Immobilization of Isochorismate Hydroxymutase. Comparison of Native Versus Immobilized Enzyme

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IMMOBILIZATION OF ISOCHORISMATE HYDROXYMUTASE. COMPARISON OF NATIVE VERSUS IMMOBILIZED ENZYME[1]

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ABSTRACT.—Partially purified isochorismate hydroxymutase (isochorismate synthase, E.C. 5.4.99.6) from *Flavobacterium* K_{3-15}, a vitamin K overproducer, was immobilized on CNBr-activated Sepharose 4B, alkylamine glass substituted with glutardialdehyde, and aminohexyl Sepharose 4B substituted with glutardialdehyde. The immobilized enzyme exhibited a lower specific activity but a broader pH tolerance and a higher thermostability than the soluble enzyme. The stability of the enzyme was greatly increased by immobilization. Isochorismic acid, which is not commercially available, was prepared by a constant flow incubation.

Investigations of isochorismate hydroxymutase and the metabolic role of isochorismic acid are hampered because the acid is not commercially available. Although a chemical multistep synthesis leading to racemic isochorismic acid has been published (1), enzymatic preparation of isochorismic acid with the aid of isochorismate hydroxymutase may be the method of choice because it is simpler and leads to the desired stereochemically defined compound. Moreover, the starting material for such an isomerization, namely chorismic acid, is commercially available. After we had purified and characterized the isochorismate hydroxymutase from *Flavobacterium* [see accompanying paper, Schaaf *et al.* (2)], it was a logical further step to explore the possibility of stabilizing the enzyme by immobilization and to generate isochorismic acid by means of the immobilized enzyme.

RESULTS AND DISCUSSION

COUPLING PROCEDURE.—The enriched enzyme (compare Experimental) was bound to three different solid supports. After binding, the suspension was poured into columns. In no case did the effluent of the columns contain detectable isochorismate hydroxymutase activity. The activity of the immobilized enzyme was 42% (CNBr activated Sepharose), 44% (alkylamine glass), or 46% (aminohexyl Sepharose) when compared to an equal amount of native enzyme.

Whereas aminohexyl Sepharose immobilizaton did not give a stable enzyme, immobilization on CNBr-activated Sepharose and alkylamine glass gave stable preparations (see later). We therefore confined ourselves to the characterization of the latter two systems.

The pH optimum of the bound enzyme was broader than that of the native enzyme (Figure 1A). Especially at alkaline pH, the stability of the bound enzyme was improved. Immobilization of the enzyme also results in increased temperature stability (Figure 1B).

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[1]This work is dedicated to the memory of Professor Edward Leete.

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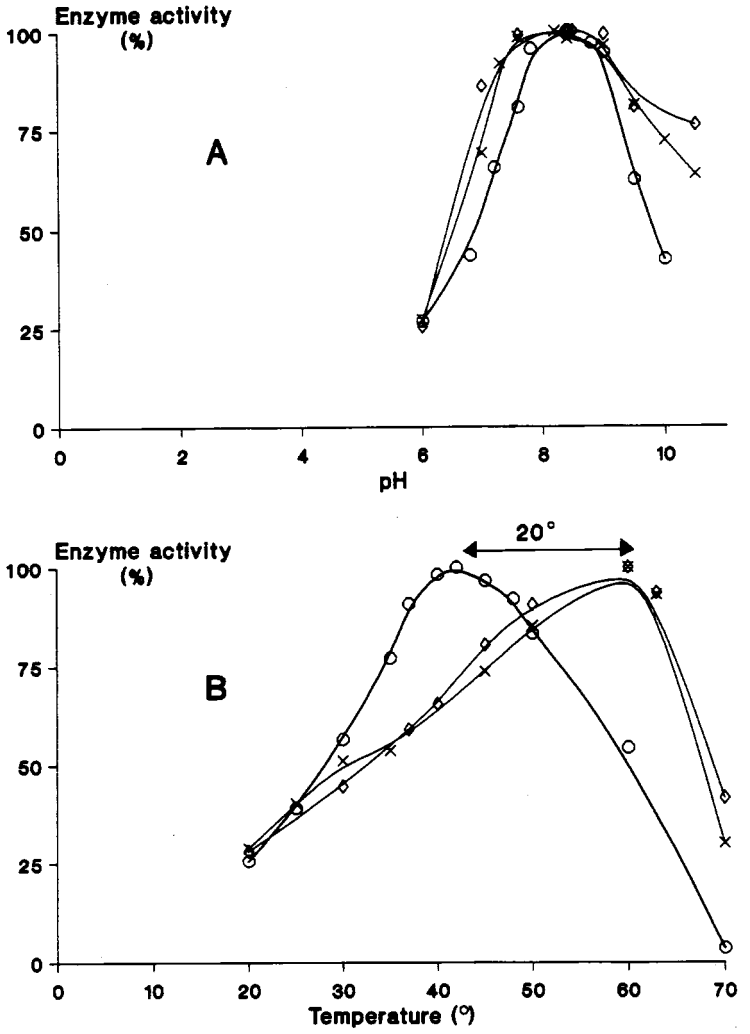

FIGURE 1. Influence of pH (A) and temperature (B) on the activity of soluble (-0-0-) and immobilized (-X-X- CNBr Sepharose; -◊-◊- alkylamine glass) isochorismate hydroxymutase. The assay in graph A was carried out in either KPi (0.1 M, pH 5–7) or Tris-HCl (0.1 M, pH 7–11) buffer under standard conditions.

The temperature optimum of the immobilized enzyme was 60°, whereas the native enzyme exhibits a maximum at 42°.

Mg^{2+} is an essential cofactor of the enzyme (3). This applies also to the immobilized enzyme which is 65% inhibited by EDTA at a 10 mM concentration. Optimum concentrations of Mg^{2+} are relatively low for the immobilized enzyme compared to those of the native enzyme (Figure 2). Metal ions other than Mg^{2+} (Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) at concentrations of 1 mM to 10 mM did not replace Mg^{2+} either in the case of the native enzyme or the immobilized enzyme systems (data not shown). These ions (10 mM) in admixture with Mg^{2+} (5 mM) reduced enzyme activity by 80 to 100%, except for Ba^{2} which led to an increase in enzyme activity by 50% (Figure 2). Inhibitors (NaF, KCNO, adamantane, aminotriazole, NaN_3) did not reduce enzyme activity at concentrations of 0.1, 1.0, 10, and 100 mM. Iodoacetamide (10 mM) inhibited the immobilized enzyme by 34% but not the native enzyme (data not shown).

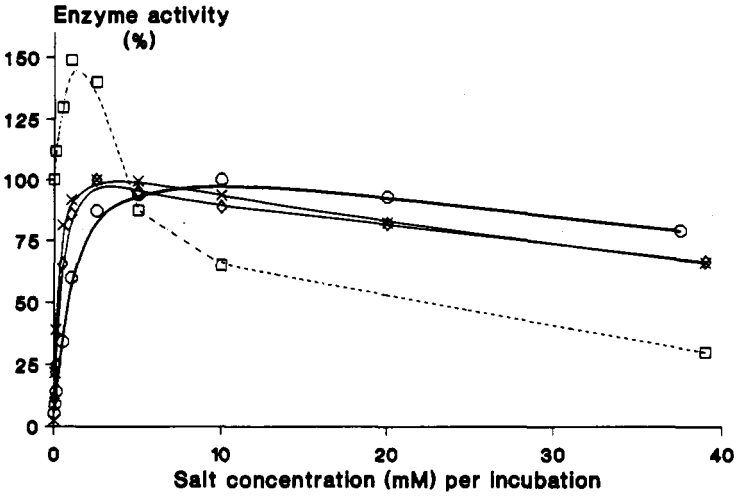

FIGURE 2. Influence of increasing Mg^{2+} ($MgCl_2$) concentrations on the activity of native -0-0-, alkylamine glass -◊-◊-, and CNBr-Sepharose -X-X- immobilized enzyme. The activity of the native enzyme changed in the presence of increasing Ba^{2+} ($BaCl_2$) concentrations -□-□- as indicated. In the latter case the incubation mixture contained both Mg^{2+} (5 mM) and Ba^{2+} ($BaCl_2$) at varying concentrations. -0-0- Native enzyme, -X-X- CNBr Sepharose, -◊-◊- alkylamine glass.

Double reciprocal plots of rate versus substrate concentrations are shown in Figure 3. The derived K_M values and V_{max} data are listed in Table 1. It can be seen that substrates have a higher affinity for the immobilized enzyme, while V_{max} values are relatively low and are higher for the forward than the reverse reaction.

The amount of protein and the time required to generate a given amount of isochorismate from chorismate in the forward reaction and of chorismate from isochorismate in the reverse reaction are shown in Figures 4 and 5. The immobilized enzyme has a half-life of about 70 days (CNBr-Sepharose) or 20 days (alkylamine glass) at room temperature, whereas the half-life of the soluble enzyme is 16 h only (Figure 6B). When the immobilized enzyme was stored at 4° about 45% (CNBr method) or 62% (glass method) of the initial activity was recovered after a period of 7 months (Figure 6 A,B). Strangely, the enzyme immobilized on CNBr-Sepharose showed an increase of activity up to 180% of the initial value (compare Figure 6).

Preparative synthesis of isochorismate was carried out using each of the immobilized enzymes. The activity was stable for 40 h at 37°. Maximum conversion of chorismate into

TABLE 1. Properties of the Soluble and the Immobilized Isochorismate Hydroxymutase.

Conditions	Immobilization on		Soluble enzyme
	CNBr-Sepharose	Alkylamine glass	
pH optimum	7.6–8.2	7.4–8.5	8.0
Temperature optimum	60°	60°	42°
Optimum Mg^{2}-concentration	2.5–5 mM	2.5–5 mM	5–20 mM
Activity without Mg^{2+}	2.5%	11%	5.5%
K_m (chorismate)	270 μM	313 μM	350 μM
V_{max} (forward reaction)	38.5 pkat/mg protein	10.8 pkat/mg protein	1160 pkat/mg protein
K_m (isochorismate)	171 μM	76 μM	254 μM
V_{max} (reverse reaction)	19 pkat/mg protein	6.2 pkat/mg protein	785 pkat/mg protein

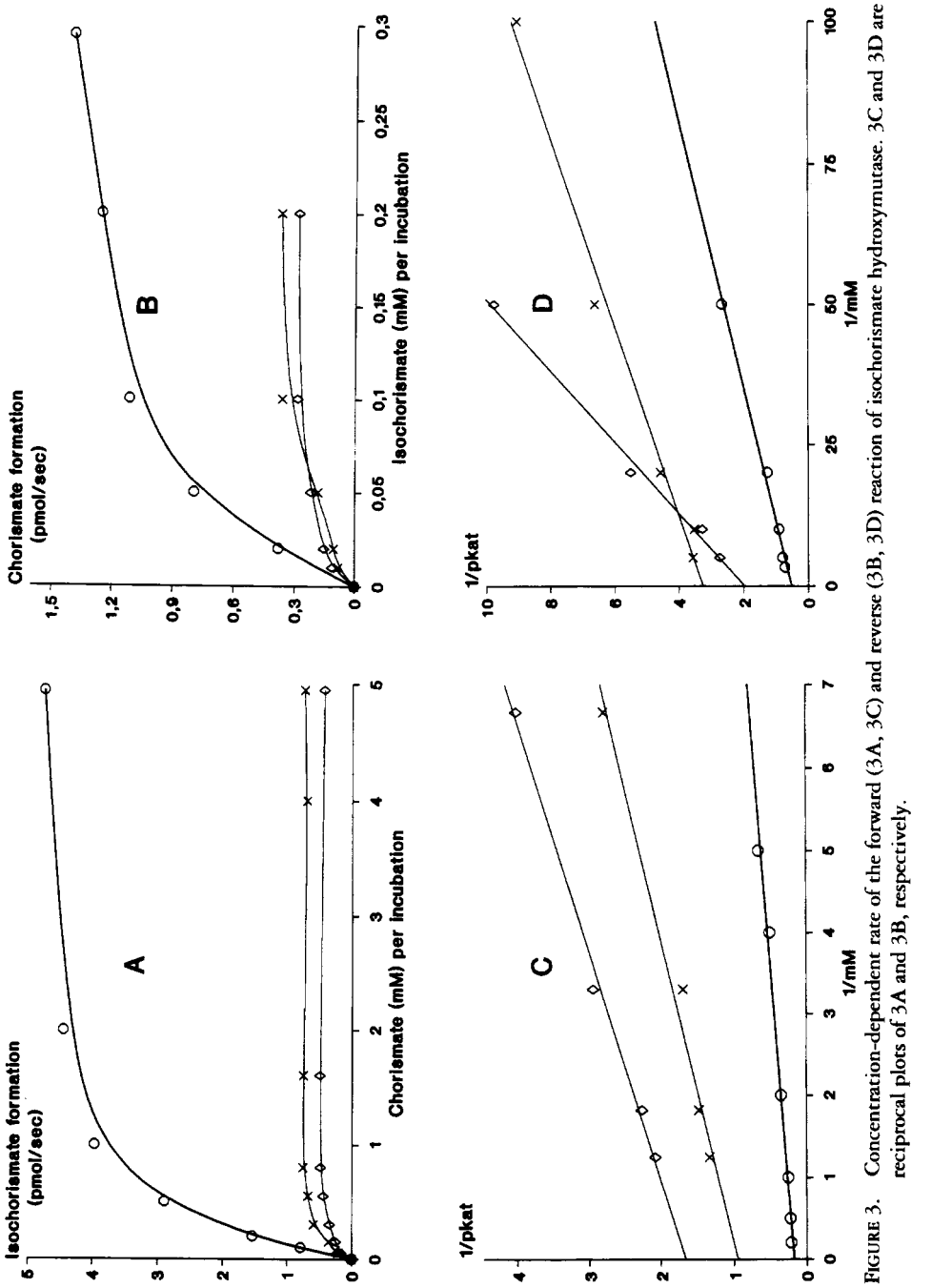

FIGURE 3. Concentration-dependent rate of the forward (3A, 3C) and reverse (3B, 3D) reaction of isochorismate hydroxymutase. 3C and 3D are reciprocal plots of 3A and 3B, respectively.

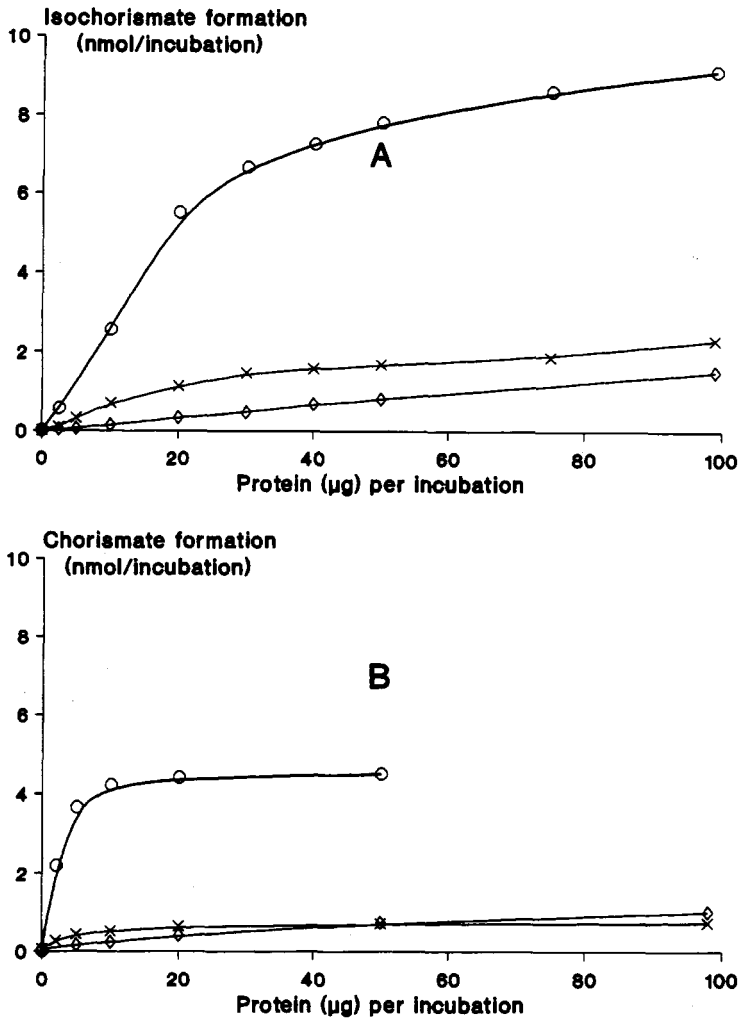

FIGURE 4. Protein dependence of the forward (A) (chorismic to isochorismic acid) and the reverse (B) (isochorismic to chorismic acid) reaction catalyzed by native (-0-0-), CNBr-Sepharose (-X-X-), and alkylamine glass (-◊-◊-) immobilized enzyme.

isochorismate was about 28% 40 h after the start of the reaction. Subsequently the activity decreased steadily to a level of 10% of the initial value. After a period of 15 days no further product formation was observed. The enzyme was irreversibly denatured (Figure 7). The two isomers were eventually separated by cc (Figure 8). The information available to date shows that the enzyme can be stabilized either by freeze drying (4) or by immobilization as shown in the present communication. Among the methods tested, immobilization on alkylamine glass may be the method of choice because the immobilized enzyme has a half-life of 210 days compared to 110 days on CNBr Sepharose (storage at 4°). The freeze-dried enzyme is stable for at least 6 months at −20° (4). The half-life, however, was not reported. Immobilization offers the advantage over the incubation of chorismic acid in admixture with native enzyme that the resulting mixture of isomers contains buffer only and no enzyme. This facilitates separation of chorismic and isochorismic acid.

Scale-up of the method here described will readily give larger amounts of isochorismic acid.

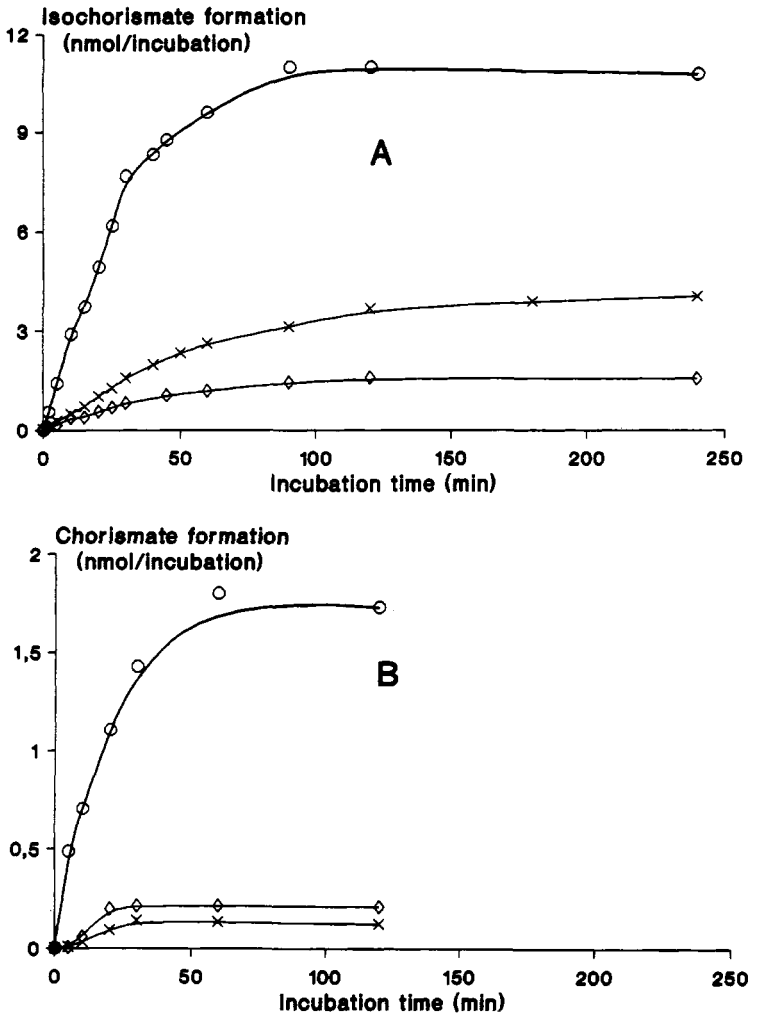

FIGURE 5. Time dependence of the forward (A) (chorismic to isochorismic acid) and the reverse (B) (isochorismic to chorismic acid) reaction catalyzed by native (-0-0-), CNBr-Sepharose (-X-X-) and alkylamine glass (-◊-◊-) immobilized enzyme.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Chemicals, organisms, growth conditions, enzyme isolation, and purification are described in the accompanying paper (2).

ENZYME ASSAY (FORWARD REACTION).—Unless otherwise stated the incubation mixture contained (in a final volume of 100 μl) chorismic acid (0.8 mM), $MgCl_2$ (5 mM), Tris HCl (pH 8.0, 100 mM), dithiothreitol (DTT, 1 mM), ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA, 1 mM), and enzyme solution [soluble enzyme 4 μg purification step 6; see accompanying paper (2), glass-immobilized enzyme 50 μg, AH-Sepharose immobilized enzyme 50 μg, CNBr-Sepharose immobilized enzyme 20 μg]. The mixture was incubated for 20 min at 37°. The incubation with the soluble enzyme was terminated by addition of MeOH (100 μl) and metahydroxybenzoic acid (1mM) as an internal standard.

For determination of the activity of bound isochorismate hydroxymutase, an aliquot of the gel was equilibrated with Tris HCl (0.1 M, pH 8.0) and assayed for product formation (see above). Incubation was carried out in Mobicol columns (2). After incubation, the effluent was filtered through a sintered glass plate at the bottom of the column, and a sample (40 μl) was analyzed by hplc (2).

ENZYME ASSAY (REVERSE REACTION).—Unless otherwise stated the incubation mixture contained (total

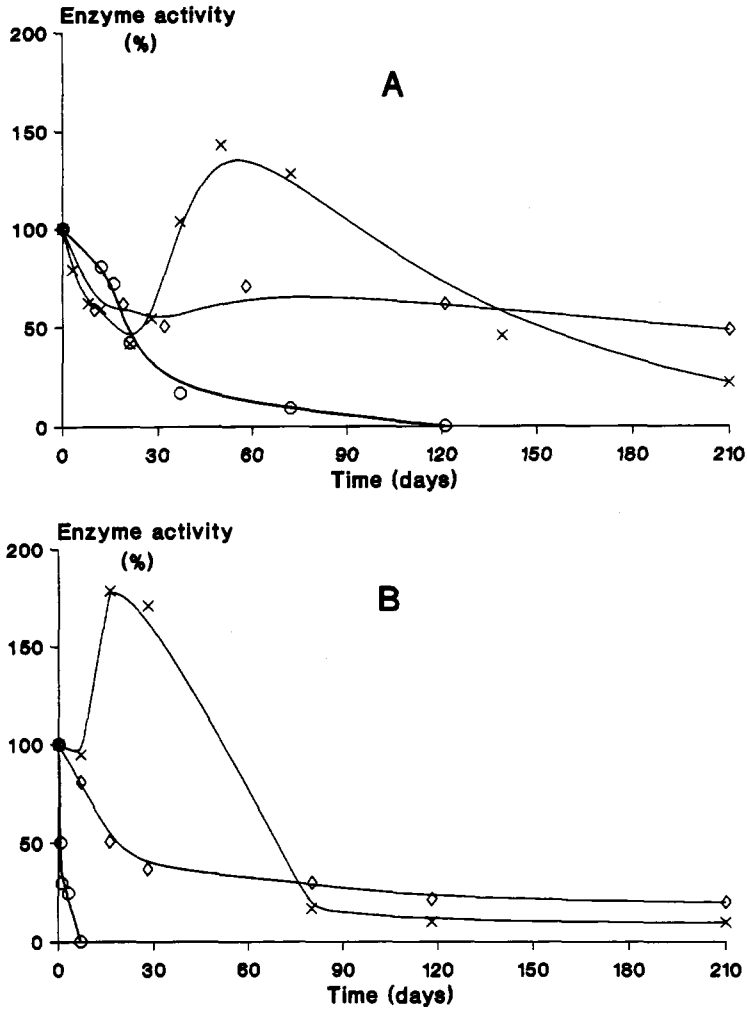

FIGURE 6. Stability of the native (-0-0-) and immobilized (-X-X- CNBr-Sepharose; -◊-◊- alkylamine glass) isochorismate hydroxymutase at 4° (A) and at room temperature (25°) (B) in the presence of NaN_3 (0.02%).

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 [soluble enzyme 2 μg, purification step 6 (2), glass-immobilized enzyme 50 μg, CNBr-immobilized enzyme 20 μg]. Subsequent manipulations were carried out as described above.

IMMOBILIZATION ON CNBR-ACTIVATED SEPHAROSE 4B.—An enzyme solution [100 mg protein, purification step 4; see accompanying paper (2)] was dialyzed against $NaHCO_3$ buffer (0.1 M, pH 8.3) containing NaCl (0.5 mM). CNBr-activated Sepharose (6 g) was soaked in HCl (1 mM). The gel was washed with HCl (2 liters, 1 mM), H_2O (400 ml), and coupling buffer ($NaHCO_3$, 0.1 M, pH 8.3; NaCl, 0.5 M, 60 ml). The gel was suspended in enzyme solution (80 ml). Coupling took place during a 2-h incubation period at room temperature with slow agitation of the mixutre. The gel containing the bound enzyme was washed with coupling buffer (120 ml). The wash was tested for isochorismate hydroxymutase activity. The gel was suspended in Tris HCl (100 ml, 0.1 M, pH 8.0) and again slowly agitated for 2 h at room temperature to block the remaining active gel groups. The supernatant was removed and the gel washed with coupling buffer (120 ml) and citrate buffer (pH 4.0) containing NaCl (0.5 M). Eventually, washing with phosphate buffer (120 ml, pH 7.6, 0.05 M) containing DTT (1 mM) and NaN_3 (0.02%) was carried out to prevent bacterial growth.

IMMOBILIZATION ON GLUTARDIALDEHYDE-SUBSTITUTED AMINOHEXYL SEPHAROSE 4B.—AH-Sepharose (1 g) was soaked in a solution of NaCl (0.5 M) for 15 min and then washed with 200 ml 0.5 M NaCl to remove

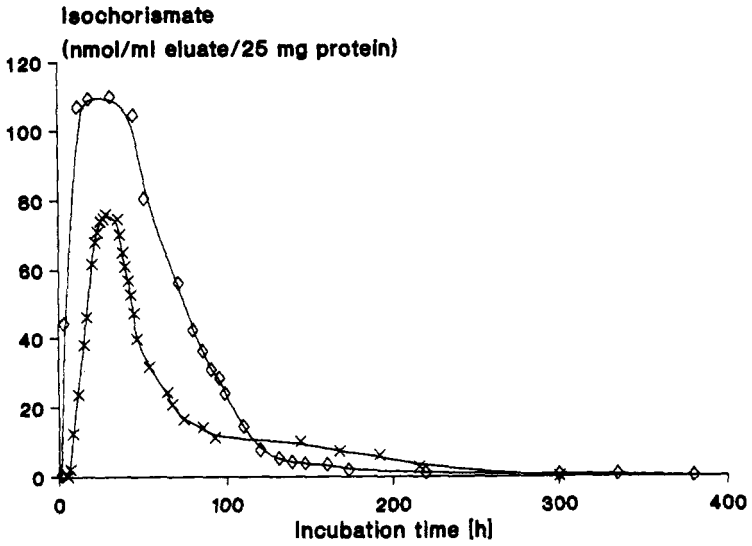

FIGURE 7. Constant flow incubation of immobilized (-X-X- CNBr-Sepharose; -◊-◊- alkylamine glass) isochorismate hydroxymutase at 37°. The half-life of CNBr immobilized enzyme is 45 h, the half-life of alkylamine immobilized enzyme is 70 h at this temperature.

additives. The gel was then washed, first with 0.5 M NaOH (20 ml) and then to neutrality with H_2O, and was suspended in sodium phosphate buffer (KPi buffer, 20 ml, 0.05 M, pH 7.0). Glutardialdehyde (2 ml, 25%) was added. The mixture was stirred for 2 h at room temperature. It was then washed with KPi buffer (100 ml, 0.05 M, pH 7.0) and finally suspended in the same buffer (8 ml). Enzyme extract (1.5 ml) [purification step 6; see accompanying paper (2)] containing 1.5 mg protein and albumine (12 mg in 1.5 ml) was added. This suspension was stirred gently for 2 h at room temperature.

After the coupling procedure was finished, the gel was washed with KPi buffer (60 ml, 0.05 M, pH 7.6) containing NaCl (1 M) to remove noncovalently bound protein. The gel was washed with KPi buffer (60 ml, 0.05 M, pH 7.6) containing DTT (1 mM) and NaN_3 (0.02%).

IMMOBILIZATION ON GLUTARDIALDEHYDE SUBSTITUTED ALKYLAMINE GLASS.—Enzyme [3.5 ml, 100 mg protein, purification step 4; see accompanying paper (2)] was dialyzed against KPi buffer (0.05 M, pH 7.6). Alkylamine glass (2.5 g) was added to a mixture of glutardialdehyde (25%, 1 ml) and KPi buffer (4 ml, 0.1

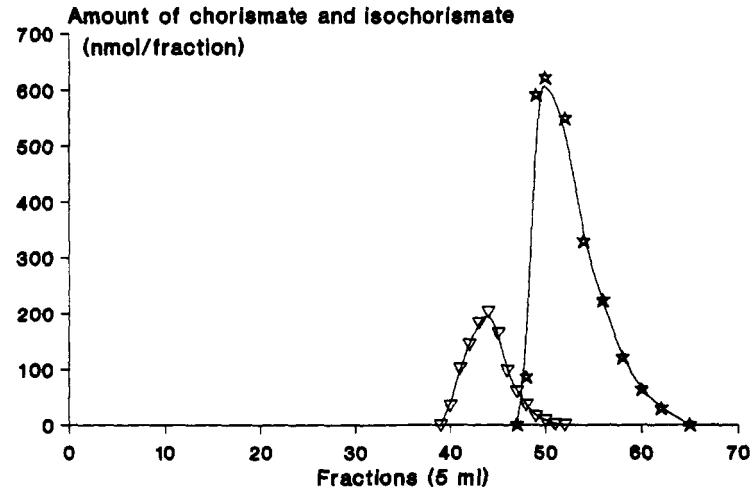

FIGURE 8. Preparative separation of isochorismic (-▽-▽-) and chorismic acid (-★-★-) on a Lobar column.

M, pH 7.6). After 1 h under vacuum, excess glutardialdehyde was removed with cold H_2O (0.5 liter). The glass was equilibrated with KPi buffer (250 ml, 0.05 M, pH 7.6). The enzyme solution was mixed with the glass and kept overnight at 4°. In order to remove all unbound and noncovalently bound enzyme the glass was washed with a solution of NaCl (0.5 liter, 1 M), cold KPi buffer (5 liters, 0.05 M, pH 7.6) and incubated with albumine solution (1%, 24 h, 4°). The immobilized enzyme was kept in KPi buffer (0.05 M, pH 7.6) containing DTT (1 mM) and NaN_3 (0.02%).

PREPARATION OF ISOCHORISMIC ACID.—A column (1.5×10 cm) was loaded with either CNBr Sepharose bound enzyme (1.5 g, 25 mg protein, 550 pkat isochorismate hydroxymutase) or with alkylamine glass bound enzyme (0.625 g, 25 mg protein, 550 pkat isochorismate hydroxymutase). The solid support was equilibrated with Tris HCl (0.1 M, pH 8.0). The substrate solution (Tris HCl, 100 mM, pH 8.0) containing chorismate (0.4 mM), $MgCl_2$ (5 mM), DTT (1 mM), and EGTA (1 mM) was pumped onto the column at a rate of 1.8 ml/h.

The column was kept at 37°. All other solutions were kept at 4°. The effluent was collected at intervals (100 min), checked for isochorismic acid by hplc, and frozen (−20°). The incubation was continued for 15 days (Figure 7). After 5 days the combined fractions contained 19.5 mg chorismic and 2.7 mg isochorismic acid. The isomers were separated by chromatography on a Lobar column (Merck, type A). The solvent system contained MeOH in aqueous HCOOH (pH 2.5). The MeOH fraction was increased in 10% steps (each 100 ml) from 10% to 50% MeOH in HCOOH. The flow rate was 60 ml/h.

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