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PROPERTIES OF ISOCHORISMATE HYDROXYMUTASE FROM FLAVOBACTERIUM $K_{3\text{-}15}$[1]

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ABSTRACT.—Isochorismate hydroxymutase (isochorismate synthase, E.C. 5.4.99.6) catalyzes the interconversion of chorismic acid [**1**] and isochorismic acid [**2**]. The enzyme was extracted from a *Flavobacterium* $K_{3\text{-}15}$ that overproduces vitamin K_2 (i.e., menaquinones) and was purified to homogeneity. The *N*-terminal amino acid sequence and the mol wt (36,240±100 daltons) were determined by ms following SDS PAG electrophoresis. The enzyme was characterized (stability, cofactor requirement, isoelectrical point), and antibodies were raised which showed no cross reactivity with isochorismate hydroxymutase from *Escherichia coli* and *Enterobacter aerogenes* 62–1. The kinetic data of the enzyme are different from those observed for the corresponding enzyme from *Escherichia coli* and *Galium mollugo*.

Isochorismate hydroxymutase catalyzes the reversible isomerization of chorismic acid [**1**] to isochorismic acid [**2**] (Scheme 1). Isochorismic acid is a link between the shikimate pathway and certain primary and secondary metabolites (1–3). Enzyme studies have shown that isochorismic acid is a substrate for the committed step in menaquinone (vitamin K_2) (4,5), phylloquinone (vitamin K_1), anthraquinone (6), and enterobactin (7–11) biosynthesis.

The enzyme has been detected in protein preparations from *Escherichia coli* (8,10), *Enterobacter aerogenes* 62–1 (12,13), *Mycobacterium smegmatis* (14), *Streptomyces aminophilus* (15), and cell suspension cultures derived from plants belonging to the families Rubiaceae, Celastraceae, and Apocynaceae (16–18).

The enzyme from *Es. coli* has been overexpressed, purified and characterized (8). In *Es. coli*, isochorismic acid has a dual function as a precursor of both enterobactin and menaquinones. Both metabolites are essential under different physiological conditions, namely iron deficiency (enterobactin) and anoxia (menaquinones). For this reason the regulation of isochorismate hydroxymutase activity is particularly interesting. The present paper describes the isolation and characterization of isochorismate hydroxymutase from *Flavobacterium* $K_{3\text{-}15}$. This organism is an overproducer of menaquinones (19) but, unlike *Es. coli*, does not grow under anaerobic conditions. Moreover, a catechol siderophore such as enterobactin does not seem to be produced by this organism because,

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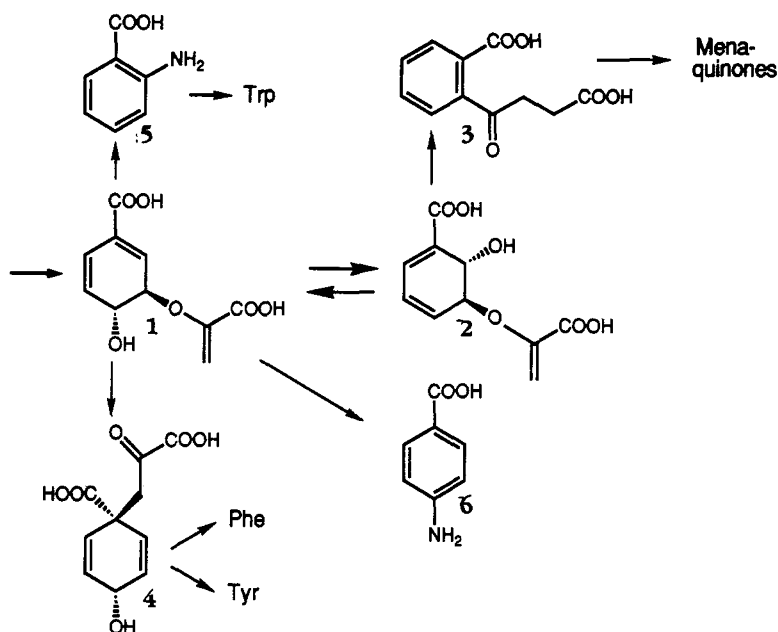

SCHEME 1. Function of isochorismic acid [**2**], chorismic acid [**1**], *o*-succinylbenzoic acid [**3**], prephenic acid [**4**], anthranilic acid [**5**], *p*-aminobenzoic acid [**6**]. Trp=tryptophan, Tyr=tyrosine, Phe=phenylalanine.

as opposed to *Es. coli*, no formation in cell-free extracts of 2,3-dihydroxybenzoic acid, a precursor of enterobactin, could be observed (P.M. Schaaf, unpublished observation). Although both *Es. coli* and *Flavobacterium* $K_{3\text{-}15}$ are Gram-negative bacteria, their GC content differs significantly [33% in the case of *Flavobacterium* (20) but 50% in the case of *Es. coli* (21)]. These observations stimulated our interest in the isochorismate hydroxymutase occurring in *Flavobacterium*. The data reported here include the characterization of the enzyme. The properties of the *Flavobacterium* enzyme are compared with those of the enzyme from *Es. coli*.

RESULTS

The product of the enzymic isomerization of chorismic acid was identified as described (16). The reaction is reversible and yields chorismic acid when isochorismic acid is the starting material. The equilibrium favors chorismic acid (8). Table 1 shows that the activity of the enzyme differs significantly in crude protein extracts from various bacteria. A time course study with *Flavobacterium* $K_{3\text{-}15}$ is shown in Figure 1. As expected, isochorismate hydroxymutase activity parallels growth and both growth and enzyme activity precede menaquinone formation (Figure 1). The mixture of menaquinones formed consists of 17% MK_4 (menaquinone with 4 isoprene units) and 83% MK_6 (menaquinone with 6 isoprene units).

The isochorismate hydroxymutase was purified by following the steps listed in Table 2. Unlike the enzyme from *Es. coli* (8), the *Flavobacterium* enzyme precipitates in a relatively narrow concentration range (45 to 70%) during ammonium sulfate fractionation. Before proceeding with the purification process, the isoelectric point was determined and found to have a very sharp maximum at pH 5.0 to 5.1. This shows that negative charges prevail on the enzyme surface. Anion exchange chromatography (DEAE Sephacel; compare Table 2) was therefore chosen as the next step (Figure 2A). The active fractions were collected and their stability tested (Figure 3). The protein was kept

TABLE 1. Comparison of Isochorismate Hydroxymutase Activity in Crude Protein Extracts from Different Bacterial Strains.

Strain	Activity of isochorismate hydroxymutase [pmol/sec/mg protein]
Escherichia coli AN 191	0.45
Escherichia coli AN 92	0.75
Flavobacterium 238–7	9.5
Flavobacterium $K_{3\text{-}15}$	12.9
Enterobacter aerogenes 62–1	129.0

in the presence of different salts, buffers, and dithiothreitol. Only the latter stabilized (and even enhanced) enzyme activity significantly. Ammonium sulfate destabilized the enzyme.

Subsequent purification steps, among which Phenylsepharose and preparative PAG electrophoresis (step 7 in Table 2) were most successful, are listed in Table 2 and are shown in Figure 2. The last purification step (SDS PAG electrophoresis) gave a protein fraction which was submitted to matrix-assisted uv-laser desorption ionization ms, which revealed the presence of two proteins with a mol wt of 36,240±100 daltons and 32,770± 100 daltons. The mass spectrum (Figure 4) also showed mass peaks corresponding to doubly charged protein molecules at half the mass of the two parent peaks. The two proteins were separable by analytical native PAGE and SDS PAG electrophoresis. Fractionation of the gel by cutting, after analytical native PAGE followed by elution of the enzyme from gel slices, showed that enzyme activity was associated with the higher

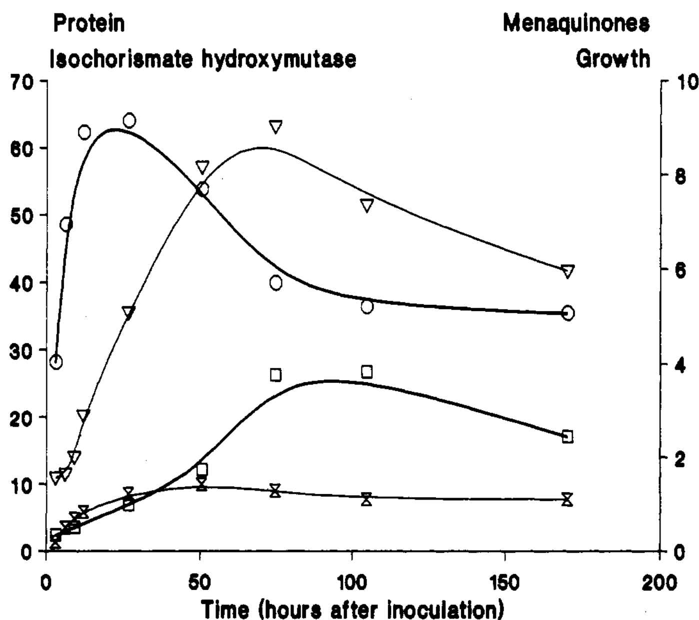

FIGURE 1. Time course study of growth (-X-, optical density of bacterial suspension at 610 nm after a 1:10 dilution with H_2O), protein extracted from cells (-□-, mg per gram wet wt), amount of menaquinones (-▽-, mg in 1 liter medium), and activity (-O-, pmol isochorismate formed per sec per mg protein) of isochorismate hydroxymutase.

TABLE 2. Purification of Isochorismate Hydroxymutase from *Flavobacterium* $K_{3\text{-}15}$.

Purification Step	Total Protein		Specific Activity (pkat/ mg protein)	Total Activity		Purification Factor
	[mg]	[%]		(pkat)	[%]	
1. Crude extract	3159	100	14	44221	100	1
2. Protamine sulfate ppt.	1680	53	35	58811	133	2.4
3. Ammonium sulfate ppt. [45–70%]	655	21	48	31385	71	3.4
4. DEAE-Sephacel	230.4	7.3	100	23035	52	7.1
5. Sephadex G-150	99.3	3.1	184	18278	41	13.2
6. Phenylsepharose	22.2	0.7	1844	41026	93	135
7. Native PAGE (10)%)	2.7	0.086	10500	28301	64	750
8. SDS-PAGE (7–25%)		not determined				

mol wt protein (36,240±100 daltons). SDS gel electrophoresis of this protein fraction indicated a mol wt of 37,000 daltons, in agreement with the mass spectral data.

The mol wt of the isochorismate hydroxymutase, determined by gel permeation chromatography on Sephadex G-150, was 45,000±4,000 daltons, however. This may indicate that the latter method is less reliable because it is based on the asumption that the enzyme and the reference proteins under investigation are ball-shaped and uncharged (22). We cannot exclude the possibility, however, that the genuine isochorismate hydroxymutase has a mol wt around 45,000 daltons and that loss of a peptide residue accounts for the difference in mol wt between 45,000 and 37,000. This would explain why we did not observe a terminal methionine residue when the N terminus of the 36,240 dalton protein was sequenced (Table 3). The 32,770±100 daltons protein in turn may be a degradation product of the 36,240±100 daltons protein for the following reasons.

Polyclonal antibodies raised against both proteins (obtained after SDS PAGE) showed reactivity and crossreactivity with both the 32,770 as well as the 36,240 dalton proteins from *Flavobacterium* $K_{3\text{-}15}$ and from *Flavobacterium* 238–7, in a Western blot. The low mol wt protein (32,770±100) is thus immunologically at least very similar to the 36,240 dalton protein and may have been generated from the 36,240 dalton protein during the purification procedures. This final degradation must have resulted in loss of catalytic activity.

We conclude that the isochorismate hydroxymutase is a labile protein.

Attempts to use the antibodies to test the immunological properties of the isochorismate hydroxymutase from different bacteria (*Es. coli* and *En. aerogenes* 62–1) gave negative results. No reactivity was observed. When pre-immune and immune sera were used, no difference was detectable in Western blots, indicating that the isochorismate hydroxymutases from different sources are immunologically distinct enzymes.

DISCUSSION

Isochorismic acid is an important metabolite which is a precursor of siderophores, menaquinones, phylloquinone, and a series of secondary natural products (1–6). The wide distribution of these metabolites in bacteria and plants reflects the importance of isochorismic acid and raises the question of the regulation of its synthesis in a highly branched pathway (Scheme 1). The enzymes involved in these reactions (Scheme 1), including the isochorismate hydroxymutase, compete for chorismic acid. The lowest K_M

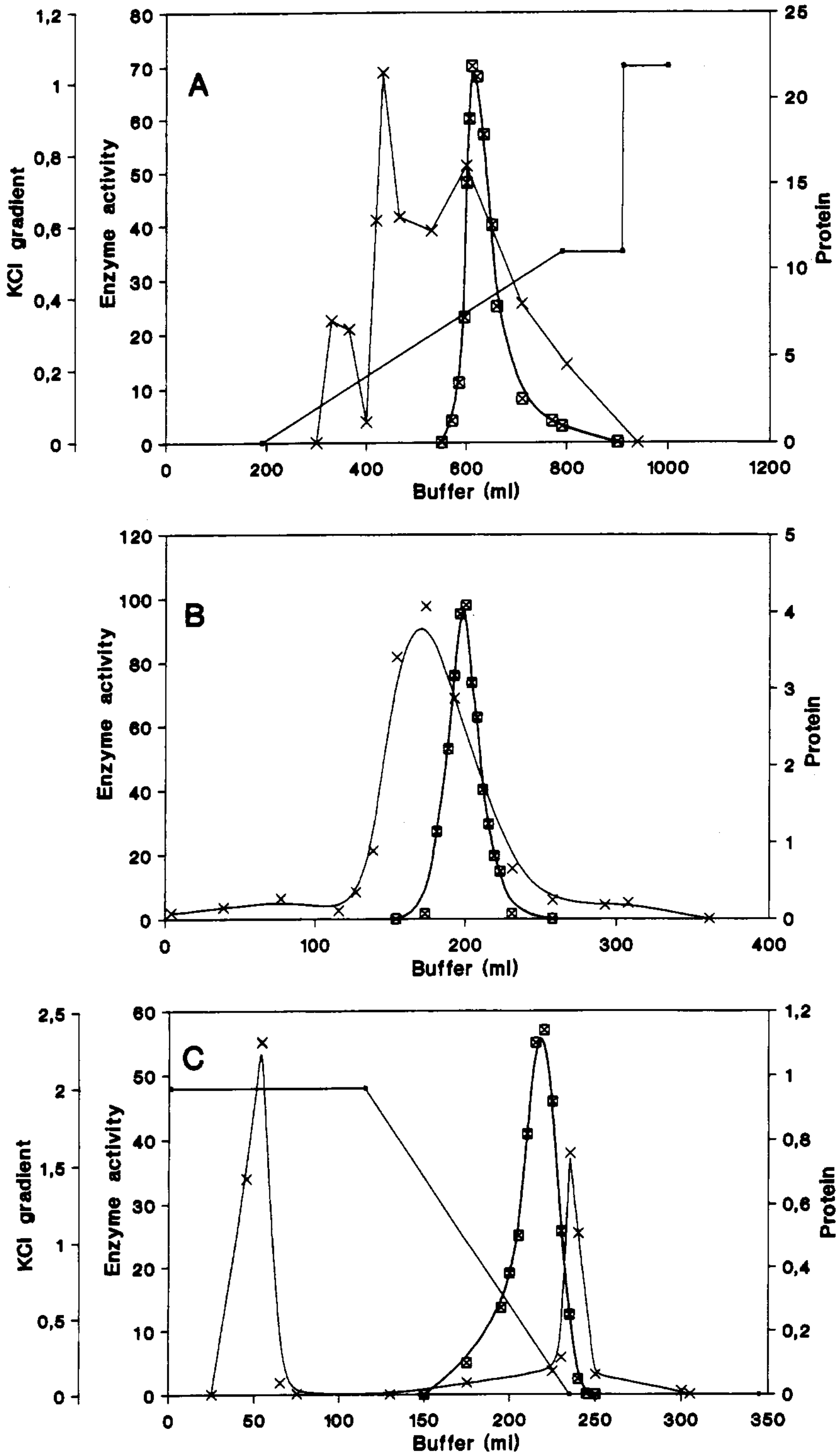

FIGURE 2. Column chromatographic steps taken to purify isochorismate hydroxymutase. A, DEAE Sephacel; B, Sephadex G-150; C, Phenylsepharose CL-4B. Protein (-X-, mg/ml), enzyme activity (-⊠-, pkat/ml), KCl gradient (---, M).

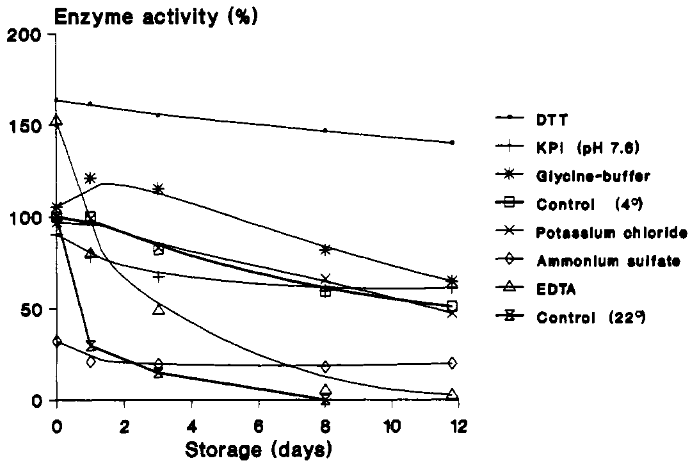

FIGURE 3. Stability of isochorismate hydroxymutase in the presence of different buffers and protecting agents. For details see Experimental.

value of a chorismate utilizing enzyme in *Es. coli* was reported for anthranilate synthase (E.C. 4.1.3.27, 1.2 μM) (23), while isochorismate hydroxymutase has a K_M value of 14 μM (8) and *p*-aminobenzoate synthase a K_M value of 23 μM [determined for *En. aerogenes* 62–1 (15)]. The lowest affinity for their substrates was found for chorismate mutase/ prephenate dehydratase (E.C. 5.4.99.5, E.C. 4.2.1.51, 45 μM) and chorismate mutase/ prephenate dehydrogenase (E.C. 5.4.99.5, E.C. 1.3.11.12, 92 μM) (23).

In spite of the rather low K_M value, drainage of substrate flow into the isochorismate utilizing reactions is prevented by a tight transcriptional control of *ent C* (24), the gene encoding isochorismate hydroxymutase in *Es. coli*. Transcription is influenced by both oxygen and iron supply (25). The situation is completely different in *Flavobacterium*, since, unlike *Es. coli*, this organism is unable to grow anaerobically and does not seem to

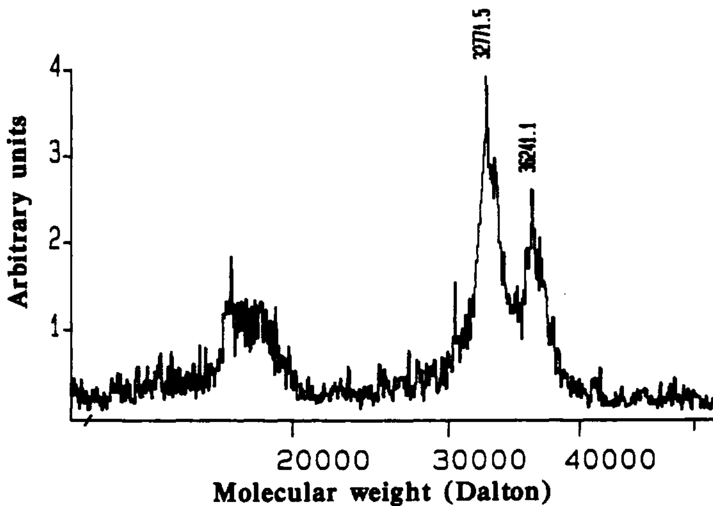

FIGURE 4. Mol wt determination of isochorismate hydroxymutase by matrix-assisted uv-laser desorption ionization ms of protein after step 8 of the purification protocol (compare Table 2).

produce a catechol sideophore from isochorismic acid. The regulation of isochorismate synthesis is therefore likely to be completely different. Indeed, the relatively high K_M value of isochorismate hydroxymutase for chorismic acid (Table 3) may prevent drainage of substrates into isochorismate-utilizing reactions (Scheme 1). A similar observation was made for an anthraquinone-producing cell suspension culture of *Galium mollugo* (16). This may indicate that isochorismate synthesis is controlled not only at the level of transcription, as is the case in *Es. coli* but also at the enzymic level.

TABLE 3. Properties of Isochorismate Hydroxymutase from *Flavobacterium* $K_{3\text{-}15}$. Comparison with the Enzyme from *Escherichia coli*.

	Strain	
	Flavobacterium $K_{3\text{-}15}$	*Escherichia coli*
Ammonium sulfate Precipitation	(at 45 to 70% saturation)	(at 0–80% saturation[a])
K_M (Chorismate)	350 µM	14 µM[a]
K_M (Isochorismate)	254 µM	5 µM[a]
Mol wt (daltons)	45,000±4,000 (Gel permeation on Sephadex G-150) 37,000 (SDS PAGE) 36,240±100 (mass spectrum)	43,000 (Gel permeation on Sephadex G-200[a] 44,000 (Gel permeation on Sephadex G-200)[b] 42,000 (SDS PAGE)[a] 42,917 (Gene sequencing)[c]
Amino acid sequence at the N-terminus	(K)-F-Q-N-A-L-E-(T)-I-G-N-T-P	M-D-T-S-L-A-[a] E-E-V-Q-Q-T[a]
Immunological properties	Polyclonal antibodies raised against the 36,240±100 dalton protein (after SDS PAGE) do not react (Western blot) with the isochorismate hydroxymutase from *Escherichia coli* or *Enterobacter aerogenes* 62–1.	

[a]From Liu *et al.* (8).
[b]From Tummuru *et al.* (10) and Ozenberger *et al.* (35).
[c]From Ozenberger *et al.* (9).
[d]This study.

EXPERIMENTAL

CHEMICALS.—Barium chorismate, dithiothreitol, glutardialdehyde, CNBr-activated Sepharose 4B, aminohexyl-Sepharose 4B, alkylamine-glass, protamine sulfate, and standard proteins for determination of the native mol wt were purchased from Sigma (Munich, FRG). The standards for mol wt determination in SDS-PAGE were from Boehringer (Mannheim, FRG). PD 10-columns, DEAE-Sephacel, Sephadex G-150, Phenylsepharose Cl-4B, and Ampholine pH 4–6 were obtained from Pharmacia (Freiburg, FRG). Polypepton was supplied by Daigo (Wako Pure Chemical Industries, LTD, Osaka, Japan) and yeast extract by Difco (Detroit). [U-^{14}C]-α-Ketoglutarate came from Amersham Buchler (Braunschweig, FRG). All other chemicals and solvents were analytical grade reagents. Menaquinone 4 (MK 4) and 6 (MK 6) were a gift of Hoffmann-LaRoche, Basle, Switzerland.

GROWTH OF *ES. COLI* AND *EN. AEROGENES*.—Growth was as previously described (26–29) (Table 4).

GROWTH OF *FLAVOBACTERIUM* $K_{3\text{-}15}$ AND 238–7.—Bacteria were grown in a medium containing glycerol (5%), polypeptone (2%), yeast extract (0.3%), K_2HPO_4 (0.6%), NaCl (0.4%), and $MgSO_4\cdot 7\ H_2O$ (0.02%) in tap H_2O. The pH was adjusted to 7.0. The strain was grown overnight in a gyratory shaker at 28° and 150 rpm in an Erlenmeyer flask (300 ml) containing 100 ml medium. The seed culture was used to inoculate the main culture (1 liter medium) in a penicillin flask. Incubations were carried out for 24 h at 28° on a reciprocal shaker (70 strokes per min). The cells were collected by centrifugation (4000×*g*, 30 min), washed with glycine/NaOH buffer (0.02 M, pH 7.6) containing dithiothreitol (1 mM) (glycine-DTT buffer) and centrifuged again at 27,500×*g* for 30 min. (See Table 4.)

TABLE 4. Bacterial Strains.

Bacterium	Genotype	Growth	Reference (Source)
Escherichia coli AN 191	thi, leuB, proC, lacY, mtL, xyL, rosl, azi, fhuA, tsx, supA, entD, entA	26	27
Escherichia coli AN 92	proA, argE, pheA, tyrA4, trp-401, aroB	26	27
Flavobacterium 238–7	wildtype	this study	19
Flavobacterium $K_{3\text{-}15}$	Menaquinone-overproducer	this study	19
Enterobacter aerogenes 62–1	blocked in Phe^-, Trp^-, Tyr^- biosynthesis	29	28

ENZYME ISOLATION AND PURIFICATION.—All operations were carried out at 4°. Cells were suspended in a 2.5-fold volume of glycine-DTT buffer (1 mM). The suspension was homogenized by sonication (Branson sonifier) and centrifuged (47,000×*g*, 30 min). A solution of protamine sulfate in H_2O (2%, pH 7.0) was added to the crude protein extract to give a final concentration of 0.3 mg protamine sulfate per mg of enzyme. After centrifugation (27,000×*g*, 30 min) the supernatant was subjected to ammonium sulfate precipitation.

The fraction precipitating between 45 and 70% saturation was collected, dissolved in glycine-DTT buffer, and desalted on a Sephadex G-25 (PD 10) column which had been equilibrated with glycine-DTT buffer.

The enzyme solution was chromatographed on a DEAE-Sephacel column (2.5×40 cm; gradient 0–0.6 M KCl in glycine-DTT buffer). Active fractions were combined, concentrated, and chromatographed on a Sephadex G-150 column (2.5×55 cm) with glycine-DTT buffer as eluent. Active fractions were combined. KCl then was added, to a concentration of 2M. This solution was applied to a Phenylsepharose Cl-4B column (2.5×8 cm) and eluted with glycine-DTT buffer, containing a gradient of KCl (2 M to 0 M). The active fractions were combined, concentrated, and desalted.

The enzyme was subjected to polyacrylamide gel electrophoresis (10%, 100 μg of protein per slot, thickness of gel 1.5 mm; distance 10 cm; 20 mA DC). The gel was cut horizontally into slices (2 mm) and the slices kept in glycine-DTT buffer for 24 h. Enzyme activity was determined in the eluent. Active fractions were separated electrophoretically on SDS PAGE gradient gel (7 to 25%) (30). After staining with Coomassie Blue (31) and electroelution, a homogeneous protein was obtained. The protein solution was eventually dialyzed against $(NH_4)HCO_3$ (0.079 g) and SDS (0.02 g) in H_2O (100 ml).

The steps of the purification process are summarized in Table 2.

STABILITY OF THE ENZYME.—Enzyme, purified up to step 4 of the purification protocol (Table 2), was dialyzed (0.01 M KPi buffer, pH 7.0) and the enzyme solution kept at 4°, either after adjustment of the pH to 7.6, or after addition of dithiothreitol (0.5 mM), KCl (1 M), $(NH_4)_2\ SO_4$ (1 M), or EDTA (0.1 mM). Enzyme was withdrawn and assayed on days 0, 1, 3, 7, and 12 after the start of the experiment.

PROTEIN ANALYSIS.—Protein was determined as described by Bradford (32), with bovine serum albumin as standard. The reference proteins for mol wt determination on Sephadex G-150 were Blue Dextran ($2\times10_6$ daltons), albumin (66,000 daltons), ovalbumin (45,000 daltons), and chymotrypsinogen (25,000 daltons). For mol wt determination with SDS PAGE, Laemmli's (30) procedure was employed. Ms determination of the enzyme was carried out with 2,5-dihydroxybenzoic acid as a matrix and a nitrogen laser at 337 nm. Calibration of signals was carried out with cytochrome C (12,360 daltons). The N-terminal amino acid sequence was determined with an ABI protein sequencer, Model 470 A.

The isoelectric point was determined as described by Harzer (33). The pH gradient was generated using Ampholine pH 4–6. The enzyme did not sediment at 100,000×*g* (2 h), indicating that it may not be bound to microsomes. Kinetic data were determined with the enzyme obtained after step 6 of the purification protocol (Table 2).

ENZYME ASSAY.—*Forward reaction (chorismic to isochorismic acid).*—The incubation mixture contained (final volume 100 μl) chorismic acid (0.8 mM), $MgCl_2$ (5 mM), Tris HCl (pH 8.0, 100 mM), dithiothreitol (1 mM), EGTA (1 mM), and enzyme (4 μg).

The mixture was incubated for 20 min at 37°. The incubation was terminated by addition of MeOH (100 μl) and 3-hydroxybenzoic acid (1 mM) as an internal standard for hplc analysis. Hplc analysis was carried out on a Multospher RP 18 column (4 μm, 2504 mm). Solvents used were (A) H_2O-HCOOH (99:1)

and (B) MeOH-H_2O-HCOOH (100:99:1). A linear gradient increasing from 45 to 100% B was used. The uv detector was set at 278 nm.

Reverse reaction (isochorismic to chorismic acid).—The incubation mixture contained (final volume 50 μl) isochorismic acid (0.1 mM), Tris HCl (100 mM, pH 8.0), $MgCl_2$ (5 mM), dithiothreitol (1 mM), EGTA (1 mM), and enzyme (2 μg). Subsequent manipulations were as described above.

POLYCLONAL ANTIBODIES.—Protein obtained by elution from SDS PAGE electrophoresis and dialysis was injected into two rabbits. During the first injection 50 μg protein and 390 μl complete Freud's adjuvans were applied. Subsequent injections of 30 μg protein with incomplete Freud's adjuvans were carried out at 3-week intervals. Ten days after the final injection, 50 ml blood was taken and centrifuged (15 min, 100×g). The serum was kept at −20°.

WESTERN BLOT.—Crude enzyme extracts from *Es. coli* and *En. aerogenes* 62–1 were separated on SDS PAGE (7.5%) for 1 h at 10 mA. Mol wt markers were from Sigma, Munich: albumin (66,000 daltons), glyceraldehyde-3-phosphate dehydrogenase (36,000 daltons), carbonanhydrase (29,000 daltons), trypsinogen (24,000 daltons), trypsin inhibitor (24,000 daltons), α-lactalbumin (14,200 daltons). Proteins were transferred (dry blot) to a nitrocellulose membrane (34).

Enzymes were made visible by immunostaining. The second antibody was anti-Rabbit IgG from goat coupled to alkaline phosphatase (Biomol Hamburg, FRG). Staining was carried out with the following buffer: boric acid 60 mM, $MgSO_4$ 5 mM, pH 9.7 (1 ml), including *o*-D-dianisidintetrazol (0.25 mg) and β-naphthylphosphate (0.25 mg).

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