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Chorismate mutase: prephenate dehydratase from *Acinetobacter calcoaceticus*

Purification, properties and immunological cross-reactivity

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The bifunctional P protein (chorismate mutase: prephenate dehydratase) from *Acinetobacter calcoaceticus* has been purified. It was homogeneous in polyacrylamide gels and was more than 95% pure on the basis of the immunostaining of purified P protein with the antibodies raised against the P protein. The native enzyme is a homodimer (M_r = 91000) composed of 45-kDa subunits. A twofold increase in the native molecular mass of the P protein occurred in the presence of L-phenylalanine (inhibitor of both activities) or L-tyrosine (activator of the dehydratase activity) during gel filtration.

Chorismate mutase activity followed Michaelis-Menten kinetics with a K_m of 0.55 mM for chorismate. L-Phenylalanine was a relatively poor non-competitive inhibitor of the mutase activity. The chorismate mutase activity was also competitively inhibited by prephenate (reaction product). Substrate-saturation curves for the dehydratase activity were sigmoidal showing positive cooperativity among the prephenate-binding sites. L-Tyrosine activated prephenate dehydratase strongly but did not abolish positive cooperativity with respect to prephenate. L-Phenylalanine inhibited the dehydratase activity, and the substrate-saturation curves became increasingly sigmoidal as phenylalanine concentrations were increased with h_{app} values changing from 2.0 (no phenylalanine) to 4.0 (0.08 mM L-phenylalanine). A sigmoidal inhibition curve of the dehydratase activity by L-phenylalanine gave Hill plots having a slope of −2.9. Higher ionic strength increased the dehydratase activity by reducing the positive cooperative binding of prephenate, and the sigmoidal substrate-saturation curves were changed to near-hyperbolic form. The h_{app} values decreased with increase in ionic strength.

Antibodies raised against the purified P protein showed cross-reactivity with the P proteins from near phylogenetic relatives of *A. calcoaceticus*. At a greater phylogenetic distance, cross-reaction was superior with P protein from *Neisseria gonorrhaeae* than with that from the more closely related *Escherichia coli*.

Multifunctional proteins having more than one catalytic center have been studied in great detail and continue to intrigue biochemists. Typically, exhaustive information exists for one organism, often *Escherichia coli*, but there is little or no information about their phylogenetic distribution [1]. A few good examples are the two bifunctional aspartokinase: homoserine dehydrogenase enzymes and the two bifunctional proteins participating in tryptophan biosynthesis [2 – 4]. In prokaryotes the evolutionary origins of two bifunctional proteins that function in phenylalanine and tyrosine biosynthesis have been pinpointed [5]. These are the P protein (chorismate mutase: prephenate dehydratase) of phenylalanine biosynthesis and the T protein (chorismate mutase : cyclohexadienyl dehydrogenase) of tyrosine biosynthesis. The P protein evolved earliest while the T protein is of much more recent origin [6]. Roughly 70% of all gram-negative bacteria possess the P protein, while only a small subcluster (enteric bacteria) additionally possesses the T protein.

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Abbreviations. CM-F, monofunctional chorismate mutase; CM-P, the chorismate mutase component of the bifunctional P protein.

Enzymes. Chorismate mutase (EC 5.4.99.5); prephenate dehydratase (EC 4.2.1.51); cyclohexadienyl dehydrogenase (EC 1.3.1.12).

The bifunctional P protein was purified from two phylogenetically widely spaced organisms, *E. coli* [7, 8] and *Alcaligenes eutrophus* [9], at a time when their phylogenetic relationships were ill-defined. The P proteins isolated from *E. coli* and *A. eutrophus* vary considerably with respect to their native molecular structure, association of native enzyme molecules in the presence of effector molecules and the kinetics of catalysis in the absence and presence of several effector molecules that affect the two component activities of the P proteins [9 – 14]. Chorismate is a novel cyclohexadienyl molecule, which serves as the last common intermediate for biosynthesis of L-tyrosine, L-phenylalanine, L-tryptophan, and a number of vitamin-like end-products. Chorismate mutase catalyzes a Claisen-type rearrangement whereby the *enol*pyruvyl side-chain of the chorismate molecule becomes the pyruvyl side-chain of prephenate. When the chorismate mutase reaction is catalyzed by the P protein, the prephenate molecules produced are spatially positioned for a second catalytic step performed by prephenate dehydratase, which transforms prephenate to phenylpyruvate.

The broad and well-defined phylogenetic distribution of the P protein provides an ideal system for comparative studies that can address the following questions. What biochemical diversity has occurred over a long evolutionary span with respect to the protein chemistry and regulatory properties of this complicated bifunctional protein? How can this diversity

be equated to coevolution with other variable features known to exist for aromatic biosynthesis, e.g. presence or absence of the T protein? Owing to the availability of well-defined phylogenetic trees of microorganisms, one can now select organisms between *E. coli* and *A. eutrophus* for detailed studies of additional P proteins that exist in systems of aromatic biosynthesis and regulation that typify known clusters of biochemical diversity. Our initial choice has been *Acinetobacter calcoaceticus*, an organism satisfying the latter criterion [15].

In this article we report the copurification of chorismate mutase and prephenate dehydratase activities to homogeneity, thus showing the presence of the bifunctional P protein in *A. calcoaceticus*. The results presented here also show that this bifunctional protein from *A. calcoaceticus* possesses unique physical, regulatory and kinetic properties.

MATERIALS AND METHODS

Materials

Chorismic acid was isolated from the accumulation medium of a triple auxotroph of *Klebsiella pneumoniae* 62-1 [16]. Prephenate was prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *S. typhimurium* [17] and was converted to the potassium salt before use. Amino acids, amino acid analogs, dithiothreitol, bovine serum albumin, molecular mass standards, Sephadex G-150 (Superfine), Sephadex G-200, Tris, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, acrylamide, bisacrylamide, ammonium persulfate, phenylalanine-Sepharose and *N,N,N′,N′*-tetramethylethylenediamine were obtained from Sigma Chemical Co. (St Louis, MO). DEAE-cellulose DE52 was purchased from Whatman Inc. (Clifton, NJ). Hydroxyapatite, DEAE-Affigel-blue, goat anti-(rabbit IgG) conjugated with horseradish peroxidase and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were standard reagent grade.

Assay procedures

Chorismate mutase and prephenate dehydratase were assayed by the method of Cotton and Gibson [18] as modified [19]. For both the activities a unit of enzyme was defined as the quantity of enzyme that catalyzed the conversion of 1 µmol substrate to product in 1 min under the assay conditions. Enzyme dilutions were made in 50 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 0.05% (mass/vol.) bovine serum albumin.

Protein concentrations in the crude extracts as well as at various steps during the purification procedure were estimated by the method of Bradford [20].

Initial steps of purification

All operations were performed at 4°C and all buffers contained 1 mM dithiothreitol. Frozen cell pellets were thawed, mixed with two volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, and the cells were broken by three 30-s bursts of ultrasound energy with a Labline ultratip sonicator at 100 W. The resulting extract was centrifuged at 30000 × *g* for 20 min to remove unbroken cells. The supernatant was centrifuged at 150000 × *g* for 1 h to remove the particulate material. The supernatant was collected and was termed crude extract.

Streptomycin sulfate (2.5 ml 10%, mass/vol., solution of streptomycin sulfate dissolved in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA/10 ml crude extract) solution was added dropwise to the crude extract. After 30 min the precipitate was collected by centrifugation at 30000 × *g* for 20 min. The supernatant was brought to 50% of saturation by adding solid ammonium sulfate, allowed to stand for 30 min, and the precipitated proteins were collected by centrifugation at 30000 × *g* for 30 min. The pellets were dissolved in 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and were dialyzed against this buffer for 15 h with at least three changes of buffer.

Molecular-mass estimations by chromatography on Sephadex G-200

The molecular mass of the purified enzyme (10 µg) was estimated by chromatography on Sephadex G-200 columns (2.5 × 87.0 cm) in the presence or absence of effectors (L-phenylalanine or L-tyrosine). The elution buffer was 50 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 0.2 mM L-phenylalanine or 1.0 mM L-tyrosine when the enzyme was run in the presence of these effectors. Protein samples in 2 ml elution buffer were applied and eluted at a flow rate of 15 ml/h. Fractions of 2.2 ml were collected. The void volume of the column was determined with blue dextran 2000. β-Amylase (200 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) were used as standards and were located by monitoring the column effluent at 280 nm. Chorismate mutase:prephenate dehydratase was located by enzyme assay.

Molecular mass estimation by gel electrophoresis

The subunit molecular mass of the purified chorismate mutase:prephenate dehydratase was estimated by polyacrylamide gel electrophoresis (7.5% acrylamide, 0.2% bisacrylamide) in the presence of SDS as described by Laemmli [21]. Protein samples were prepared as described [21] and bromophenol blue was used as the tracking dye. Myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, albumin (egg) and carbonic anhydrase were used as standard proteins.

Bacterial strains and growth conditions

Acinetobacter calcoaceticus ATCC 14987 was obtained from the American Type Culture Collection (Rockville, MD). The organisms were grown in the minimal medium described by Kaplan and Rosenberg [22] supplemented with 0.2% (mass/vol.) Casamino acids (Difco). Cells in late exponential phase of growth were harvested by centrifugation, washed once with 50 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and were stored at −80°C until used. All the organisms used in the immunological cross-reactivity experiments were obtained from ATCC. Growth conditions and preparation of cell-free extracts are described elsewhere [23, 24].

Preparation of antibodies specific for P protein

Antibodies to the P protein from *A. calcoaceticus* were obtained by injecting the homogeneous P protein into a male New Zealand White rabbit. A 55-µg amount of P protein, dissolved in 10 mM potassium phosphate (pH 7.0) and mixed

with three volumes of complete Freund's adjuvant, was injected intramuscularly in the four limbs. Six weeks later the rabbit was injected with an additional 25 μg P protein by the same route, prepared by mixing the protein solution in 10 mM potassium phosphate (pH 7.0) with incomplete and complete Freund's adjuvant in volume ratio of 1:2:1. The rabbit was bled on the 7th, 8th and 9th days following the booster injection. The collected blood was allowed to clot overnight at 4°C. After removal of the clot, the immunoglobulin fraction was isolated from the serum by ammonium sulfate fractionation at 0°C. The precipitate (0–50% ammonium sulfate saturation) was dissolved in a minimal volume of 10 mM potassium phosphate (pH 7.0) and dialyzed against the same buffer for 14 h with at least three changes of the buffer. The immunoglobulin fraction was then loaded onto a DEAE-Affigel-blue column (1.5 × 20.0 cm) equilibrated in 10 mM potassium phosphate (pH 7.0). Immunoglobulins do not bind to the resin under these conditions. Fractions containing antibodies were combined and concentrated by ultrafiltration on a PM-10 membrane. This purified antibody preparation was used in inhibition of enzyme activities and in Western blotting and immunostaining experiments.

Titration of P protein activities with antibodies

The P protein activities from various organisms were isolated following DEAE-cellulose DE52 column chromatography of the crude extracts as described previously [18, 23]. The P protein activities and the monofunctional chorismate mutase (CM-F) from *A. calcoaceticus* for titration were also obtained following DE52 chromatography of the crude extracts (see Fig. 1). Antibody dilutions were made in 50 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 0.05% (mass/vol.) bovine serum albumin. The chorismate mutase and prephenate dehydratase activities were diluted to 0.06 unit/ml with the same dilution buffer. An appropriate dilution of the immunoglobulin fraction was added in progressive amounts to 50 μl diluted enzyme, and the final volume was adjusted to 150 μl with the same dilution buffer. The mixture was incubated at 37°C for 15 min and then assayed for chorismate mutase or prephenate dehydratase activities by the procedure described above.

Western blotting and immunostaining

The P protein samples from various organisms, prepared as described above, and the CM-F isolated from *A. calcoaceticus* were subjected to polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulfate by the method of Laemmli [21]. After electrophoresis the gels were blotted onto nitrocellulose (Schleicher & Schüll). The blots were then blocked with gelatin, treated with the antibodies raised against the P protein from *A. calcoaceticus* followed by goat anti-(rabbit IgG) conjugated with horseradish peroxidase and 4-chloro-1-naphthol as the substrate for the peroxidase. The immunostaining procedure was performed exactly as specified by Bio-Rad, supplier of the anti-(rabbit IgG) and the substrate.

RESULTS

Purification of chorismate mutase: prephenate dehydratase

The dialyzed protein sample obtained in the initial steps of purification (see Materials and Methods) was loaded onto a DEAE-cellulose (DE52) column (2.5 × 40.0 cm) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 60 ml/h. After washing the column with two bed volumes of the equilibration buffer, the bound proteins were eluted by a linear potassium phosphate gradient (0.05–0.18 M) (pH 7.0) in a total volume of 1400 ml. Fractions of 6.0 ml were collected and were assayed for absorbance at 280 nm and for chorismate mutase and prephenate dehydratase activities. The elution profiles of the enzyme activities are shown in Fig. 1. Two peaks of chorismate mutase activity eluted, one peak of activity washing through the column without retardation (CM-F) while the second peak of activity eluted in the gradient fractions. The latter peak of activity (CM-P) coeluted with the prephenate dehydratase peak of activity.

Fractions 232–252 were combined, concentrated by ultrafiltration on a PM-10 membrane (Amicon Inc. Deerfield, MA), dialyzed to 50 mM potassium phosphate (pH 7.0) and loaded onto a second DE52 column (1.5 × 20 cm) equilibrated in 50 mM potassium phosphate (pH 7.0) at a flow rate of 60 ml/h. After washing the column with one bed volume of the equilibrating buffer, the bound proteins were eluted with a linear potassium phosphate gradient (0.05 M, pH 7.0–0.18 M, pH 5.8) in a total volume of 500 ml. Fractions of 3.0 ml were collected and were monitored for A_{280} and the chorismate mutase and prephenate dehydratase activities. Both of the P-protein enzyme activities eluted in gradient fractions 110–160.

The proteins recovered from the second DE52 column (fractions 117–142) were combined, brought to pH 7.0 by the addition of 50 mM potassium phosphate (dibasic), concentrated on a PM-10 membrane, dialyzed to 25 mM potassium phosphate (pH 7.0) and loaded onto a phenylalanine-Sepharose column (1.5 × 7.0 cm) equilibrated in 25 mM potassium phosphate (pH 7.0) at a flow rate of 60 ml/h. After washing the column extensively with the equilibrating buffer, the bound proteins were eluted by applying 5 mM potassium phosphate (pH 7.0). Fractions of 2.0 ml were collected and were assayed for absorbance at 280 nm and for chorismate mutase and prephenate dehydratase activities. The chorismate mutase and prephenate dehydratase activities eluted with 5 mM potassium phosphate (pH 7.0) in fractions 63–140.

The active fractions from the phenylalanine-Sepharose column (fractions 68–120) were combined, concentrated on a PM-10 membrane, dialyzed to 50 mM potassium phosphate (pH 7.0) and loaded onto a Sephadex G-150 (Superfine) column (2.5 × 93.0 cm) equilibrated with 50 mM potassium phosphate (pH 7.0) at a flow rate of 3.0 ml/h. The proteins were eluted with the equilibration buffer. Fractions of 1.05 ml were collected and were monitored for absorbance at 230 nm and for chorismate mutase and prephenate dehydratase activities. Both chorismate mutase and prephenate dehydratase activities coeluted as a sharp peak with an elution volume of 244 ml.

The fractions containing the enzyme activities from the G-150 column (fractions 226–243) were combined, concentrated on a PM-10 membrane, dialyzed to 20 mM potassium phosphate (pH 7.0) and loaded onto a hydroxyapatite column (1.5 × 20.0 cm) equilibrated with 20 mM potassium phosphate (pH 7.0) at a flow rate of 30 ml/h. After washing the column with one bed volume of the equilibrating buffer, the bound proteins were eluted with a linear potassium phosphate gradient (0.02–0.20 M) at pH 7.0 in a total volume of 300 ml. Fractions of 1.55 ml were collected and were screened for absorbance at 230 nm and for the chorismate mutase and prephenate dehydratase activities. Both the enzyme activities

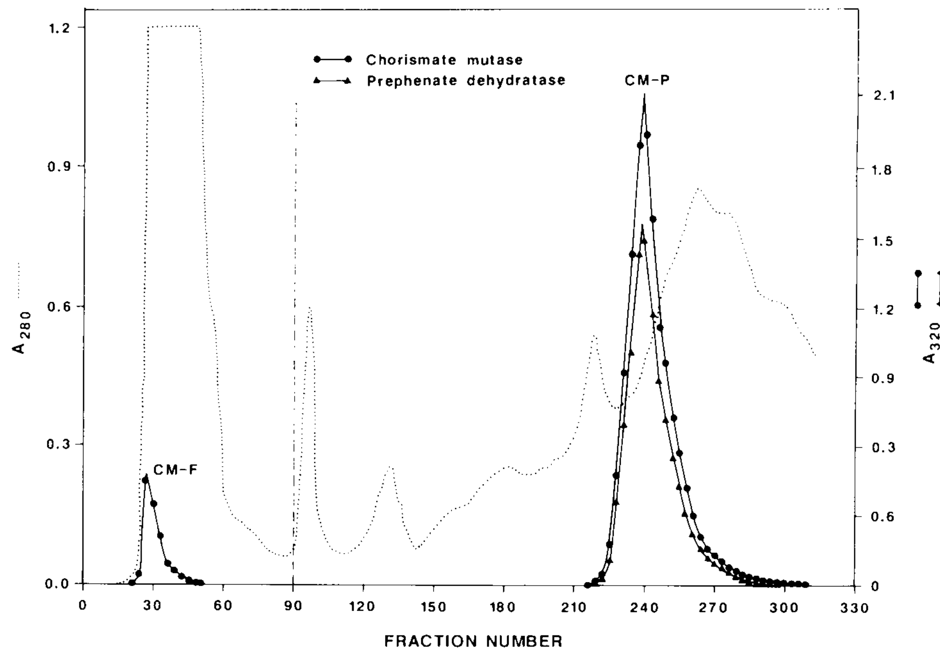

Fig. 1. *Elution profiles of chorismate mutase and prephenate dehydratase from the initial DEAE-cellulose chromatography step using the proteins that precipitated between 0 and 50% saturation with ammonium sulfate.* Prephenate dehydratase activity was measured in the presence of 1 mM L-tyrosine. The chorismate mutase activity that washed through the column is termed the monofunctional chorismate mutase (CM-F) while the mutase activity that eluted in the gradient fractions together with the prephenate dehydratase activity is denoted CM-P. Both activities are expressed as phenylpyruvate absorbance at 320 nm. An A_{320} of 1.0 corresponds to 2.9 nmol phenylpyruvate formed/min. The distribution of the proteins was monitored at 280 nm. (- -) The onset point of gradient elution

coeluted in the gradient fractions. Fractions 87–99 were combined, concentrated on a PM-10 membrane, dialyzed to 10 mM potassium phosphate (pH 7.0) and were used for further studies.

The purified chorismate mutase: prephenate dehydratase preparation at this stage of purification was homogeneous when subjected to polyacrylamide gel electrophoresis in the presence of SDS (Fig. 2), with a molecular mass of 45 kDa, thus showing the presence of the bifunctional P protein in *A. calcoaceticus*. The purification procedure is summarized in Table 1.

Molecular mass estimations

The molecular mass of the native chorismate mutase: prephenate dehydratase was determined in the absence or presence of effector molecules (L-phenylalanine or L-tyrosine) using a Sephadex G-200 column. In the absence of effector molecules, the chorismate mutase: prephenate dehydratase eluted at a molecular mass of 91 kDa. However, in the presence of phenylalanine or tyrosine it eluted at an estimated molecular mass position of 175 kDa. This indicates a twofold increase in the native molecular mass of the bifunctional P protein in the presence of L-phenylalanine or L-tyrosine. The molecular mass of 45 kDa, obtained from polyacrylamide gel electrophoresis of the purified P protein in the presence of SDS, suggests that the native P protein is composed of two subunits of identical molecular mass.

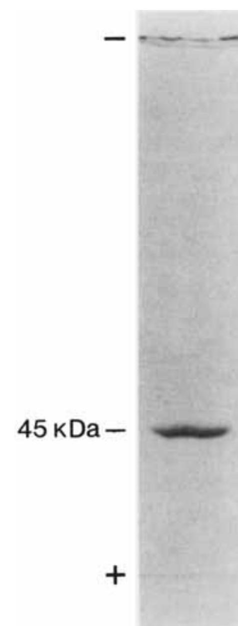

Fig. 2. *Biochemical characterization of chorismate mutase: prephenate dehydratase from* A. calcoaceticus. Purified P protein was subjected to electrophoresis in 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate and then stained with Coomassie blue as described in Materials and Methods. The indicated molecular mass was estimated from the mobility of standard proteins

Substrate saturation curves for chorismate mutase

The chorismate mutase activity exhibited disproportionality of velocity at low protein concentrations, and thus all kinetic determinations was carried out at protein concentrations within the range giving proportionality. The plot of reaction velocity of chorismate mutase as a function of chorismate concentration was hyperbolic, giving a linear

Table 1. *Purification of chorismate mutase: prephenate dehydratase from* A. calcoaceticus
Experimental details are described in the text; 65 g wet cells were used. Prephenate dehydratase assays were performed in 50 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 1 mM L-tyrosine. Prephenate concentrations of 10.0 mM were used for the enzyme assay in steps I and III and 2.0 mM for steps IV – VIII. n. d., not determined

Purification step	Volume	Total protein	Prephenate dehydratase				Chorismate mutase				Ratio (mutase : dehydratase)
			total	specific activity	yield	purification	total	specific activity	yield	purification	
	ml	mg	units	units/mg	%		units	units/mg	%		
I. Crude extract	116.5	1711.0	163.1	0.09	100	1.0	223.4	0.13	100	1.0	1.36
II. After streptomycin sulfate treatment	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
III. 0 – 50% ammonium sulfate fraction	57.1	1480.0	146.8	0.09	90.0	1.0	180.1	0.12	81.0	0.91	1.25
IV. After first DE52 chromatography	126.0	79.1	99.9	1.26	61.3	13.2	111.0	1.40	49.7	10.8	1.11
V. After second DE52 chromatography	78.0	14.6	41.3	2.84	25.3	29.8	48.5	3.3	21.7	25.7	1.15
VI. After phenylalanine-Sepharose chromatography	106.0	1.0	19.9	20.3	12.2	213.9	24.6	23.3	11.0	179.6	1.14
VII. After Sephadex G-150 chromatography	18.9	0.34	17.4	50.8	10.7	533.8	19.9	58.2	8.9	448.1	1.14
VIII. After hydroxyapatite chromatography	20.1	0.08	6.8	84.2	4.2	884.4	7.5	92.8	3.4	714.2	1.10

double-reciprocal plot (Fig. 3, bottom panel, open circles). The K_m value obtained for chorismate was 0.55 mM.

The Hill plot of these data was linear over the concentration range studied and had a slope of 1.0.

The chorismate mutase activity was only partially inhibited by L-phenylalanine with 50% inhibition achieved at 1.0 mM L-phenylalanine (Fig. 3, top panel) while L-tyrosine had no effect. The inhibition curve for the chorismate mutase activity by L-phenylalanine was hyperbolic, and the Hill plot yielded a slope (h') of -1.0 (Fig. 3, top panel). Plots of $1/v$ against $1/s$ at several fixed concentrations of phenylalanine (Fig. 3, bottom panel) showed L-phenylalanine to act as a relatively poor non-competitive inhibitor of the chorismate mutase activity.

The chorismate mutase activity was also inhibited by prephenate, the product of the chorismate mutase reaction (Fig. 4, inset). 40% inhibition was obtained at 0.5 mM prephenate. Inhibition at higher prephenate concentrations could not be determined accurately as less and less product must be determined against a greater and greater background of added prephenate. Double-reciprocal plots of velocity against chorismate at several fixed concentrations of prephenate (Fig. 4) showed prephenate to be a competitive inhibitor of the chorismate mutase activity, a K_i value of 0.61 mM (slope replot) being obtained for prephenate.

Substrate-saturation curves for prephenate dehydratase

The prephenate dehydratase activity was dramatically activated by L-tyrosine (Fig. 5). Maximum activation of nearly 24-fold was observed at 1.0 mM L-tyrosine concentration.

The prephenate dehydratase activity exhibited an even greater disproportionality of velocity at low protein concentrations than did chorismate mutase activity even in the presence of L-tyrosine. Therefore, all kinetic studies were performed at enzyme concentrations where velocity was a proportional function of enzyme concentration.

The substrate-saturation curves constructed for prephenate dehydratase at various fixed concentrations of L-tyrosine were sigmoidal. When the data were plotted in double-reciprocal form, the plots were not linear. However, the plots of $1/v$ against $1/s^2$ were linear (Fig. 6) at all concentrations of L-tyrosine, suggesting cooperative binding of prephenate at two prephenate-binding sites ($h = 2.0$). L-Tyrosine acts by decreasing the intrinsic dissociation constant (K_s) of the enzyme for prephenate, the value of K_s decreasing with increasing L-tyrosine concentrations. Since the enzyme has a very high K_m for prephenate in the absence of tyrosine and since L-tyrosine activates the dehydratase activity by lowering K_m for prephenate without affecting cooperativity, all further studies were carried out at the L-tyrosine concentration that causes maximal activation (1.0 mM).

Inhibition by L-phenylalanine

The prephenate dehydratase activity was strongly inhibited by L-phenylalanine, with 90% inhibition achieved at 0.2 mM L-phenylalanine (Fig. 7). A Hill plot of the inhibition data was linear between L-phenylalanine concentrations of 0.05 mM and 0.2 mM, with a slope (h') of -2.9 (Fig. 7, inset). Thus, L-phenylalanine also exhibits cooperative binding in its inhibition of the prephenate dehydratase activity.

Substrate-saturation curves constructed at several different, fixed concentrations of L-phenylalanine gave a family of sigmoidal curves, with sigmoidicity increasing with increasing L-phenylalanine concentration (Fig. 8). Hill plots of these data yielded an h_{app} value for prephenate of 2.0 in the absence of effector, 3.2 at low L-phenylalanine concentrations (0.04 mM) and a value of 4.0 at all other L-phenylalanine concentrations used (Fig. 8, inset).

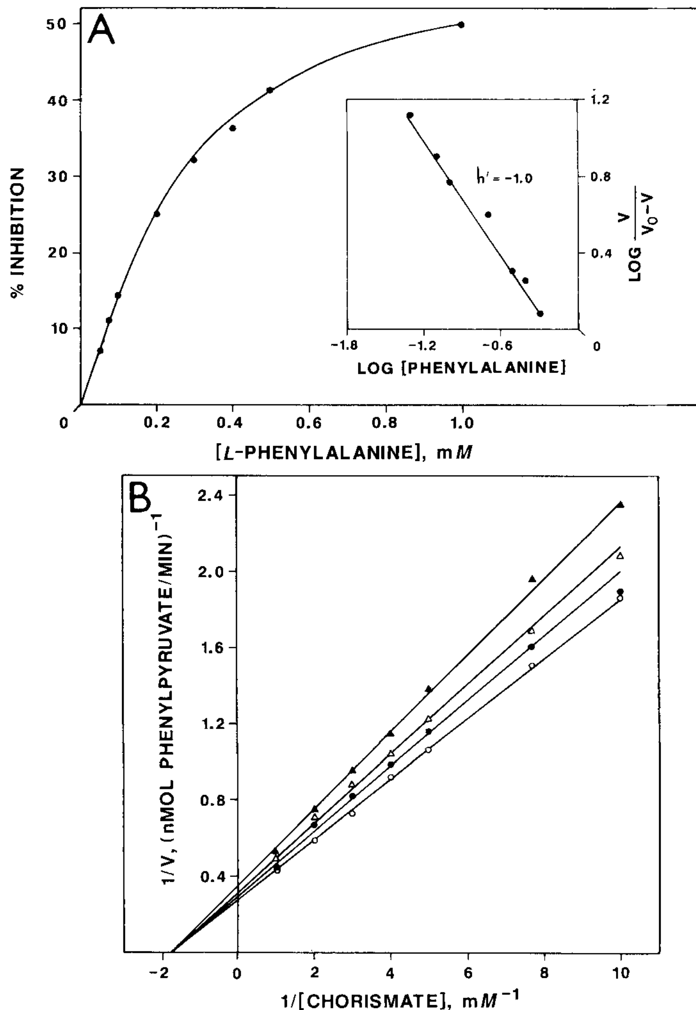

Fig. 3. *(A) Inhibition of the mutase activity by L-phenylalanine. (B) Substrate-saturation data plotted in double-reciprocal form for chorismate mutase activity at L-phenylalanine concentrations of 0.0 mM (○), 0.05 mM (●), 0.1 mM (△) and 0.2 mM (▲).* (A) The assay was performed with 1 mM chorismate and 33 ng enzyme protein. Inset: Hill plot of the data obtained from the inhibition of the chorismate mutase activity by increasing concentrations of L-phenylalanine

Inhibition of the P-protein activities by analogs of phenylalanine

The inhibition of the prephenate dehydratase and chorismate mutase activities by two analogs of phenylalanine, *m*-fluorophenylalanine and *p*-fluorophenylalanine, was also studied. Both *m* and *p*-fluorophenylalanine strongly inhibited the dehydratase activity (though less effectively than L-phenylalanine), and the inhibition curves were sigmoidal. The chorismate mutase activity was only partially inhibited by *m*- and *p*-fluorophenylalanine, and the inhibition curves were hyperbolic. Since the analogs are a racemic mixture, the inhibition is probably twice as good as indicated (assuming L isomers are the effective molecules). *m*-Fluorophenylalanine was more effective than *p*-fluorophenylalanine as an inhibitor of both the dehydratase and the mutase activities. Hill plots of these data gave slopes of nearly identical values to those obtained with L-phenylalanine for both the dehydratase and the mutase activities, suggesting analog function at the allosteric site for L-phenylalanine.

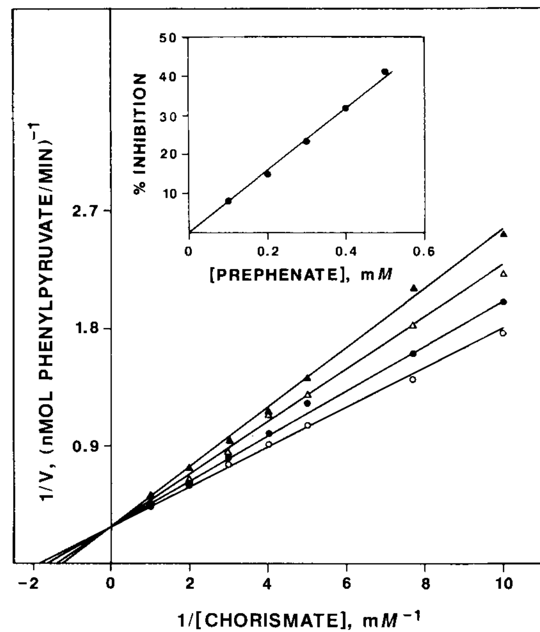

Fig. 4. *Inhibition of chorismate mutase activity by prephenate.* The assays were performed with 1 mM potassium chorismate and 33 ng enzyme protein. The inhibition curve is shown in the inset. The substrate saturation data (plotted in double-reciprocal form) were obtained in the absence of potassim prephenate (○), and in the presence of 0.1 mM (●), 0.2 mM (△) or 0.3 mM (▲) potassium prephenate

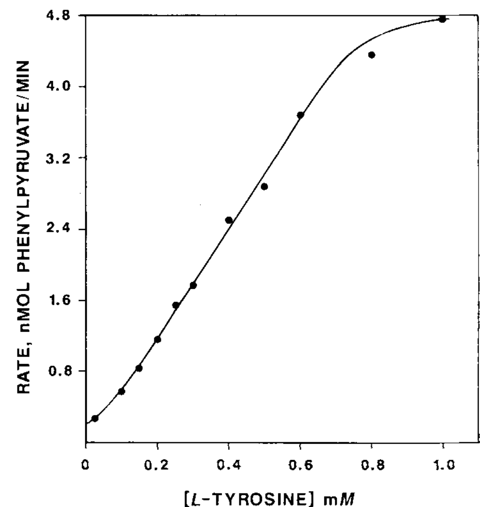

Fig. 5. *Activation of the prephenate dehydratase activity by L-tyrosine.* The assay was performed in 25 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol, 2 mM potassium prephenate and 66 ng protein

Activation of the dehydratase activity at high ionic strength

The prephenate dehydratase activity, but not the chorismate mutase activity, was stimulated by higher salt concentrations in the assay. The activation was independent of the cation or anion used (data not shown) and was totally dependent on ionic strength. The substrate saturation curves, constructed at several different fixed concentrations of potassium phosphate (pH 7.0) (used to increase ionic strength), were sigmoidal at low potassium phosphate concentrations. How-

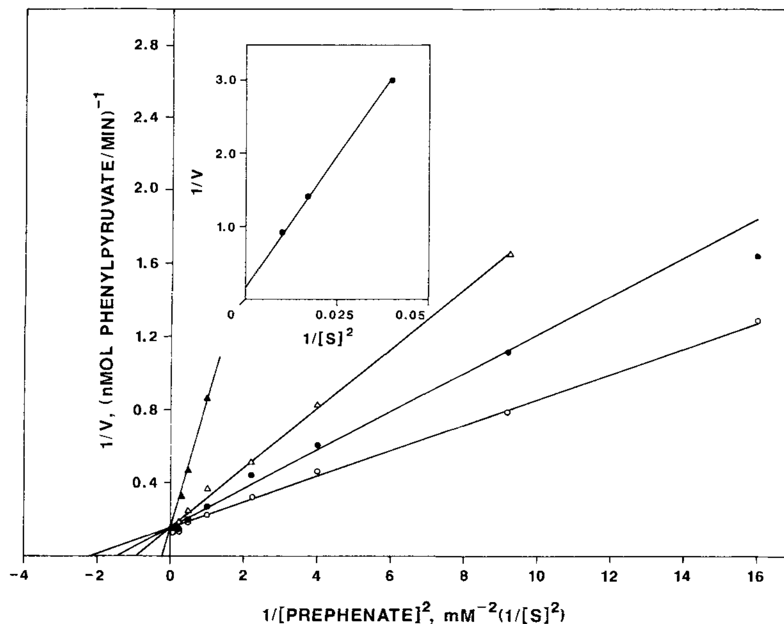

Fig. 6. *Substrate-saturation data for prephenate dehydratase plotted in double-reciprocal form.* The enzyme assay was performed in 37 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol and 66 ng protein. Results were obtained at various fixed concentrations of L-tyrosine, these being 1.0 mM (○), 0.75 mM (●), 0.5 mM (△) and 0.25 mM (▲). The data obtained in the absence of L-tyrosine are shown in the inset

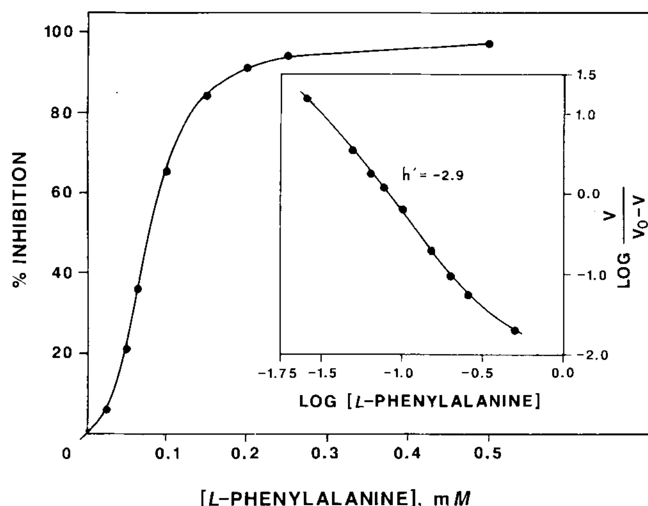

Fig. 7. *Inhibition of the prephenate dehydratase activity by L-phenylalanine.* The enzyme assays were performed in 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 2 mM potassium prephenate, 1 mM L-tyrosine and 66 ng protein. A Hill plot is shown in the inset

ever, the cooperativity decreased with increasing potassium phosphate concentrations (Fig. 9). The plots were nearly hyperbolic at 0.2 M potassium phosphate. Hill plots of these data yielded a slope value (h_{app}) of 2.0 at low potassium phosphate concentrations, the h_{app} values decreasing progressively as potassium phosphate concentrations were increased, reaching a value of 1.4 at 0.2 M potassium phosphate (Fig. 9, inset).

The different physical, regulatory and kinetic properties of the P proteins isolated from *E. coli. A. eutrophus* and *A. calcoaceticus* are highlighted in Table 2.

Immunological cross-reactivity

A. calcoaceticus belongs to the gram-negative assemblage of purple sulfur bacteria (superfamily B) [5] that branches off at a phylogenetic position that is relatively distant from the *E. coli* lineage [25]. Since all superfamily-B organisms appear to possess the bifunctional P protein [6], the cross-reactivity of the antibodies raised against the purified P protein from *A. calcoaceticus* was checked with the partially purified P proteins from organisms occupying phylogenetic positions between *E. coli* and *A. calcoaceticus*. The antibodies reacted effectively with the P-protein activities from *A. calcoaceticus* (Fig. 10) in the absence of effectors, in the presence of L-tyrosine, in the presence of L-phenylalanine or at high salt concentration (conditions that result in the dimeric or the tetrameric form of the protein and increase or decrease the cooperative binding of prephenate for the dehydratase activity) (data not shown). Significant cross-reactivity was also observed with the P-protein activities isolated from *Pseudomonas stutzeri* and *P. aeruginosa*. However, the antibodies reacted very weakly with the P-protein activities isolated from the enteric bacteria, *Proteus mirabilis* and *E. coli*. No cross-reactivity was observed with the monofunctional chorismate mutase (CM-F) isolated from *A. calcoaceticus* (Fig. 10, bottom panel). Similar results were obtained by Western blotting and immunostaining procedures (data not shown).

DISCUSSION

The bifunctional P protein

A. calcoaceticus belongs to a phylogenetic cluster, denoted as superfamily B [5], that also includes *E. coli* and other enteric bacteria. Although a rationale has been advanced in support of the contention that all members of superfamily A and superfamily B possess the P protein [5, 6, 26], only the P proteins from *A. eutrophus* and *E. coli* have been purified. The

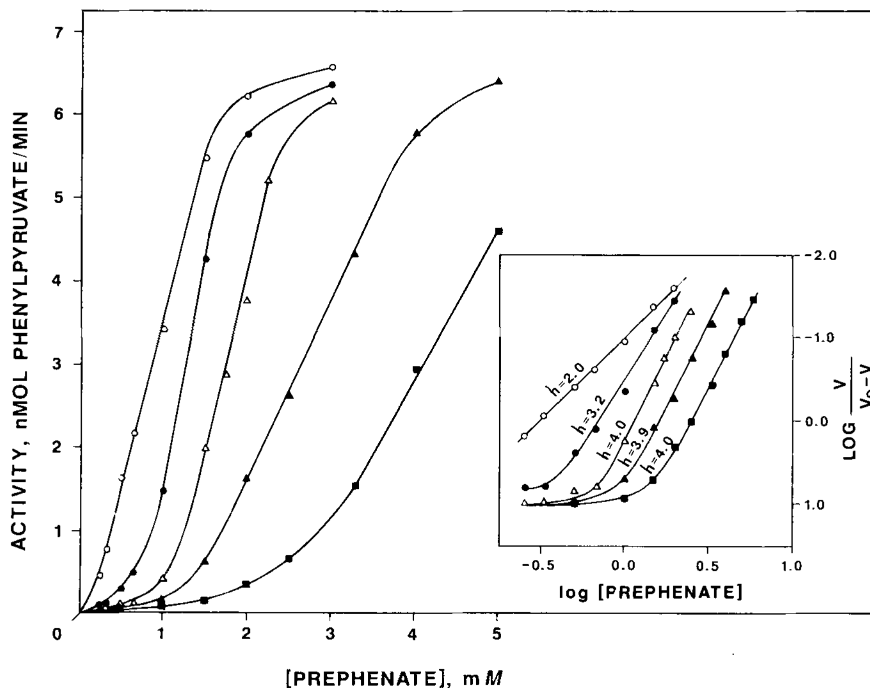

Fig. 8. *Substrate-saturation curves for prephenase dehydratase activity in the presence of varying fixed concentrations of L-phenylalanine*. The enzyme assays were performed in 25 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol, 1 mM L-tyrosine and 66 ng protein. L-Phenylalanine concentrations were fixed at 0.0 mM (○), 0.04 mM (●), 0.08 mM (△), 0.12 mM (▲) and 0.25 mM (■). Inset: Hill plot of the data obtained from the substrate-saturation curves in the presence of varying concentrations of L-phenylalanine (symbols indicated above)

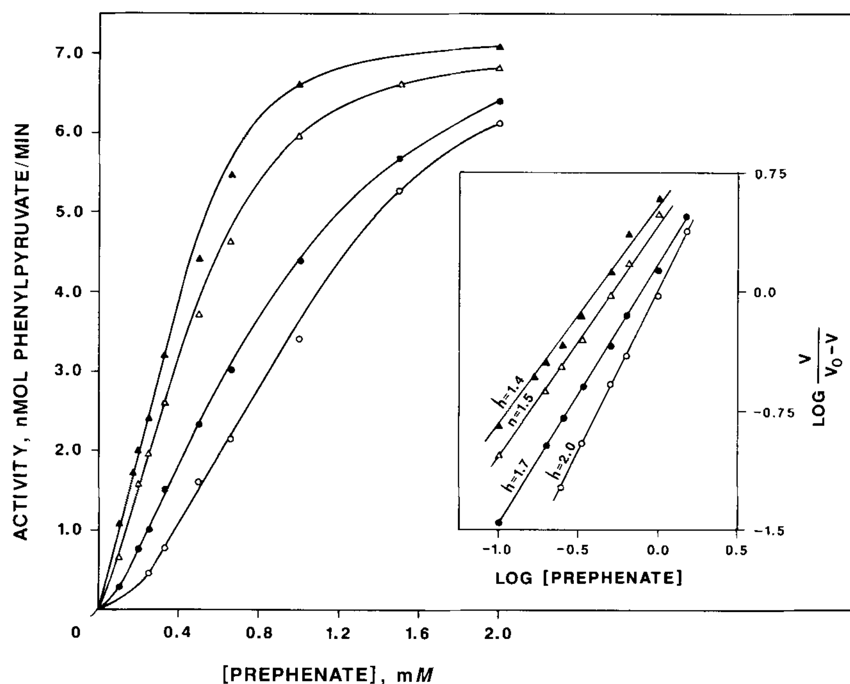

Fig. 9. *Activation of the prephenate dehydratase activity as a function of ionic strength*. Substrate saturation curves were constructed at various fixed concentrations of potassium phosphate (pH 7.0) as a function of varying concentrations of prephenate. The assays were performed in the presence of 1 mM dithiothreitol, 1 mM L-tyrosine and 66 ng enzyme protein. Potassium phosphate concentrations were 25 mM (○), 50 mM (●), 100 mM (△) and 200 mM (▲). The ionic strength of 25 mM potassium phosphate (pH 7.0) is 0.11. Inset: Hill plot of the data shown on the left

prephenate dehydratase activity form *A. calcoaceticus* was inseparable from one isozyme of chorismate mutase activity during all the steps of purification, and the final protein sample, which possessed both the activities of prephenate dehydratase and chorismate mutase, was homogeneous upon electrophoresis. Further, after the separation of chorismate mutase isozymes on a DEAE-cellulose DE52 column, the ratio of the mutase to dehydratase activity remained nearly constant during further chromatographic runs. These findings shows that a bifunctional P protein is present in *A.*

Table 2. *Comparison of purified P proteins isolated from* E. coli, A. eutrophus *and* A. calcoaceticus
E. coli data are from [7, 10, 11, 13]; *A. eutrophus* data are from [9, 14]. Values in parentheses indicate number of subunits. Extent of positive cooperativity in cases of sigmoidal substrate saturation is indicated by Hill coefficient values. n.d., not determined

Property	*E. coli*	*A. eutrophus*	*A. calcoaceticus*
Subunit M_r	40000 (1)	47000 (1)	45000 (1)
Native M_r	85000 (2)	144000 (4)	91000 (2)
M_r in the presence of Phe	137000 (4)	158000 (4)	175000 (4)
M_r in the presence of Tyr	85000 (2)	158000 (4)	175000 (4)
Properties of mutase component			
K_m for chorismate	0.04 mM	0.20 mM	0.55 mM
Substrate saturation kinetics			
No effector	linear	linear	linear
In the presence of Phe	linear	sigmoidal: 1.7	linear
At high ionic strength	sigmoidal: 1.6	n.d.	linear
Hill coefficient for Phe inhibition	2.3	2.3	1.0
Effect of high ionic strength	inhibition	n.d.	none
Effect of Tyr or Trp	none	none	none
Properties of dehydratase component			
K_m for prephenate	1.0 mM	0.67 mM	>100 mM
Substrate saturation kinetics			
No effector	linear	linear	sigmoidal: 2.0
In the presence of Phe	sigmoidal: 2.5	linear	sigmoidal: 4.0
At high ionic strength	sigmoidal: 1.5	n.d.	sigmoidal: 1.4
Hill coefficient for Phe inhibition	2.3	1.0	2.9
Effect of high ionic strength	inhibition	n.d.	activation
Effect of Tyr	none	activation	activation
Effect of Trp	none	inhibition	activation

calcoaceticus. Since *A. calcoaceticus* is relatively distant in phylogenetic position from *E. coli* [25], the thesis that all members of superfamily B possess the bifunctional P protein is further supported.

Molecular mass transitions

The native molecular mass of 91 kDa and a subunit molecular mass of 45 kDa calculated for the P protein from *A. calcoaceticus* compares to the molecular mass values obtained for the *E. coli* P protein, which has a native molecular mass of 85 kDa and is composed of two subunits of 40 kDa [7]. In contrast, the P protein isolated from *A. eutrophus* has a native molecular mass of 187 kDa and a subunit molecular mass 47 kDa [9]. These data suggest that the P proteins isolated from *E. coli* and *A. calcoaceticus* are dimeric proteins composed of two subunits of identical molecular mass while the native P protein from *A. eutrophus* is a tetramer.

The P protein isolated form *A. calcoaceticus* underwent molecular mass transitions from dimer to tetramer in the presence of L-phenylalanine. L-Tyrosine, which activates the dehydratase but not the mutase activity in *A. calcoaceticus*, also resulted in a twofold increase in the native molecular mass of the P protein, while protein concentration had no effect on the native molecular mass. The native dimeric P protein isolated from *E. coli* has been shown to undergo tetramerization in the presence of L-phenylalanine or at high protein concentrations [10, 12] while L-tyrosine had no effect on the P-protein activities or the native molecular mass. In *A. eutrophus* L-tyrosine is an activator of only the dehydratase activity, but neither L-tyrosine nor L-phenylalanine cause significant change in the native molecular mass of the P protein [9].

Kinetic properties

Both the chorismate mutase and the prephenate dehydratase activities of the bifunctional P protein from *A. calcoaceticus* exhibited disproportionality of velocity at low protein concentrations, and the addition of inhibitor (L-phenylalanine) or activators of the dehydratase activity (L-tyrosine, increase in ionic strength) did not abolish this hysteretic response. The component activities of the P protein from *A. calcoaceticus* showed varied kinetic behavior. The mutase activity followed Michaelis-Menten kinetics, while the substrate saturation curves for the dehydratase activity were sigmoidal. The h_{app} value of 2.0 suggests that the dimeric P protein possesses two binding sites for prephenate which show cooperative interactions. In contrast, both the enzyme activities of the P protein isolated from *E. coli* [10], *S. typhimurium* [27] and *A. eutrophus* [14] follow linear kinetics in the absence of effector molecules.

The inhibition of the mutase activity also followed Michaelis-Menten kinetics in *A. calcoaceticus* and was additionally inhibited by prephenate (competitive inhibitor), while L-tyrosine had no effect. Product inhibition of the mutase activity (either monofunctional or as a component of the bifunctional P protein) has been reported from several organisms [14, 18, 27]. In contrast to the mutase activity, the dehydratase activity was dramatically activated by L-tyrosine. Substrate-saturation curves for prephenate dehydratase were sigmoidal at all concentrations of L-tyrosine used and showed

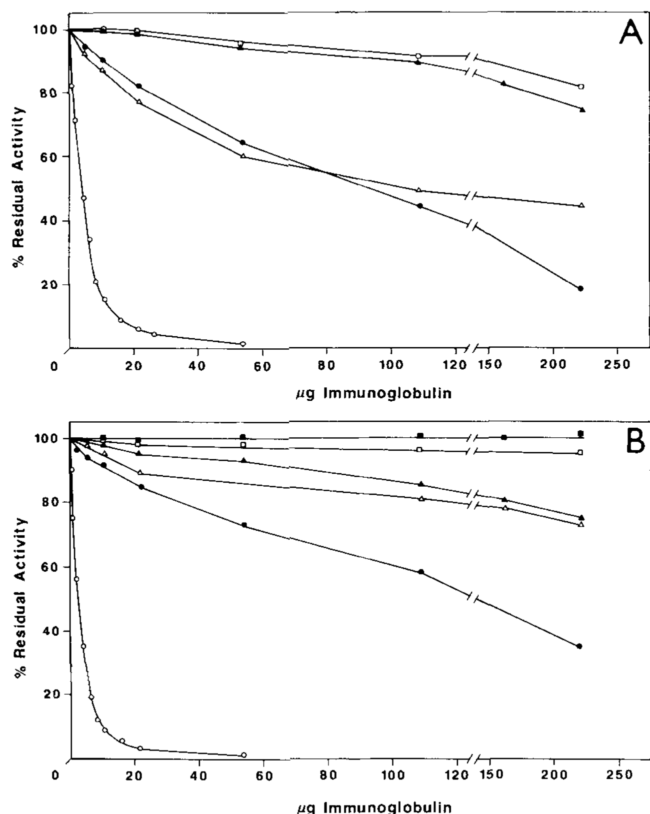

Fig. 10. *Immunological cross-reactivity of purified P protein antibodies from* A. calcoaceticus *against the dehydratase activity (A) and mutase activities (B) of the P proteins isolated from several superfamily-B organisms.* The monofunctional chorismate mutase (CM-F) isolated from *A. calcoaceticus* is also included. Enzyme assays were performed as described in Materials and Methods and 1 mM L-tyrosine was added for the dehydratase assay from *A. calcoaceticus*. The enzyme activity in the absence of any antibodies was taken as control. *A. calcoaceticus* (○), *P. stutzeri* (●), *P. aeruginosa* (△), *P. mirabilis* (▲), *E. coli* (□). CM-F isolated from *A. calcoaceticus* (■)

two prephenate-binding sites (h_{app}) at all L-tyrosine concentrations. The inhibition of the dehydratase activity increased sigmoidally with L-phenylalanine concentrations, and the Hill plot yielded a slope of −2.9 indicating homotropic cooperativity in the binding of L-phenylalanine. Substrate-saturation curves for prephenate dehydratase yielded h_{app} values of 2.0 (no phenylalanine), 3.2 (0.04 mM phenylalanine) and 4.0 (at higher phenylalanine concentrations used), suggesting that the number of prephenate-binding sites increases from 2 to 4 in the presence of L-phenylalanine, undoubtedly because of tetramerization of the native dimeric P protein (note that L-phenylalanine causes a twofold increase in the native molecular mass of the P protein). The h_{app} value of 2.0 obtained in the absence of L-phenylalanine but with L-tyrosine present (which also causes tetramerization) suggests that the two tetramers brought about by L-phenylalanine and L-tyrosine are different molecular species. Perhaps in the tetramer brought about by L-tyrosine, the two active sites of one dimer are independent of the two active sites of the other dimer with respect to prephenate binding.

However, the native dimer and the two tetramer species obtained in the presence of L-phenylalanine or L-tyrosine were indistinguishable immunologically since the antibodies raised against the native dimer inhibited the P-protein activities in the absence or presence of either L-phenylalanine or L-tyrosine. In *E. coli*, the inhibition by L-phenylalanine exhibited Michaelis-Menten kinetics for the mutase activity and sigmoidal kinetics for the dehydratase activity [10], while in *S. typhimurium* both the activities followed sigmoidal kinetics of inhibition by phenylalanine [27]. In contrast, of the P-protein activities isolated from *A. eutrophus*, the inhibition by phenylalanine followed sigmoidal kinetics for the mutase activity while the dehydratase activity exhibited Michaelis-Menten kinetics [14].

The prephenate dehydratase activity but not the mutase activity of the P protein isolated from *A. calcoaceticus* was stimulated by increase in ionic strength, and the cooperativity diminished with increasing ionic strength. The chorismate mutase as well as the prephenate dehydratase activity of the P protein from *E. coli*, on the other hand, are inhibited by increased ionic strength [11, 13]. This increase in ionic strength promotes positive cooperativity in binding of substrate and also facilitates the binding of L-phenylalanine, enhancing the inhibition by L-phenylalanine in *E. coli* [11–13].

The results from *A. calcoaceticus* presented in this paper, showing the molecular mass transitions, kinetic properties of the component activities in the absence and presence of effector molecules and the effect of ionic strength, reflect the unique physical and kinetic properties of bifunctional P protein from *A. calcoaceticus*. The individuality of P proteins presumably reflects coevolutionary pressures to maximize efficient metabolic integration within the distinctive gestalt of overall aromatic biosynthesis that is present in any given organism. A viable approach to test this assumption would be to use recombinant DNA methods for creation of new P protein/host cell combinations in order to examine physiological and biochemical effects upon growth and metabolism. Such effects may very well be subtle, but still far-reaching in evolutionary implications.

Immunological cross-reactivity

The antibodies raised against the purified P protein from *A. calcoaceticus* showed considerable cross-reactivity with the P proteins isolated from two near relatives of *A. calcoaceticus*, namely *P. aeruginosa* and *P. stutzeri*. On the other hand, the antibodies reacted poorly with the P-protein activities isolated from *E. coli* and other enteric bacteria suggesting that these organisms possess P proteins that differ structurally from the P protein of *A. calcoaceticus*. This is consistent with the knowledge that *A. calcoaceticus* diverged from *E. coli* and other enteric bacteria at a relatively deep phylogenetic level [25]. However, the antibodies reacted strongly (data not shown) with the P protein isolated from *Neisseria gonorrhoeae* (a superfamily A member [28]), which is phylogenetically even more distant from *Acinetobacter* than is *Escherichia*. Thus, the P protein from *A. calcoaceticus* is immunologically closer to the P protein from *N. gonorrhoeae* than to *E. coli*. (The P-protein dehydratase activity from *N. gonorrhoeae* also resembles the *Acinetobacter* enzyme in the property of dramatic activation by L-tyrosine [28].)

It was surprising to note that the antibodies raised against the purified P protein did not react with the monofunctional chorismate mutase (CM-F) from *A. calcoaceticus* since it is believed that P-protein mutase and CM-F that later evolved into CM-T (chorismate mutase component of the bifunctional chorismate mutase: prephenate dehydrogenase) are homologous and arose from a single monofunctional chorismate mutase following gene duplication [5, 6, 29, 30]. Perhaps the lack of cross-reactivity is due to differing conformations of the

mutase active sites in CM-F and in the P protein (possibly brought about by the fusion of chorismate mutase and prephenate dehydratase genes). If so, then antibodies raised against denatured P protein may exhibit the expected cross-reactivity.

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