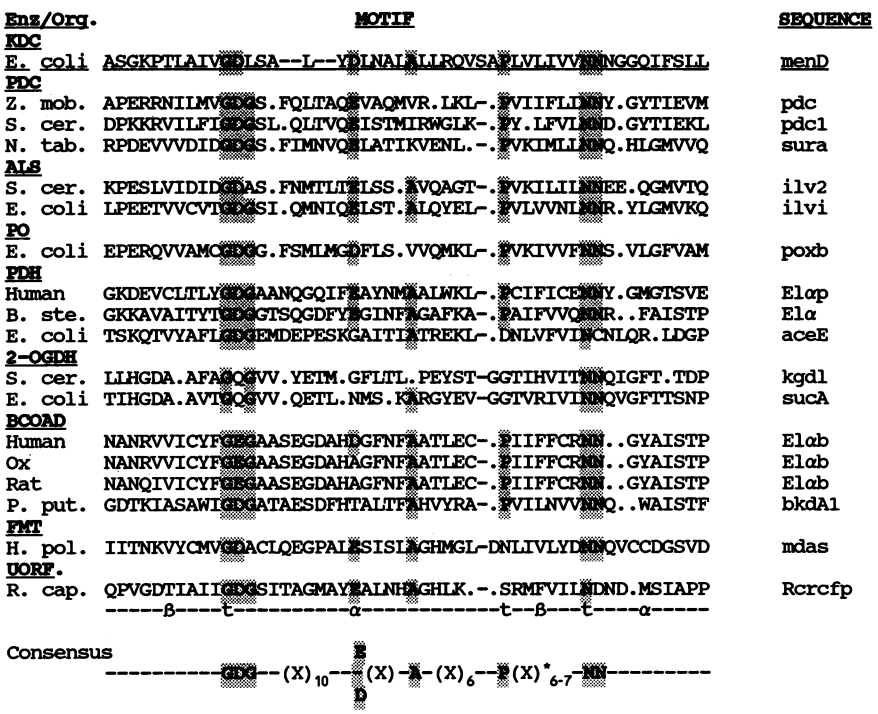

FIG. 6. Presence of a TPP-binding motif in the KDC coding region of *menD*. The amino acid sequence of residues 498 through 545 of the *menD* product are aligned with equivalent regions of other TPP-binding enzymes (15). The conserved regions are shown by boldface letters; the spacing introduced by Hawkins et al. (15) is indicated by dots, and the spacing introduced in this article is shown by hyphens. Enzyme (Enz) abbreviations: PDC, pyruvate decarboxylase; ALS, acetolactate synthase; PO, pyruvate oxidase; PDH, pyruvate dehydrogenase (E1); 2-OGDH, 2-oxoglutarate dehydrogenase (E1); BCOAD, branched-chain 2-oxoacid dehydrogenase (E1); FMT, formaldehyde transketolase; UORF., unidentified open reading frame from *Rhodobacter capsulatus*. Organism (Org.) abbreviations: B. ste., *Bacillus stearothermophilus*; H. pol., *Hansenula polymorpha*; N. tab., *Nicotiana tabacum*; P. put., *Pseudomonas putida*; R. cap., *Rhodobacter capsulatus*; Z. mob., *Zymomonas mobilis*. The consensus sequence is as described previously (15). (X) indicates a variable amino acid, with the numbers indicating approximate numbers of residues. The asterisk identifies amino acids with hydrophobic side chains.

termed E1 (EC 1.2.4.2, oxoglutarate dehydrogenase [lipoamide]), was proposed (8). Subsequently, it was demonstrated that cell extracts of *E. coli* required TPP for the biosynthesis of OSB from chorismate and α-ketoglutarate (23). Further studies showed that there is a prearomatic intermediate before OSB called SHCHC and that it is formed from isochorismate and α-ketoglutarate (13).

Menaquinone functions in a number of anaerobic reactions in *E. coli* (17, 34), such as the reductions of fumarate (14), trimethylamine *N*-oxide (21), and tetrahydrothiophene *N*-oxide (24), etc., and since it was known that the KGDH complex is repressed under anaerobic conditions (1, 16), it was reasoned that the KGDH complex is probably not involved in the biosynthesis of menaquinone. This assumption was further strengthened by the observation that in an *sucA* mutant which lacks the decarboxylase activity of the KGDH complex, the formation of OSB in vitro was unaffected (23). These observations prompted us to search for a decarboxylase specifically involved in menaquinone biosynthesis in *E. coli* extracts. The presence of such an enzyme was reported previously (19).

In a preliminary report, we showed that a 3-kb fragment previously shown to contain the *menD* gene and to overproduce SHCHC synthase was capable of producing amplified levels of decarboxylase (25). By completing the sequence of the remainder of this fragment and by reexamining part of the previously reported sequence, we found that the *menD* gene includes a single open reading frame of 1.857 kb capable of encoding a 69-kDa protein. In previous studies, it was shown that when a strain containing the expression vector pJP101 (with the entire 2.65-kb fragment) was labeled with [^{35}S]methionine and the proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, a 61-kDa protein was detected (26). The discrepancy between the molecular mass of the translated protein obtained from the newly formulated extended reading frame (69 kDa) and the molecular mass of 61 kDa obtained by polyacrylamide gel electrophoresis suggested that the protein might be hydrophobic. It is well established that SDS-polyacrylamide gel electrophoresis of hydrophobic proteins results in underestimates of molecular weights compared with protein sizes derived from DNA sequencing (2, 38). Hydropathic analysis of the deduced protein sequence of the *menD* gene revealed that the protein is relatively hydrophobic (data not shown).

Weische et al. (35) studied the enzymes involved in the conversion of isochorismate and α-ketoglutarate to OSB. Those authors did not consider the involvement of SHCHC as an intermediate in the biosynthesis of OSB. They reported the presence of a complex capable of forming OSB from isochorismate and α-ketoglutarate in the presence of TPP. This complex necessarily contains SHCHC synthase and KDC (product of the *menD* gene) and OSB synthase (product of the *menC* gene) (23, 26, 27). Weische et al. were also able to separate a decarboxylating subunit from the complex which was capable of decarboxylating α-ketoglutarate (35).

The protein designated by them as the decarboxylase subunit presumably contains both SHCHC synthase and KDC activities (they assayed only decarboxylase activity). If this assumption is correct, then their reported molecular mass of 66.5 kDa obtained by gel filtration studies of the decarboxylase subunit alone is remarkably close to the 69 kDa obtained in this study for the translated protein of the *menD* gene.

On the bases of the sequence data and the deletion analyses, it is concluded that the product of a single gene (*menD*), encoding a single open reading frame, is necessary for both SHCHC synthase and KDC activities.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM42137-01 from the National Institutes of Health.

We thank W. Epstein and Janet L. Popp for some of the strains and plasmids used in this study.

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