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# Regulation of Chorismate Mutase-Prephenate Dehydratase and Prephenate Dehydrogenase from *Alcaligenes eutrophus*

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Highly purified enzymes from *Alcaligenes eutrophus* H 16 were used for kinetic studies. Chorismate mutase was feedback inhibited by phenylalanine. In the absence of the inhibitor, the double-reciprocal plot was linear, yielding a $K_m$ for chorismate of 0.2 mM. When phenylalanine was present, a pronounced deviation from the Michaelis-Menten hyperbola occurred. The Hill coefficient ($n$) was 1.7, and Hill plots of velocity versus inhibitor concentrations resulted in a value of $n' = 2.3$, indicating positive cooperativity. Chorismate mutase was also inhibited by prephenate, which caused downward double-reciprocal plots and a Hill coefficient of $n = 0.7$, evidence for negative cooperativity. The pH optimum of chorismate mutase ranged from 7.8 to 8.2; its temperature optimum was 47 C. Prephenate dehydratase was competitively inhibited by phenylalanine and activated by tyrosine. Tyrosine stimulated its activity up to 10-fold and decreased the $K_m$ for prephenate, which was 0.67 mM without effectors. Tryptophan inhibited the enzyme competitively. Its inhibition constant ($K_i = 23$ $\mu$M) was almost 10-fold higher than that determined for phenylalanine ($K_i = 2.6$ $\mu$M). The pH optimum of prephenate dehydratase was pH 5.7; the temperature optimum was 48 C. Prephenate dehydrogenase was feedback inhibited by tyrosine. Inhibition was competitive with prephenate ($K_i = 0.06$ mM) and noncompetitive with nicotinamide adenine dinucleotide. The enzyme was further subject to product inhibition by *p*-hydroxyphenylpyruvate ($K_i = 0.13$ mM). Its $K_m$ for prephenate was 0.045 mM, and that for nicotinamide adenine dinucleotide was 0.14 mM. The pH optimum ranged between 7.0 and 7.6; the temperature optimum was 38 C. It is shown how the sensitive regulation of the entire enzyme system leads to a well-balanced amino acid production.

Chorismic acid plays a strategic role in the multibranched pathway of aromatic compounds. It is the precursor of phenylalanine, tyrosine, and tryptophan and leads to metabolites such as vitamin K, folate, ubiquinone, and hydroxybenzoate (16, 24). To balance the flow of intermediates, a sensitive regulation, especially at the branch points, is required. A wide variety of control mechanisms have been discovered (26), including multivalent allosteric feedback control as well as interactions between metabolites and enzymes that are located in quite different metabolic pathways (27).

Regarding aromatic amino acid biosynthesis, the physical association of the enzymes is often part of the regulatory system. In particular, the synthesis of phenylalanine and tyrosine provides several examples for multifunctional enzymes and different organization patterns (Fig. 1) (6, 8, 20, 21, 29). The new type of organization discovered in *Alcaligenes eutrophus* H 16 (13) led to a study of the regulation of the bifunctional enzyme chorismate mutase (EC 5.4.99.5)-prephenate dehydratase (EC 4.2.1.51) and prephenate dehydrogenase (EC 1.3.1.12). During the overall reaction from chorismate to phenylpyruvate, catalyzed by the bifunctional protein, prephenate accumulated. The dissociation of the intermediate product allows prephenate dehydrogenase to compete with prephenate dehydratase for substrate.

## MATERIALS AND METHODS

**Enzymes.** Chorismate mutase-prephenate dehydratase and prephenate dehydrogenase from *A. eutrophus* H 16 were purified as described (11). Pure enzyme from step 7 of Table 1 (37 mutase units per mg of protein [11]) was used for all experiments.

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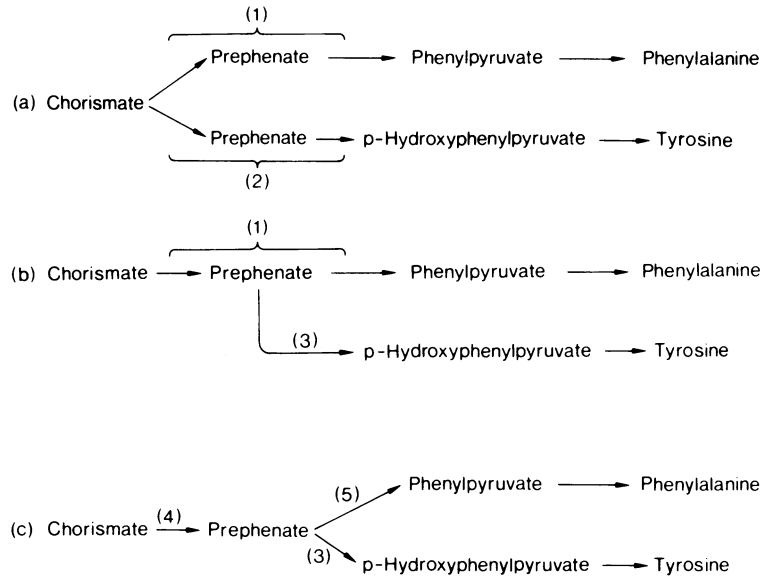


FIG. 1. *Organization of the enzymes involved in phenylalanine and tyrosine biosynthesis in Escherichia coli (a), Alcaligenes eutrophus H 16 (b), and Neurospora crassa (c). Enzymes: Chorismate mutase-prephenate dehydratase (1), chorismate mutase-prephenate dehydrogenase (2) (brackets indicate bifunctional nature of these proteins); prephenate dehydrogenase (3); chorismate mutase (4); prephenate dehydratase (5).*

**Enzyme assays.** One enzyme unit catalyzes the conversion of 1 μmol of substrate per min. The specific activity is expressed as units per milligram of protein.

**Chorismate mutase.** Purified enzyme was assayed according to the method of Cotton and Gibson (7), using an optical test. The rate of chorismate disappearance was followed continuously with a Servogor recorder throughout an interval of 3 min at 275 nm, the absorbance maximum for chorismate. The reaction mixture contained, in a final volume of 1.0 ml, 1 mM potassium chorismate and 50 mM potassium phosphate (pH 7.8). After preincubation of the reaction mixture at 37 C, the reaction was initiated by the addition of the enzyme. The amount of chorismate converted was calculated by using a molar extinction coefficient of 2,630 for chorismate.

**Prephenate dehydratase.** The assay was carried out according to the method of Cotton and Gibson (6) with slight modifications (11).

**Prephenate dehydrogenase.** The activity of prephenate dehydrogenase was determined by the method of Champney and Jensen (4) as described (11).

**Protein.** The protein of the enzyme preparations was determined as described (11).

**Chemicals.** Nicotinamide adenine dinucleotide (NAD) and reduced NAD were obtained from Boehringer Mannheim GmbH (Mannheim, West Germany). L-Isomers of the amino acids of the highest purity grade available were purchased from Merck (Darmstadt, West Germany). Analogous compounds of phenylalanine and intermediates of the pathway were obtained as follows: D-phenylalanine and 3,4-dihydroxyphenylalanine from Fluka AG (Switzerland); and DL-*m*- and *p*-fluorophenylalanines, β-phenylpyruvic acid, and *p*-hydroxyphenylpyruvic acid from Sigma Chemical Co. (St. Louis, Mo.).

Chorismic acid was prepared as barium salt according to the method of Gibson (15). Barium prephenate was prepared by using the auxotrophic mutant strain 6B-1 of *A. eutrophus* H 16 as previously described (13). Barium chorismate and barium prephenate were converted to the potassium salts with a twofold molar excess of potassium sulfate before use. The concentration of stock solutions of both substrates was corrected for purity. All the other chemicals were of analytical reagent grade and obtained from commercial sources.

## RESULTS

**Effect of pH on the regulatory properties of the purified enzymes.** The optimum pH for chorismate mutase activity in potassium phosphate buffer was pH 7.8 and in tris(hydroxymethyl)aminomethane-hydrochloride buffer was pH 8.2 (Fig. 2a). This difference may have been due to changes in ionic strength of the respective buffers at a given pH. However, the enzyme activity was not affected in phosphate buffer, whose molarity ranged between 25 and 100 mM.

Chorismate mutase was inhibited by phenylalanine only in alkaline solution. At a pH of 7.2 no inhibition occurred, and below this pH the activity measured in the presence of phenylalanine was even higher than without the effector

(Fig. 2a). This observation leads to the assumption that under acid conditions phenylalanine protects the enzyme against inactivation.

Prephenate dehydratase activity reached a maximum at pH 5.7 (Fig. 2b). However, the assays were conducted at pH 7.8 due to the acid lability of the substrate prephenate (14), which is represented in Fig. 2b. At an alkaline pH, both activation by tyrosine and inhibition by phenylalanine were most pronounced. Within an acid pH range, the regulatory properties of the enzyme were scarcely visible (Fig. 2b).

Prephenate dehydrogenase exhibited a broad pH optimum between 7.0 and 7.6. This pH represents a range where maximal inhibition by tyrosine occurred (Fig. 2c).

**Effect of temperature.** The temperature optimum for chorismate mutase was found to be 47 C and was almost identical with the value determined for prephenate dehydratase activity (48 C). The value for prephenate dehydrogenase differed significantly; the temperature optimum was 38 C.

**Effect of phenylalanine on chorismate mutase activity.** In the absence of phenylalanine, the substrate saturation curve for chorismate was hyperbolic. Its double-reciprocal plot was linear and resulted in a $K_m$ for chorismate of 0.20 mM (Fig. 3). In the presence of phenylalanine, the substrate saturation curves were sigmoid, and the extent of sigmoidicity increased with increasing inhibitor concentrations up to 1 mM, at which point the inhibition was maximum. The concave upward double-reciprocal plot (Fig. 3) and the Hill plot of these data (Fig. 3, insert) with slopes of 1.3 to 1.7 were consistent with positive cooperative interactions of chorismate binding sites in the presence of the feedback inhibitor.

Plots of chorismate mutase activity versus varying concentrations of phenylalanine were sigmoid, indicating cooperativity of the inhibitor (Fig. 4a). However, cooperativity was dependent upon chorismate concentration as shown by the Hill plots, which were linear in a range of 0.1 to 0.4 mM phenylalanine (Fig. 4b). The values of $n'$ deduced from these data ranged from 1.1 to 2.3.

**Prephenate inhibition of chorismate mutase.** Chorismate mutase activity was inhibited by its product prephenate. Double-reciprocal plots of the substrate saturation curve in the presence of prephenate were concave downward, indicating negative cooperativity (Fig. 5). The reaction rate of unireactant enzymes is the result of forward and reverse velocity. However, under the assay conditions the reverse reaction, the formation of chorismate from prephenate, was not detectable. The following reaction, the conversion of prephenate to phen-

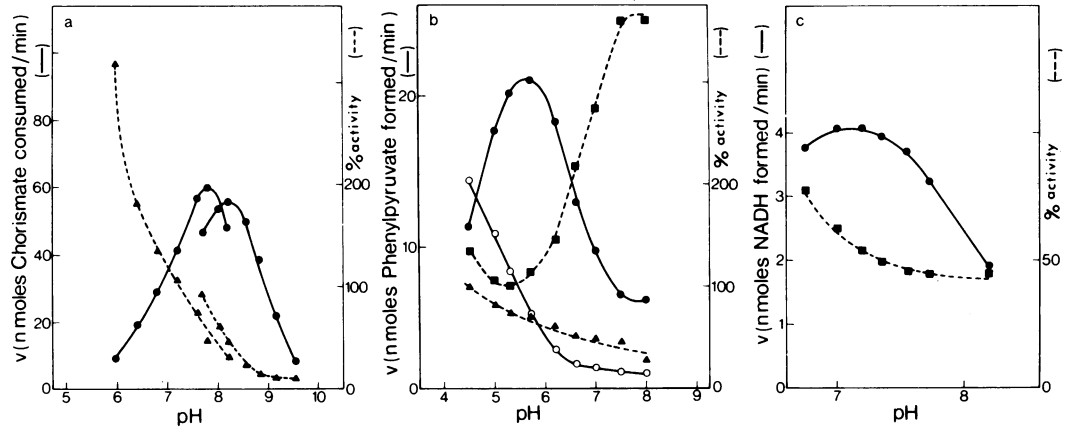


FIG. 2. *pH dependence of chorismate mutase (a), prephenate dehydratase (b), and prephenate dehydrogenase (c) activity in the presence of effectors. Symbols: (●) Without any effector; (▲) with phenylalanine at the concentrations of (a) 1 mM and (b) 8 μM; (■) with tyrosine at concentrations of (b) 5 μM and (c) 0.5 mM. The chemical decay of prephenate is shown in (b) (○) by the formation of phenylpyruvate. Enzyme activities determined in the presence of effectors are expressed as percentage of activity and related to that activity obtained without any effector at a given pH, set as 100%. (a) The reaction mixture contained in 1.0 ml; 50 mM potassium phosphate or 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer; 1 mM potassium chorismate; and 1.7 μg of purified chorismate mutase-prephenate dehydratase. (b) The reaction mixture contained, in 0.50 ml: 50 mM potassium phosphate, 1 mM potassium prephenate, and 3 μg of purified chorismate mutase-prephenate dehydratase. In the presence of tyrosine only 0.8 μg of enzyme was used. (c) The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, 0.2 mM potassium prephenate, 1 mM NAD, and 0.5 μg of purified prephenate dehydrogenase.*

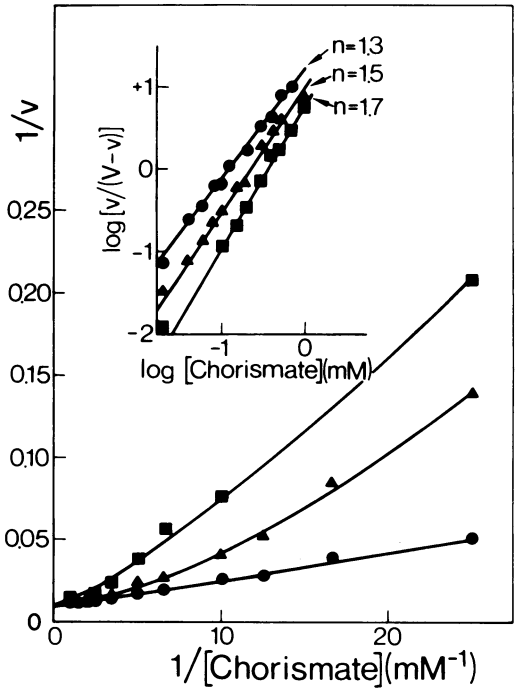


FIG. 3. *Double-reciprocal plot of the substrate saturation of chorismate mutase. The reaction mixture contained, in 1.0 ml: 50 mM potassium phosphate, pH 7.8; varying concentrations of potassium chorismate; and 2.5 μg of purified chorismate mutase-prephenate dehydratase. Phenylalanine was included as indicated. Symbols: (●) No phenylalanine; (▲) 0.1 mM phenylalanine; and (■) 0.5 mM phenylalanine. Hill plots of these data are given in the insert.*

ylpyruvate, was insignificant because initial rates were taken. The prephenate concentration used never exceeded the $K_m$ of prephenate dehydratase. Therefore the amount of phenylpyruvate formed was minimal during the time required to measure the initial rates of chorismate utilization.

Further evidence for negative cooperative interactions was obtained from linear Hill plots, with $n$ values ranging from 1.04 to 0.74. Plots of $1/v$ versus inhibitor concentrations also exhibited concave downward curvature.

Although the negative cooperative effects need further investigation, it is very likely that the catalytic site of prephenate dehydratase functions as a regulatory site for chorismate mutase.

**Effectors influencing prephenate dehydratase.** The substrate saturation curve of prephenate dehydratase for prephenate was hyperbolic. The double-reciprocal plot was linear, yielding a $K_m$ of 0.67 mM (Fig. 6). Neither the inclusion of the activator tyrosine nor the presence of the inhibitors phenylalanine and tryptophan in the reaction mixture modified the Michaelis-Menten type of substrate saturation kinetics. As shown for phenylalanine (Fig. 6), the $K_m$ for prephenate increased. Tyrosine, however, exhibited just the opposite effect. The reaction rate of the enzyme increased in direct response to the activator concentration. Tyrosine affected both maximal velocity and $K_m$ for prephenate, indicated by different intercepts and slopes of the double-reciprocal plots (Fig. 7). Similar kinetic data are reported for prephenate dehydrogenase from *Bacillus subtilis* in the presence of the inhibitors tryptophan and *p*-hydroxyphenylpyruvate (4). The kinetic data were further analyzed by replotting the slopes obtained from the double-reciprocal plots versus inhibitor and activator concentrations, respectively (5). The resulting slope replots for phenylalanine and tryptophan inhibition were linear (Fig. 8). The inhibition constant ($K_{is}$) of such replots was determined from the horizontal intercept, resulting in a value for phenylalanine of 2.6 μM and an almost 10-fold higher value for tryptophan (23 μM). The activation of prephenate dehydratase by tyrosine (Fig. 8a) was in accordance with a hyperbolic activation curve (5).

In crude extracts, prephenate dehydratase was significantly less inhibited by phenylalanine, and almost no inhibitory effect of tryptophan could be detected. However, compared with purified enzyme preparations, the protein was more strongly activated by tyrosine. These observations and the finding that the total activity of the enzyme increased during the purification, unless activated (11), led to the assumption that prephenate dehydratase was partially inhibited in the crude extract. Although this effect was not released by dialysis, it is likely that the inhibitor was tightly bound to the protein and dissociated during the course of purification. It is unlikely that the low activity of prephenate dehydratase in crude extracts was due to another prephenate-consuming reaction. Prephenate dehydrogenase did not function under the assay conditions because of its cofactor dependency.

**Effect of phenylalanine analogues on chorismate mutase and prephenate dehydratase activity.** The effect of phenylalanine analogues on chorismate mutase-prephenate dehydratase is shown in Table 1. The D-isomer of phenylalanine did not exhibit any remarkable effect on either activity; *p*-fluorophenylalanine inhibited only prephenate dehydratase and left the mu-

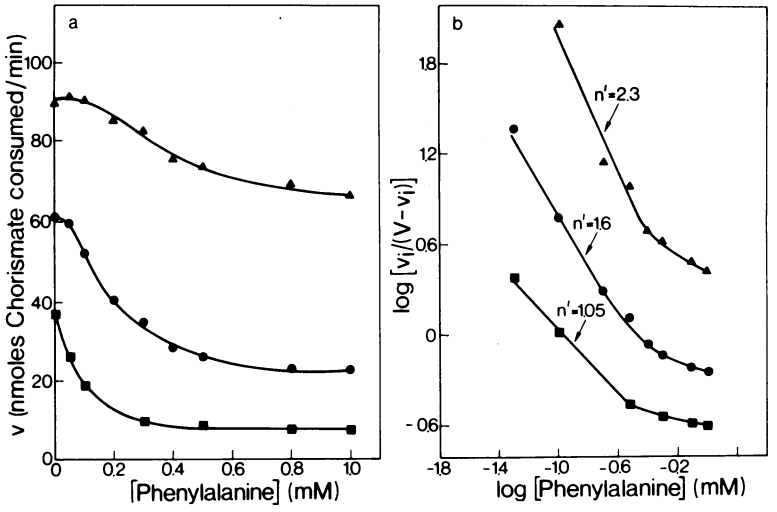


FIG. 4. *Effect of phenylalanine on chorismate mutase activity. (a) The reaction mixture contained, in 1.0 ml: 50 mM potassium phosphate, pH 7.8, varying concentrations of phenylalanine and 2.5 μg purified enzyme. Potassium chorismate was used at following concentrations: (▲) 0.7 mM; (●) 0.2 mM; and (■) 0.08 mM. The corresponding Hill plots of these data are shown in (b).*

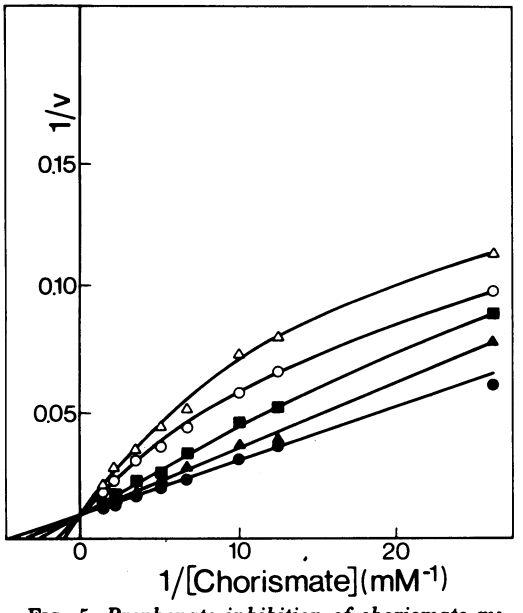


FIG. 5. *Prephenate inhibition of chorismate mutase. The data are presented in a double-reciprocal plot. The reaction mixture contained, in 1.0 ml: 50 mM potassium phosphate, pH 7.8; different concentrations of potassium chorismate; and 2.5 μg of purified enzyme. Potassium prephenate was included as indicated. Symbols: (●) No potassium prephenate; (▲) 0.1 mM; (■) 0.2 mM; (○) 0.4 mM; and (△) 0.7 mM potassium prephenate.*

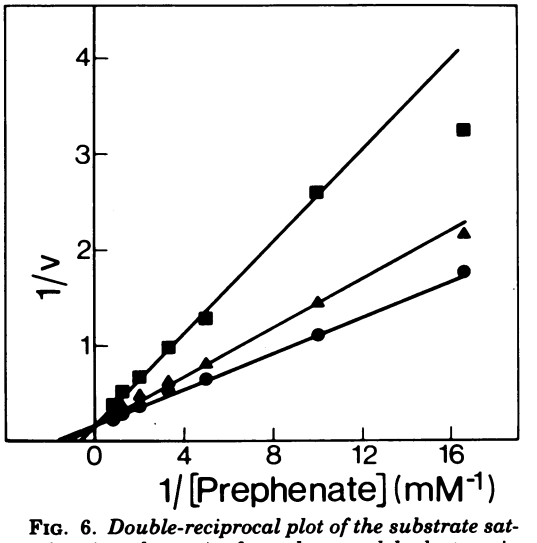


FIG. 6. *Double-reciprocal plot of the substrate saturation (prephenate) of prephenate dehydratase in the presence of phenylalanine. The reaction mixture contained, in 0.50 ml; 50 mM potassium phosphate, pH 7.8; varying concentrations of potassium prephenate; and 1.7 μg of purified enzyme. Phenylalanine was included at fixed concentrations as indicated. Symbols: (●) No phenylalanine; (▲) 1 μM; and (■) 4 μM phenylalanine.*

tase activity unaffected. Both enzyme activities were sensitive to inhibition by *m*-fluorophenylalanine. Intermediates such as phenylpyruvate and *p*-hydroxyphenylpyruvate did not influence chorismate mutase activity. They interfere with the assay procedure for prephenate dehydratase, and therefore they could not be included in the investigation on this enzyme activity.

**Effect of tyrosine on prephenate dehydrogenase activity.** The substrate saturation curve

of prephenate dehydrogenase was measured with prephenate as the variable substrate at a fixed saturating concentration of the second substrate, NAD; the resulting curve was hyperbolic. The $K_m$ for prephenate extrapolated from the linear double-reciprocal plot was found to be 0.045 mM. The inclusion of tyrosine in the reaction mixture caused sigmoidicity of the prephenate saturation curve (Fig. 9a). The double-reciprocal plot was concave upward. Hill plots of these data yielded straight lines with slopes of 1.0 to 1.4, indicating positive cooperativity of prephenate (Fig. 9b). Tyrosine did not affect the linearity of the double-reciprocal plot when NAD was the variable substrate. In the presence of tyrosine the $K_m$ for NAD remained constant (0.14 mM), indicating a noncompetitive type of inhibition (Fig. 10).

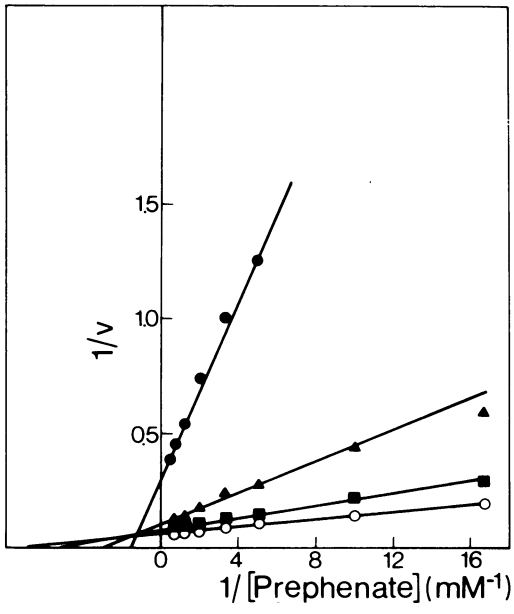


FIG. 7. *Activation of prephenate dehydratase by tyrosine. Data are presented in a double-reciprocal plot. The reaction mixture contained, in 0.50 ml: 50 mM potassium phosphate, pH 7.8; varying concentrations of potassium prephenate; and 0.85 μg of purified enzyme. Tyrosine was included at fixed concentrations as indicated. Symbols: (●) No tyrosine; (▲) 2 μM; (■) 8 μM; and (○) 20 μM tyrosine.*

The inhibition of prephenate dehydrogenase by tyrosine was competitive with respect to prephenate. Plots of $1/v$ versus tyrosine concentration were linear (Fig. 11). The inhibitor constant for tyrosine was determined as 0.06 mM. Although tyrosine exhibited heterotropic cooperative effects on prephenate binding, homotropic cooperative interactions were missing. The Hill coefficient $n'$ for the inhibition curve did not exceed a value of 1.0. A similar kinetic

TABLE 1. *Inhibition of chorismate mutase-prephenate dehydratase by phenylalanine analogues*

| Inhibitor[a] | Mutase activity (%) | Dehydratase activity (%) |
|---|---|---|
| None | 100 | 100 |
| D-Phenylalanine | 98 | 93 |
| *p*-Fluorophenylalanine | 100 | 49 |
| *m*-Fluorophenylalanine | 62 | 13 |
| Phenylpyruvate | 95 | ND[b] |
| *p*-Hydroxyphenylpyruvate | 100 | ND |

[a] Effector concentration was 1 mM.
[b] ND, Not determined.

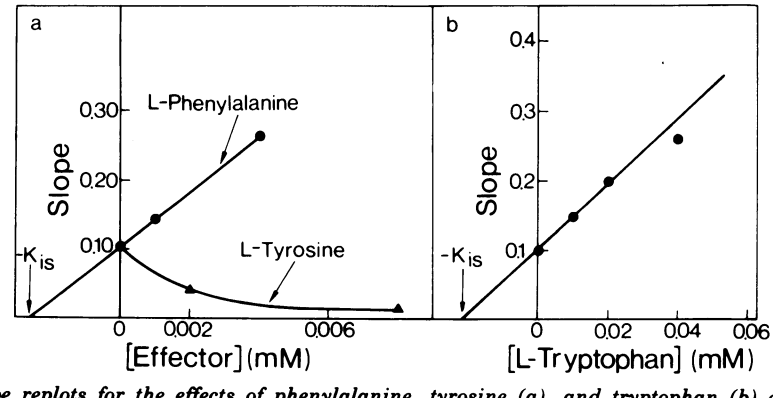


FIG. 8. *Slope replots for the effects of phenylalanine, tyrosine (a), and tryptophan (b) on prephenate dehydratase activity. The data from Fig. 6 and 7 are replotted as slopes that were taken from the different lines of the Lineweaver-Burk plots with respect to effector concentration. The double-reciprocal plots obtained in the presence of tryptophan are not shown. However, the assay mixture was the same as described in Fig. 6 except that tryptophan was used instead of phenylalanine. The data from Fig. 7 were calculated for a protein concentration of 1.7 μg.*

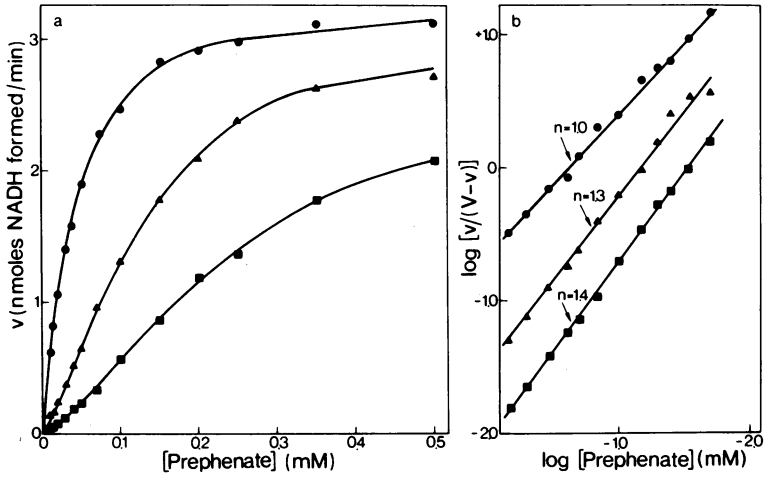


FIG. 9. *Substrate saturation curves of prephenate dehydrogenase for prephenate in the presence of tyrosine. (a) The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, pH 7.8; 1 mM NAD; different concentrations of potassium prephenate; 0.35 μg of purified enzyme; and tyrosine as indicated. Symbols: (●) No tyrosine; (▲) 0.2 mM; and (■) 1 mM tyrosine. (b) Hill plots of the data obtained from (a).*

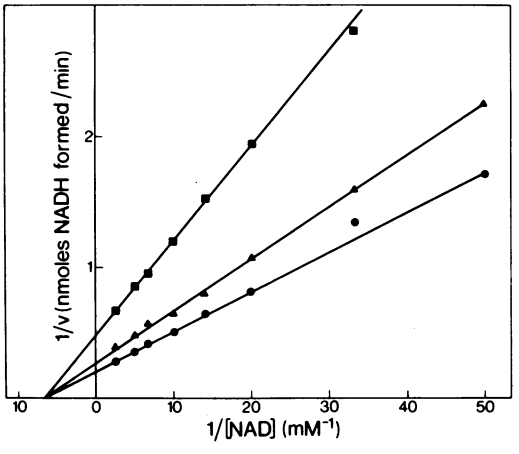


FIG. 10. *Double-reciprocal plot of NAD saturation for prephenate dehydrogenase. The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, pH 7.8; 0.2 mM potassium prephenate; varying concentrations of NAD; and 0.45 μg of purified enzyme. Tyrosine was included at fixed concentrations as indicated. Symbols: (●) No tyrosine; (▲) 0.1 mM; and (■) 0.5 mM tyrosine.*

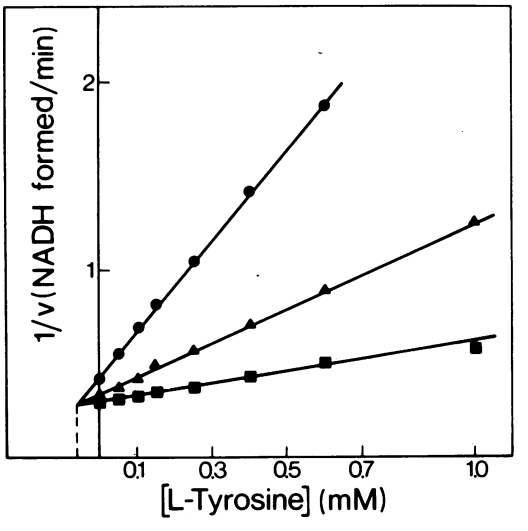


FIG. 11. *Effect of tyrosine on prephenate dehydrogenase activity. The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, pH 7.8; 1 mM NAD; varying concentrations of tyrosine; and 0.35 μg of purified enzyme. Potassium prephenate was used at the following concentrations: (●) 0.05 mM; (▲) 0.1 mM; and (■) 0.2 mM.*

behavior is reported for prephenate dehydrogenase from *B. subtilis* (4). Furthermore, prephenate dehydrogenase was inhibited by its product *p*-hydroxyphenylpyruvate, and inhibition was competitive with respect to prephenate. The $K_i$ for *p*-hydroxyphenylpyruvate was 0.13 mM. Structurally related compounds such as dihydroxyphenylalanine and cumaric acid inhibited the enzyme competitively. The inhibition constants were determined as 0.47 mM and 0.20 mM, respectively.

**Competition for prephenate.** Experiments with crude extracts indicated that tyrosine not only inhibited the activity of prephenate dehydrogenase but also affected the linearity of the reaction rate. Without tyrosine the time course

was linear for at least 5 min (Fig. 12a [1]). When tyrosine was added the reaction rate became nonlinear with respect to time (Fig. 12a [2]). This effect was even more pronounced at a higher concentration of tyrosine (Fig. 12a [3]). Purified enzyme, although still inhibited by tyrosine, revealed a linear reaction rate (Fig. 12b [1, 3]). This observation led to the conclusion that the apparent inactivation of prephenate dehydrogenase by tyrosine in crude extract was an indirect effect and primarily caused by prephenate dehydratase. Prephenate dehydratase was activated in the presence of tyrosine; its affinity for prephenate increased so that less substrate became available for prephenate dehydrogenase. As a result of this, the reaction rate of the latter enzyme slowed down. The effect of tyrosine was reversible by phenylalanine (not shown).

The observations made with crude extract obviously reflect the balanced synthesis of phenylalanine and tyrosine in vivo. The concerted action of enzymes and effectors that got partially lost by separating prephenate dehydratase from prephenate dehydrogenase was reconstructed by mixing both purified enzyme preparations (Fig. 12b [4]).

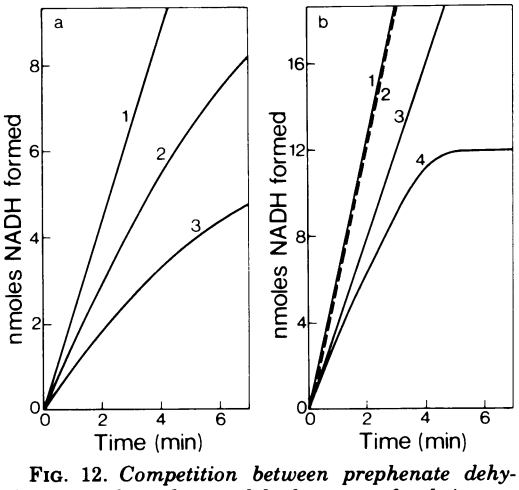


FIG. 12. *Competition between prephenate dehydratase and prephenate dehydrogenase for their common substrate. (a) The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, pH 7.8; 1 mM NAD; 0.2 mM potassium prephenate; and crude extract of 187 μg of protein (1). Tyrosine was added at the following concentrations: (2) 0.2 mM; (3) 1 mM. (b) The reaction mixture contained, in 1.0 ml: 100 mM potassium phosphate, pH 7.8; 1 mM NAD; 0.2 mM potassium prephenate; and 0.8 μg of purified prephenate dehydrogenase (8.9 U/mg) (1). To this assay mixture was either added (2) 1.7 μg of purified chorismate mutase-prephenate dehydratase (37 mutase units/mg) or (3) 0.2 mM tyrosine. Assay (4) contained a mixture of prephenate dehydrogenase, chorismate mutase-prephenate dehydratase, and tyrosine at the same concentrations as mentioned above.*

## DISCUSSION

In *A. eutrophus* H 16, chorismate mutase and prephenate dehydratase activities reside in a single protein, whereas prephenate dehydrogenase is not associated with other enzyme activities of the phenylalanine-tyrosine pathway (11). This fact favors the assumption that phenylalanine formation is facilitated and the flow of chorismic acid directed toward phenylalanine. Furthermore, phenylalanine is a precursor of tyrosine in tyrosine auxotrophic mutants. The bacterium is able to hydroxylate phenylalanine (12). However, phenylalanine hydroxylase as an inducible enzyme is probably involved in the catabolism of phenylalanine as similarly reported for *Pseudomonas aeruginosa* (2). Therefore, under normal conditions of growth, tyrosine is synthesized by prephenate dehydrogenase followed by transamination. The highly sensitive control of the three enzymes specifically involved in phenylalanine and tyrosine formation allows a well-balanced production of both amino acids (Fig. 13).

The first point of regulation is chorismate mutase, which is allosterically inhibited by phenylalanine. Phenylalanine increased the cooperativity of chorismate and revealed homotropic cooperative interactions, indicated by a Hill coefficient of $n' = 2.3$. The presence of phenylalanine decreased the apparent affinity of chorismate mutase for chorismate and inhibited the enzyme up to a maximum of 70%, which still provides enough prephenate to guarantee tyrosine synthesis. The enzyme activity was also inhibited by its product prephenate. Prephenate caused negative cooperativity of chorismate, evidence for interaction between different binding sites. Positive as well as negative cooperativity exhibited by one enzyme are also reported for cytidine 3'-triphosphate synthetase (22). It is likely that in the case of chorismate mutase the negative cooperativity is due to the second catalytic function of the enzyme, which binds prephenate as a substrate. The catalytic site of prephenate dehydratase could be a regulatory site for chorismate mutase. Evidence for chorismate and prephenate binding sites not being identical is as follows: (i) mutants were isolated that were only missing prephenate dehydratase while retaining normal chorismate mutase activity (11); and (ii) in the overall reaction, prephenate dissociated from the protein before conversion

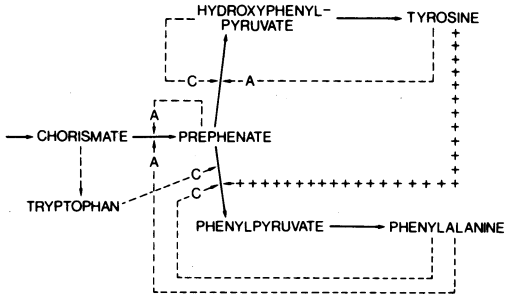


FIG. 13. *Regulation of phenylalanine and tyrosine biosynthesis in Alcaligenes eutrophus H 16. Symbols: (- - -) Feedback inhibition; (+++) activation. The mechanisms of inhibition are indicated by (A) allosteric or (C) competitive.*

to phenylpyruvate (11). Functionally distinct sites for chorismate mutase and prephenate dehydratase are also described for *Salmonella typhimurium* (28). The dissociation of prephenate from the protein during the overall reaction provides substrates for both prephenate dehydrogenase and prephenate dehydratase. The accumulation of prephenate was experimentally shown (11). However, this was already evident from the turnover numbers of the enzyme activities concerned. Chorismate mutase revealed a turnover of 2,210 mol/min per mol of enzyme. Prephenate dehydratase exhibited a turnover number of 350, which increased up to 980 when the enzyme was activated by tyrosine. The turnover number for prephenate dehydrogenase could not be determined because the enzyme had not been purified to homogeneity.

The most sensitive response to even small changes in effector concentration was shown by prephenate dehydratase. The activity was multivalently controlled by phenylalanine, tyrosine, and tryptophan. Prephenate dehydrogenase, however, was only feedback inhibited by tyrosine and inhibited by its product *p*-hydroxyphenylpyruvate ($K_i$ = 0.13 mM). The inhibition of tyrosine as reported for *B. subtilis* (4) and *Aerobacter aerogenes* (7) was competitive with respect to prephenate ($K_i$ = 0.06 mM) and noncompetitive with respect to NAD. Tyrosine, in addition to inhibiting its own synthesis, stimulated phenylalanine formation manyfold at extremely low concentrations (8 $\mu$M). This was achieved by an increased affinity of prephenate dehydratase for prephenate and a simultaneous increase in maximal velocity. In the absence of effectors, prephenate dehydrogenase had a more than 10-fold higher affinity for prephenate ($K_m$ = 0.045 mM) than prephenate dehydratase ($K_m$ = 0.67 mM), thus allowing prephenate to be channeled toward tyrosine. This effect was even more pronounced when phenylalanine was present because phenylalanine inhibited prephenate dehydratase competitively ($K_i$ = 2.6 $\mu$M). Increasing the concentration of tyrosine gave just the opposite effect. By activating prephenate dehydratase, the flow of prephenate was shifted toward phenylalanine.

Furthermore, prephenate dehydratase was competitively inhibited by tryptophan ($K_i$ = 23 $\mu$M). Although the physiological significance of this effect is not fully understood, other examples of cross-pathway regulations are known. Prephenate dehydratase from *B. subtilis* is inhibited by tryptophan and activated by leucine and methionine (4, 27). Chorismate mutase from *Neurospora crassa* (1), *Euglena gracilis* (31), and *Saccharomyces cerevisiae* (30) is activated by tryptophan. Prephenate dehydrogenase from both *N. crassa* (3) and *S. cerevisiae* (23) is subject to cross-pathway activation by phenylalanine.

The regulation pattern presented in Fig. 13 fits well with the organization of the enzymes concerned. The high sensitivity of the system obviously reflects the situation in vivo. In vitro the competition between the two prephenate-utilizing enzymes could be demonstrated in crude extracts and in a reconstructed system containing the purified enzymes and, as effectors, tyrosine and phenylalanine. The reconstructed regulation system functioned as predicted from the kinetic data.

## LITERATURE CITED

1. **Baker, T. I.** 1968. Phenylalanine-tyrosine biosynthesis in *Neurospora crassa*. Genetics 58:351-359.
2. **Calhoun, D. H., D. L. Pierson, and R. A. Jensen.** 1973. Channel shuttle mechanism for the regulation of phenylalanine and tyrosine synthesis of a metabolic branchpoint in *Pseudomonas aeruginosa*. J. Bacteriol. 113:241-251.
3. **Catcheside, D. E. A.** 1969. Prephenate dehydrogenase from *Neurospora:* feedback activation by phenylalanine. Biochem. Biophys. Res. Commun. 36:651-656.
4. **Champney, W. S., and R. A. Jensen.** 1970. The enzymology of prephenate dehydrogenase in *Bacillus subtilis*. J. Biol. Chem. 245:3763-3770.
5. **Cleland, W. W.** 1970. Steady state kinetics, p. 1-65. *In* P. D. Boyer (ed.), The enzymes, vol. 2, 3rd ed. Academic Press Inc., New York.
6. **Cotton, R. G. H., and F. Gibson.** 1965. The biosynthesis of phenylalanine and tyrosine; enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. Biochim. Biophys. Acta 100:76-88.
7. **Cotton, R. G. H., and F. Gibson.** 1967. The biosynthesis of tyrosine in *Aerobacter aerogenes:* partial purification of the protein. Biochim. Biophys. Acta 147:222-237.
8. **Davidson, B. E., E. H. Blackburn, and T. A. A. Dopheide.** 1972. Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12. 1. Purification, molecular weight, and amino acid composition. J. Biol. Chem. 247:4441-4446.
9. **Dayan, J., and D. B. Sprinson.** 1971. Enzyme altera-

tions in tyrosine and phenylalanine auxotrophs of *Salmonella typhimurium*. J. Bacteriol. 108:1174–1180.

10. **Dopheide, T. A. A., P. Crewther, and B. E. Davidson.** 1972. Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12. II. Kinetic properties. J. Biol. Chem. 247:4447–4452.
11. **Friedrich, B., C. G. Friedrich, and H. G. Schlegel.** Purification and properties of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase from *Alcaligenes eutrophus*. J. Bacteriol. 126:712–722.
12. **Friedrich, B., and H. G. Schlegel.** 1972. Die Hydroxylierung von Phenylalanin durch *Hydrogenomonas eutropha H 16*. Arch. Mikrobiol. 83:17–31.
13. **Friedrich, B., and H. G. Schlegel.** 1975. Aromatic amino acid biosynthesis in *Alcaligenes eutrophus H 16*. II. The isolation and characterization of mutants auxotrophic for phenylalanine and tyrosine. Arch. Microbiol. 103:141–149.
14. **Gibson, F.** 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. 90:256–261.
15. **Gibson, F.** 1968. Chorismic acid. Biochem. Prep. 12:94–97.
16. **Gibson, F., and J. Pittard.** 1968. Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bacteriol. Rev. 32:465–492.
17. **Görisch, H., and F. Lingens.** 1974. Chorismate mutase from *Streptomyces*. Purification, properties, and subunit structure of the enzyme from *Streptomyces aureofaciens* Tü 24. Biochemistry 13:3790–3794.
18. **Jensen, R. A.** 1969. Metabolic interlock. Regulatory interactions exerted between biochemical pathways. J. Biol. Chem. 244:2816–2823.
19. **Jensen, R. A., D. Nasser, and E. W. Nester.** 1967. Comparative control in microorganisms. J. Bacteriol. 94:1582–1593.
20. **Koch, G. L. E., D. C. Shaw, and F. Gibson.** 1970. Studies on the subunit structure of chorismate mutase-prephenate dehydrogenase from *Aerobacter aerogenes*. Biochim. Biophys. Acta 212:387–395.
21. **Koch, G. L. E., D. C. Shaw, and F. Gibson.** 1971. The purification and characterization of chorismate mutase-prephenate dehydrogenase from *E. coli* K-12. Biochim. Biophys. Acta 229:795–804.
22. **Levitzki, A., and D. E. Koshland, Jr.** 1969. Negative cooperativity in regulatory enzymes. Proc. Natl. Acad. Sci. U.S.A. 62:121–128.
23. **Lingens, F.** 1968. Die Biosynthese aromatischer Aminosäuren und deren Regulation. Angew. Chem. 80:384–394.
24. **Lingens, F., W. Goebel, and H. Uesseler.** 1967. Regulation der Biosynthese der aromatischen Aminosäuren in *Saccharomyces cerevisiae*. 2. Repression, Induktion und Aktivierung. Eur. J. Biochem. 1:363–374.
25. **Monod, J., J. Wyman, and J. P. Changeux.** 1965. On the nature of allosteric transitions: a plausible model. J. Mol. Biol. 12:88–118.
26. **Pittard, J., and F. Gibson.** 1970. The regulation of biosynthesis of aromatic amino acids and vitamins. Curr. Top. Cell. Regul. 2:29–63.
27. **Rebello, J. L., and R. A. Jensen.** 1970. Metabolic interlock. The multi-metabolite control of prephenate dehydratase activity in *Bacillus subtilis*. J. Biol. Chem. 245:3738–3744.
28. **Schmit, J. C., S. W. Artz, and H. Zalkin.** 1970. Chorismate mutase-prephenate dehydratase. Evidence for distinct catalytic and regulatory sites. J. Biol. Chem. 245:4019–4027.
29. **Schmit, J. C., and H. Zalkin.** 1969. Chorismate mutase-prephenate dehydratase. Partial purification and properties of the enzyme from *Salmonella typhimurium*. Biochemistry 8:174–181.
30. **Sprössler, B., U. Lensen, and F. Lingens.** 1970. Eigenschaften der Chorismat-Mutase aus *Saccharomyces cerevisiae* S 288 C. Hoppe-Seyler's Z. Physiol. Chem. 351:1178–1182.
31. **Weber, H. L., and A. Böck.** 1970. Chorismate mutase from *Euglena gracilis:* purification and regulatory properties. Eur. J. Biochem. 16:244–251.