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Menaquinone (Vitamin K_2) Biosynthesis: Overexpression, Purification, and Characterization of a New Isochorismate Synthase from *Escherichia coli*

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The first committed step in the biosynthesis of menaquinone (vitamin K_2) is the conversion of chorismate to isochorismate, which is mediated by an isochorismate synthase encoded by the *menF* gene. This isochorismate synthase (MenF) is distinct from the *entC*-encoded isochorismate synthase (EntC) involved in enterobactin biosynthesis. MenF has been overexpressed under the influence of the T7 promoter and purified to homogeneity. The purified protein was found to have a molecular mass of 98 kDa as determined by gel filtration column chromatography on Sephacryl S-200. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a molecular mass of 48 kDa. Thus, the enzyme is a homodimer. The purified enzyme showed a pH optimum of 7.5 to 8.0 and a temperature optimum of 37°C. The enzyme carries out the irreversible conversion of chorismate to isochorismate in the presence of Mg^{2+}. The enzyme was found to have a K_m of 195 ± 23 μM and a k_{cat} of 80 min^{-1}. In the presence of 30 mM β-mercaptoethanol (BME), the k_{cat} increased to 176 min^{-1}. The reducing agents BME and dithiothreitol stimulated the enzymatic activity more than twofold. Treatment of the enzyme with the cysteine-specific modifying reagent *N*-ethylmaleimide (NEM) resulted in the complete loss of activity. Preincubation of the enzyme with the substrate, chorismate, before NEM treatment resulted in complete protection of the enzyme from inactivation.

Menaquinone (vitamin K_2), or 2-methyl-3-prenyl-1,4-naphthoquinone, plays an essential role in several anaerobic electron transport systems in *Escherichia coli*. It is derived from the shikimate pathway from chorismate (1). Chorismate is a common precursor for the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan; folate coenzymes; the benzenoid and naphthalenoid coenzymes ubiquinone and menaquinone, respectively; and the catechol siderophore enterobactin (enterochelin) (1, 10, 14). The first committed step in the formation of both enterobactin and menaquinone is the formation of isochorismic acid {*trans*-5-[(1-carboxyethyl)oxy]-6-hydroxy-1,3-cyclohexadiene-1-carboxylic acid} (1, 10, 14). It is well established that the isochorismate synthase encoded by the *entC* gene is responsible for supplying the isochorismate required for enterobactin biosynthesis (11, 14). It was subsequently reported that the *entC*-encoded isochorismate synthase plays a dual role in supplying the isochorismate for both enterobactin and menaquinone biosynthesis (5). In 1993, on the basis of regulatory studies, evidence against the involvement of the *entC* gene in menaquinone biosynthesis was obtained (7). This information enabled us to search for and identify a new gene (*menF*) encoding an alternate isochorismate synthase (4). Recently, we reported the complete nucleotide sequence of the *menF* gene and showed that this gene encoded a new isochorismate synthase (3). The roles of EntC and MenF in the biosynthesis of enterobactin and menaquinone, respectively, are shown in Fig. 1.

In this paper, the purification and properties of the overexpressed *menF*-encoded isochorismate synthase are reported.

MATERIALS AND METHODS

Materials. Barium chorismate, DEAE–Sephadex A-50, reactive yellow 86-agarose, protamine sulfate, β-mercaptoethanol (BME), dithiothreitol (DTT), *N,N,N′,N′*-tetramethylethylenediamine, *N*-ethylmaleimide (NEM), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Mo.). High-pressure liquid chromatography (HPLC)-grade methanol, ethyl acetate, and acetonitrile were from Aldrich Chemical Co. (Milwaukee, Wis.). The low-molecular-weight protein standard was from Bio-Rad Laboratories (Hercules, Calif.). Centriprep and Centricon concentrators were products of Amicon (Beverly, Mass.). All other reagents used were of the highest purity commercially available.

Preparation of isochorismate. The incubation mixture consisted of 30 mg of barium chorismate, 1 mM $MgCl_2$, and the enzyme (40 mg of protein) from the DEAE–Sephadex A-50 column (see below). The total volume was made up to 20 ml with 200 mM Tris-HCl (pH 7.9) and incubated at 37°C for 1 h. The reaction was terminated by the addition of concentrated HCl until the pH reached 2.0. The precipitated protein was removed by centrifugation at 15,000 × *g* for 30 min. The supernatant was extracted with 40 ml of ice-cold ethyl acetate four times and dried in a rotary evaporator. The residue was dissolved in 100 μl of cold ethyl acetate, 20-μl aliquots were injected into a C_{18} Nucleosil HPLC column (150 by 4.6 mm) and eluted with an isocratic gradient of water-acetonitrile (95:5, vol/vol) containing 0.05% trifluoroacetic acid, and the column output was monitored at 278 nm. Fractions were assayed for isochorismate after conversion to salicylic acid (see below). Fractions containing isochorismate were pooled, acidified, and extracted with twice the volume of cold ethyl acetate four times. The ethyl acetate fractions were pooled and evaporated to dryness in a Savant AES 1000 Speed Vac and stored at −80°C.

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

Overexpression of the enzyme and preparation of cell extracts. For MenF, a 5-ml overnight LB culture of *E. coli* BL21(DE3) containing the plasmid pMKS33 (3) 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 absorbance at 600 nm reached 1.5. At this time, the T7 RNA polymerase was induced by the addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and after 30 min, rifampin was added at a concentration of 200 μg/ml and growth was continued for a further 4 h. The cells were harvested by centrifugation at 5,000 × *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 cell pellet was suspended in a buffer of the same composition containing 20 mM BME (buffer A) at a ratio of 1 g/1.5 ml and passed through a French pressure cell twice 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.

For the preparation of EntC-containing extracts, the *entC*$^+$ plasmid pITS557 (13) was transformed into the overexpression strain BL21(DE3), and the cells

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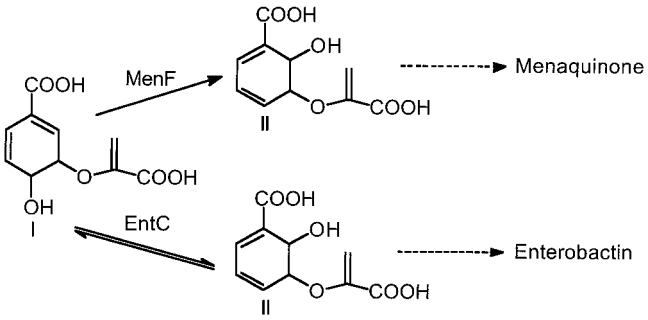

FIG. 1. Conversion of chorismate to isochorismate by the *entC*- and *menF*-encoded isochorismate synthases, leading to the ultimate formation of enterobactin and menaquinone, respectively. Only the pertinent reactions are shown. I, chorismate; II, isochorismate; MenF, isochorismate synthase encoded by the *menF* gene; EntC, isochorismate synthase encoded by the *entC* gene.

were grown and induced and cell extracts were prepared exactly as described above for MenF. The 30,000 × *g* supernatant was used without further purification.

Protamine sulfate precipitation. The protein content of the crude cell extract was adjusted to a concentration of 20 mg/ml. A 2% protamine sulfate solution in buffer A was added to the extract dropwise with constant stirring until the volume increased by 50%, and the stirring was continued for another 30 min. The entire operation was carried out in an ice bath. 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. An 18- by 0.8-cm column of DEAE–Sephadex A-50 was equilibrated with buffer A. The protamine sulfate supernatant (10.5 ml) was applied to the column and washed with 10 column volumes of buffer A until all the unbound protein was removed. The bound enzyme was eluted with 140 ml of a linear gradient of 0 to 600 mM KCl in buffer A, and the absorbance of the eluent was monitored at a wavelength of 280 nm. Fractions (1.8 ml) were collected, and the active fractions (no. 17 to 25) were pooled and concentrated with Centriprep C-30 concentrators.

Reactive yellow 86-agarose affinity chromatography. The concentrated protein from the DEAE–Sephadex A-50 column was applied to a 6- by 0.5-cm reactive yellow 86 column, equilibrated with buffer A, and allowed to bind for 30 min. The column was washed with 10 column volumes of buffer A to remove unbound protein. The enzyme was eluted with 140 ml of a linear gradient of 0 to 600 mM KCl in buffer A, and 1.5-ml fractions were collected. Fractions (no. 12 to 24) containing the enzyme activity were pooled and concentrated with Centriprep C-30 concentrators. DMSO was added to a final concentration of 20%, and the mixture was stored at −20°C until used.

Preparation of BME-free enzyme. The BME and DMSO in the enzyme were removed by using Centricon C-50 concentrators as recommended by the manufacturer, and the 20 mM Tris-HCl buffer containing BME was replaced four times with 20 mM Tris-HCl buffer (pH 7.2) to ensure that all traces of BME and DMSO were removed.

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

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the enzyme preparation was monitored on SDS–12% polyacrylamide gels as described by Laemmli (8). The mini-PROTEAN II cell apparatus from Bio-Rad Laboratories was used according to the instructions of the manufacturer. The following low-molecular-mass Bio-Rad protein standards were used: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

Native molecular mass determination. The native molecular mass of the enzyme was determined by Sephacryl S-200 gel filtration column chromatography as described previously (12a). A 1.5- by 100-cm column was equilibrated with 50 mM Tris-HCl buffer (pH 7.2) and calibrated by using the gel filtration kit from 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 isochorismate synthase was determined from the calibration curve.

Spectroscopic methods. The UV absorption spectrum was recorded with a Perkin-Elmer lambda 4A spectrophotometer at 25°C.

Assay of enzyme activity. (i) Assay by conversion to salicylic acid. The enzyme was routinely assayed by converting the isochorismate formed to salicylic acid as described before (12, 13) with modifications. The assay mixture contained 200 mM Tris-HCl (pH 7.9), 1.12 mM chorismate, 1 mM $MgCl_2$, and 1 to 2 μg of enzyme in a total volume of 1.0 ml. After the mixture was incubated at 37°C for 30 min, a 0.2-ml aliquot was withdrawn, added to 2.8 ml of 0.1 M sodium phosphate buffer (pH 7.2), heated at 100°C for 10 min, and cooled to room temperature. The fluorescence of the salicylate formed was measured (emission, 300 nm; excitation, 410 nm) on a Perkin-Elmer L3B fluorescence spectrophotometer. The salicylate measured represents 25% of the isochorismate formed (12). Specific activity is expressed as nanomoles of isochorismate formed $minute^{-1}$ milligram of $protein^{-1}$.

(ii) Assay by HPLC. (a) Forward direction. The concentrations of various components in the incubation mixture were identical to those described above for the salicylate assay except that the total volume was 0.5 ml. The reaction was carried out at 37°C for 60 min, the mixture was cooled on ice, and a drop of concentrated HCl was added. The cooled and acidified incubation mixture was extracted with 1.0 ml of ice-cold ethyl acetate four times and dried in a Savant AES 1000 Speed Vac. The residue was dissolved in ethyl acetate and analyzed by HPLC with a C_{18} reverse-phase column (150 by 4.6 mm) with an isocratic gradient of water and acetonitrile (95:5, vol/vol) containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The eluent was monitored with a spectrophotometric detector set at 278 nm. Authentic chorismate and isochorismate with retention times of 21.1 and 11.9 min, respectively, were used as standards.

(b) Reverse direction. The reaction mixture and the conditions of incubation were identical to those described above for the forward reaction except that the substrate, chorismate, was replaced by 500 μM isochorismate. The reaction was terminated and the product was extracted and analyzed as described above.

Determination of pH optimum. For the determination of the pH optimum, the isochorismate synthase assay was performed in the pH range of 6.5 to 9.0. For pH 6.5 to 8.0, 200 mM phosphate buffer was used, and for pH 7.0 to 9.0, 200 mM Tris-HCl buffer was used. Control assays without enzyme were performed at each pH.

RESULTS

Purification of isochorismate synthase. Cell extracts of the amplified *E. coli* strain produced 45 nmol of isochorismate min^{-1} mg of $protein^{-1}$ (Table 1). With cell extracts of this overexpression strain as the starting point, the enzyme was purified by various methods (Table 1). Treatment of the cell extract with protamine sulfate resulted in a twofold purification of the enzyme with a 96% recovery. The protamine sulfate supernatant was chromatographed on DEAE–Sephadex A-50 anion-exchange resin, resulting in an eightfold purification with a 76% yield. The eightfold-purified enzyme was chromatographed on a reactive yellow 86-agarose column, resulting in an 18-fold-purified homogeneous preparation with a yield of 28%. The purity of the enzyme during various stages of purification was monitored by SDS-PAGE (Fig. 2).

The purified enzyme was found to be unstable and lost approximately 70% of the activity in 24 h when stored at 4°C, while at −20°C, 40% of the activity was lost in 5 days. Attempts were made to stabilize the enzyme by the addition of 1% bovine serum albumin, 10% glycerol, or 20% DMSO. DMSO was found to stabilize the enzyme for up to 10 days at −20°C. Hence, the purified enzyme was stored under these conditions and used for all of the studies reported in this paper.

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 98 kDa (data not shown). The subunit molecular mass as determined by SDS-PAGE was found to be 48 kDa (Fig. 2).

pH and temperature optima. The pH optimum for the enzyme was determined by using the range of pH 6.5 to 9.0. The enzyme exhibited optimum activity between pH 7.5 and 8.0.

TABLE 1. Purification of isochorismate synthase of *E. coli*

Purification step	Total protein (mg)	Sp act (nmol min^{-1} mg of $protein^{-1}$)	Total activity (nmol min^{-1})	Yield (%)	Purification (fold)
Crude extract	140	45	6,316	100	1
Protamine sulfate	64	95	6,050	96	2
DEAE–Sephadex A-50	14	347	4,850	76	8
Reactive yellow 86-agarose	2.2	808	1,766	28	18

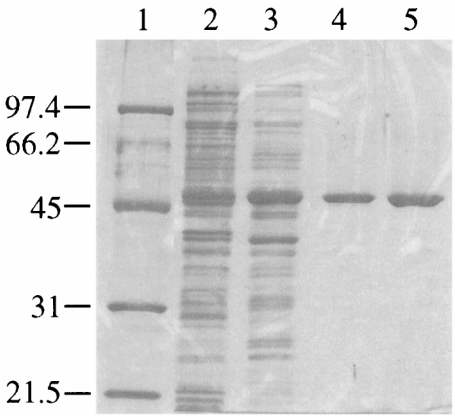

FIG. 2. SDS-PAGE analysis of protein fractions from various stages of purification. Proteins were separated on an SDS–12% polyacrylamide gel and stained with Coomassie blue. Lanes (numbers in parentheses indicate the amount of protein loaded): 1, low-molecular-weight markers from Bio-Rad Laboratories; 2, crude extract (10 μg); 3, protamine sulfate fraction (6 μg); 4, DEAE–Sephadex A-50 fraction (5 μg); 5, reactive yellow 86-agarose fraction (5 μg). Numbers on the left are molecular weights in thousands.

The enzyme activity decreased sharply at pH 6.5 or 9.0, to about 20% of that observed at the optimum pH. There was no difference in the activity whether Tris-HCl or phosphate buffer was used (data not shown).

The optimum temperature for the reaction was determined by incubating the assay mixture at various temperatures ranging from 15 to 60°C. The enzyme showed maximum activity at 37°C, while at 60°C the activity was completely lost. At 30 and 40°C, the enzyme showed about 80% of the activity at 37°C, and at 15 and 20°C, the activities were 72 and 74%, respectively, of the activity at 37°C. At 50°C, the enzyme retained only 42% of the activity (data not shown).

Kinetic constants of the enzyme. The isochorismate synthase activity increased with increasing concentration of the substrate, chorismate, in a typical hyperbolic fashion. The corresponding Hanes plot showed an apparent K_m of 195 ± 23 μM. The enzyme had k_{cat} values of 80 min^{-1} in the absence of BME and 176 min^{-1} in the presence of 30 mM BME.

Absorption spectrum of the enzyme. The absorption spectrum of the purified enzyme was recorded for 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 enzyme showed no detectable activity in the absence of metal ions. Addition of Mg^{2+} resulted in a sharp increase in enzymatic activity. Hence, the optimum concentration of Mg^{2+} required for enzyme activity was determined. The enzyme showed no detectable activity from 1 to 20 μM Mg^{2+}. However, when the Mg^{2+} concentration was increased further, the enzyme showed a dramatic increase in activity from 100 μM, reaching a maximum at 1 mM. At concentrations above 1 mM, the enzyme activity was inhibited.

The effects of Mn^{2+} and Co^{2+} were tested at concentrations of 0.5, 1.0, and 5 mM. In the presence 0.5 mM Mn^{2+}, the enzyme showed only 20% of the activity observed at the optimum concentration (1 mM) of Mg^{2+}, while at 1 and 5 mM, the activity was 17 and 7%, respectively (data not shown). Compared to the activity with Mg^{2+}, the presence of Co^{2+} led to 24, 20, and 9% of the activity at 1.0, 0.5, and 5 mM concentrations, respectively. In the presence of other metal ions, i.e., Ca^{2+}, Fe^{2+}, Zn^{2+}, and Na^{+}, at 1 mM, the enzyme showed only 4 to 5% of the activity with 1 mM Mg^{2+}. No enzymatic activity was observed in the presence of K^{+}, Cu^{2+}, or Hg^{2+} (data not shown).

Effect of reducing agents. After removal of the reducing agent BME from the purified enzyme (see Materials and Methods), the effects of BME and DTT on the enzymatic activity were investigated. In the presence of DTT or BME at 1 mM, the enzymatic activity was stimulated approximately twofold. Hence, the effects of various concentrations of these two reducing agents on the enzymatic activity were tested (Fig. 3). Increasing concentrations of BME resulted in increasing stimulation of enzymatic activity, reaching a maximum of 2.25-fold at 20 to 30 mM. In contrast, DTT caused a twofold stimulation at 1 mM; further increases resulted in progressive inhibition of enzymatic activity. However, even at the inhibitory concentration of 30 mM, the enzymatic activity was 1.5-fold higher than that of the control.

Effect of NEM. The stimulation of enzymatic activity by BME and DTT strongly suggested that this stimulation might be due to protection of sulfhydryl groups. Hence, the effect of the cysteine-specific modifying agent NEM on the enzyme was investigated (Fig. 4). The enzyme was inactivated in a concentration-dependent manner. At a concentration of 100 μM, the enzyme was completely inactivated. Lower concentrations of 1 and 10 μM resulted in 25 and 50% inactivation, respectively, compared to the control values. When the enzyme was preincubated with the substrate, chorismate, before treatment with NEM, the inactivation was completely prevented. Even high concentrations of NEM (100 μM) failed to inactivate the substrate-protected enzyme.

Reversibility of the reaction. To determine the reversibility of the reaction, the enzyme assays were carried out in parallel under identical conditions. For the forward reaction, chorismate was used as the substrate, and for the reverse reaction, isochorismate replaced the chorismate in the assay mixture. The product(s) of the reaction was extracted and analyzed by HPLC. Chorismate and isochorismate eluted from the column well separated, with retention times of 21.1 and 11.9 min, respectively (Fig. 5). When chorismate was the substrate, isochorismate was the sole product (Fig. 5B). In the absence of

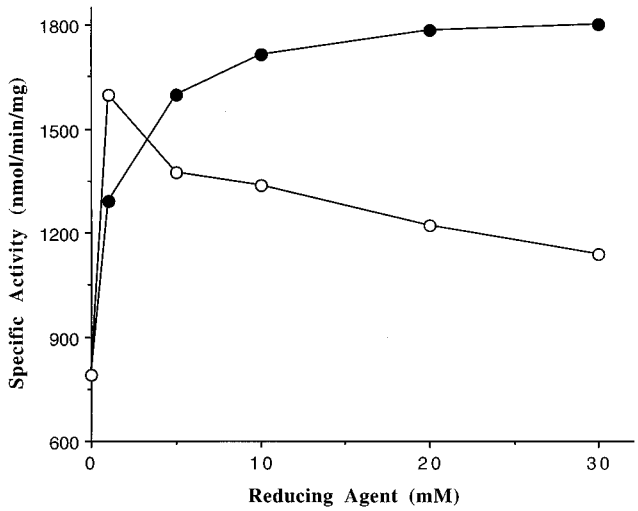

FIG. 3. Effect of reducing agents on the enzymatic activity. To an incubation mixture containing enzyme, $MgCl_2$, and buffer, various concentrations of either DTT (open circles) or BME (closed circles) were added, and the mixture was incubated at room temperature for 5 min. The reaction was initiated by the addition of chorismate, the incubation was continued at 37°C for 30 min, and the isochorismate formed was determined.

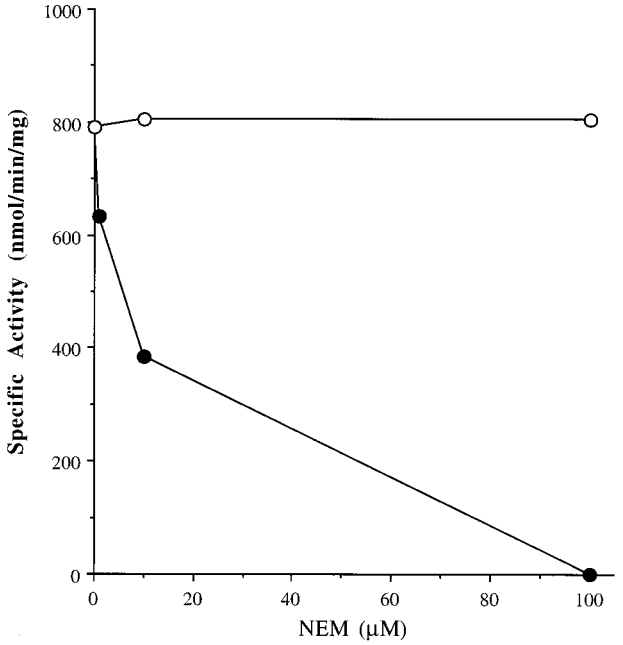

FIG. 4. Inactivation of isochorismate synthase by NEM and protection by the substrate, chorismate. (Closed circles) To the enzyme in 20 μl of 20 mM Tris-HCl buffer (pH 7.2), NEM (dissolved in water) was added at the indicated concentrations, and the mixture was incubated for 30 min at 37°C. To this mixture, 200 mM Tris-HCl buffer (pH 7.9), 1 mM $MgCl_2$, and 1.12 mM chorismate were added. The volume was made up to 1.0 ml, incubated for 30 min at 37°C, and assayed for isochorismate. (Open circles) To the enzyme in 20 μl of 20 mM Tris-HCl buffer (pH 7.2), 0.6 mM chorismate was added, and the mixture was incubated for 30 min at 37°C. NEM was then added at the indicated concentrations, and incubation was continued for 30 min. Subsequently, 200 mM Tris-HCl buffer (pH 7.9), 1 mM $MgCl_2$, and 0.6 mM chorismate were added. The volume was made up to 1.0 ml, incubated for 30 min at 37°C, and assayed for isochorismate.

enzyme, in the control tube, isochorismate was not formed (Fig. 5A).

When the reverse reaction was performed with isochorismate as the substrate, chorismate was not detected. However, an unidentified peak was present (Fig. 5D). In a control tube containing isochorismate without the enzyme under identical conditions, only the unidentified peak was formed (Fig. 5C). As a positive control for the reverse reaction, an extract of EntC (which is known to carry out the reversible reaction) was incubated with isochorismate as the substrate. In this case, chorismate was formed as the product (Fig. 5E). When chorismate was the substrate, as expected, isochorismate was formed (Fig. 5F).

DISCUSSION

The pathway for the biosynthesis of menaquinone involves six identified genes designated *menA*, *-B*, *-C*, *-D*, *-E*, and *-F* (10). The various enzymes involved in the menaquinone biosynthetic pathway are present at extremely low levels, and among these, the *menF*-encoded isochorismate synthase is the least active. In the *entC* mutant (which lacks the EntC isochorismate synthase), the MenF activity was reported to be 0.6 nmol h^{-1} mg of $protein^{-1}$ (6). The strain in which the protein was overexpressed produced 45 nmol of isochorismate min^{-1} mg of $protein^{-1}$, which is about 4,500-fold higher than that produced by the wild-type strain. The enzyme was purified 18-fold, resulting in a protein with a specific activity of 808 nmol min^{-1} mg of $protein^{-1}$.

The two isochorismate synthases, EntC and MenF, present in *E. coli* catalyze identical reactions (Fig. 1). Hence, it is important to compare the properties of the two proteins. The deduced molecular mass of EntC was reported to be 42.9 kDa, which is in agreement with a molecular mass of 43 kDa obtained by SDS-PAGE and a native molecular mass of 42 kDa obtained by gel filtration, establishing that the enzyme is a monomer (9, 14). In contrast, the purified MenF had a molecular mass of 48 kDa by SDS-PAGE and a calculated molecular mass of 48.777 kDa. Since the native molecular mass as determined by gel filtration was 98 kDa, the enzyme is a homodimer.

MenF was found to have a K_m for chorismate of 195 ± 23 μM with k_{cat} values of 80 min^{-1} in the absence of BME and 176 min^{-1} in the presence of 30 mM BME, while EntC had a K_m of 14 μM and a k_{cat} of 173 min^{-1} for the forward reaction. EntC carries out a reversible reaction with an equilibrium constant of 0.56, with the equilibrium lying towards chorismate (9).

In contrast, the MenF-catalyzed reaction was found to be not reversible. Since during the assay of the enzyme in the reverse direction with isochorismate as the substrate, the unidentified peak is observed, it could be argued that this peak inhibits the reaction.

This unidentified peak appeared whenever isochorismate was incubated in the buffer, whether the enzyme was present or not. However, if the isochorismate was injected into the HPLC column without incubation, the unidentified peak did not appear. These observations established that this peak is a nonenzymatic degradation product of isochorismate.

It is well established that isochorismate is highly unstable, and it decomposes at room temperature and neutral pH to salicylate and 3-(carboxyphenyl)pyruvate. It was further reported, citing a personal communication from G. A. Berchtold, that the major breakdown route in water at 30°C as determined by nuclear magnetic resonance was via isoprephenate, and this rearrangement was reported to be three times faster than the rearrangement of chorismate to prephenate (14). We have determined in this study that the unidentified peak is not that of salicylate, and hence it may be the unstable isoprephenate or the commercially unavailable 3-(carboxyphenyl)pyruvate.

To circumvent the difficulties enumerated above, we determined the reaction with EntC, which carries out an identical but reversible reaction (9). As seen from Fig. 5E, the EntC reaction was reversible in spite of the presence of the unidentified peak. Hence, it can be concluded that the irreversibility of MenF is not due to inhibition by the unidentified peak.

The stimulatory effect of the reducing agents BME and DTT on the activity of the enzyme suggests the involvement of sulfhydryl groups. The inactivation of the enzyme by NEM and the protection from inactivation by the substrate strongly suggest that a cysteine residue(s) in or near the substrate binding site is critical for activity.

The effect of reducing agents on the purified EntC protein has not been reported (9). However, when BME or DTT was added to the incubation mixtures containing cell extracts of EntC, the enzyme activity was stimulated about 25%. If purified EntC is used, greater stimulation might be obtained.

The chemical structure of chorismate differs from that of isochorismate in the locations of the hydroxyl group and the double bonds (Fig. 1). In chorismate, the hydroxyl group is located at C-4, while in isochorismate, it is located at C-2. Three possible mechanisms can be envisioned for the origin of the hydroxyl group in isochorismate: (i) intramolecular transfer of the hydroxyl from chorismate, (ii) incorporation of molecular oxygen, or (iii) transfer from the solvent water. The first mechanism, involving intramolecular transfer, has been considered

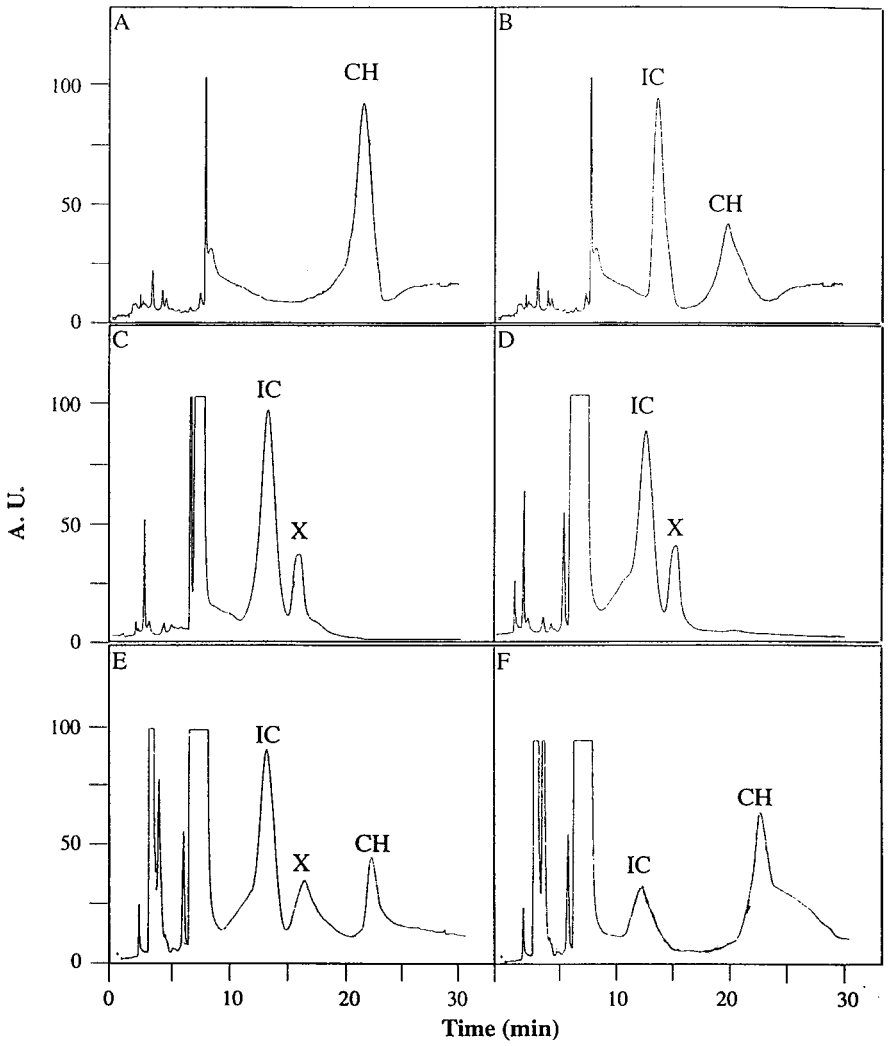

FIG. 5. Determination of the reversibility of the reaction. For the forward reaction, the standard reaction mixture with chorismate was used, and for the reverse reaction, the chorismate in the reaction mixture was replaced by isochorismate. After incubation, the product(s) formed was determined as described in Materials and Methods. (A) Chorismate without enzyme; (B) complete reaction mixture with MenF and chorismate; (C) isochorismate without enzyme; (D) complete reaction mixture with MenF and isochorismate; (E) complete reaction mixture with EntC and isochorismate; (F) complete reaction mixture with EntC and chorismate. CH, chorismate; IC, isochorismate; X, unidentified peak. A. U., arbitrary units.

unlikely (9). The second mechanism, involving molecular oxygen, can be ruled out, since menaquinone has to be synthesized under anaerobic conditions where oxygen is unavailable. The third mechanism, involving solvent water, is the most likely, since it can function under both aerobic and anaerobic conditions.

It has been shown that EntC catalyzes the 1,5 double-S_N2' displacement of the 4-hydroxyl group of chorismate with water, resulting in the formation of isochorismate (9, 14). Consistent with this observation is the recent demonstration that enterobactin is synthesized both aerobically and anaerobically (6). Since menaquinone is synthesized both aerobically and anaerobically (at a higher level) and functions anaerobically, the reaction carried out by MenF will be similar in the utilization of water as the source of the hydroxyl group.

Support for this argument comes from the fact that the enzyme-catalyzed S_N2' reactions have an absolute requirement for a divalent metal ion, with the most probable natural candidate being Mg^{2+}. Four different mechanisms have been proposed for the S_N2' reaction carried out by EntC. In all the four proposed mechanisms, Mg^{2+} plays a critical role (14). Consistent with this is the fact that MenF shows an absolute requirement for Mg^{2+} similar to that of EntC.

Work is in progress to establish the origin of the hydroxyl group in isochorismate and the mechanism of the reaction.

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