Chorismate Mutase–Prephenate Dehydratase. Partial Purification and Properties of the Enzyme from *Salmonella typhimurium**

Joseph C. Schmit† and H. Zalkin

ABSTRACT: The protein or protein complex carrying chorismate mutase and prephenate dehydratase activities has been partially purified from *Salmonella typhimurium*. End-product inhibition of both activities by phenylalanine is obtained. Inhibition by phenylalanine is partially competitive with chorismate and with prephenate. Analysis of inhibition by the Hill equation yields values of n' of 2.0 and 1.7 for chorismate mutase and prephenate dehydratase activities, respectively, and suggests cooperative interaction of phenylalanine sites. Sucrose gradient centrifugation experiments show that phenylalanine and dithiothreitol cause large changes in the $s_{20,w}$ value of the enzyme. Treatment with 1 mM phenylalanine causes a change in $s_{20,w}$ from 5.3 to 6.3 S; treatment with 0.1 mM dithiothreitol causes a change from 5.3 to 6.9 S. Analysis of substrate saturation data, obtained in the absence of phenylalanine, by the Hill equation yields a value of n of 1.1 for both chorismate mutase and prephenate dehydratase activities and suggests little or no cooperative interaction of substrate sites under these conditions. Cooperativity is suggested by maximal values of n of 2.0 and 1.9 for chorismate mutase and prephenate dehydratase activities obtained in the presence of 0.24 mM phenylalanine. In the over-all conversion of chorismate into phenylpyruvate, prephenate dissociates from the enzyme and accumulates in the reaction mixture. After a lag prephenate is converted into phenylpyruvate.

Three soluble multiactivity enzymes or enzyme complexes appear to function in the utilization of chorismate for phenylalanine, tyrosine, and tryptophan biosynthesis in *Escherichia coli*, *Salmonella typhimurium*, and *Aerobacter aerogenes*. An enzyme complex containing anthranilate synthetase activity and anthranilate–5-phosphoribosyl pyrophosphate phosphoribosyltransferase activity catalyzes the first two reactions of tryptophan synthesis as shown in eq 1 and 2 (Ito and Yanofsky, 1966; Bauerle and Margolin, 1966; Egan and Gibson, 1966). An enzyme or enzyme complex containing chorismate mutase and prephenate dehydrogenase activities catalyzes the over-all conversion of chorismate to 4-hydroxyphenylpyruvate, the precursor of tyrosine (Cotton and Gibson, 1967, 1968), as shown by eq 3 and 4.

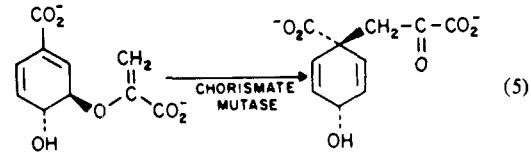
$$\text{chorismate} + \text{glutamine} \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} + \text{glutamate} \quad (1)$$

$$\text{anthranilate} + \text{5-phosphoribosyl 1-pyrophosphate} \xrightarrow{Mg^{2+}} N\text{-(5'-phosphoribosyl)anthranilate} + PP_i \quad (2)$$

$$\text{chorismate} \longrightarrow \text{prephenate} \quad (3)$$

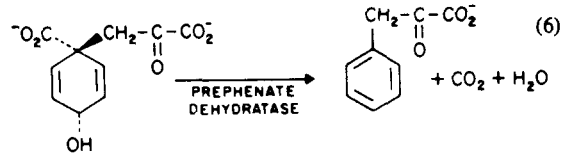
$$\text{prephenate} + NAD^+ \longrightarrow \text{4-hydroxyphenylpyruvate} + CO_2 + NADH + H^+ \quad (4)$$

Finally, an enzyme or enzyme complex containing chorismate mutase and prephenate dehydratase activities catalyzes the over-all conversion of chorismate into phenylpyruvate, the precursor of phenylalanine (Cotton and Gibson, 1965), as shown by eq 5 and 6. That these activities were physically associated was deduced from the coincidence of chorismate mutase and prephenate dehydratase activities following chromatography of extracts of *E. coli* or *A. aerogenes* on columns of DEAE-cellulose. Furthermore, phenylalanine auxotrophs were found to lack both prephenate dehydratase activity and its associated chorismate mutase activity. These results are in accord with the genetic data which indicate a single locus, for phenylalanine synthesis, on the *E. coli*



(5)

CHORISMATE PREPHENATE



(6)

PREPHENATE PHENYLPYRUVATE

* From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907. *Received September 22, 1968.* Supported by Grant GB-5841 from the National Science Foundation. Journal Paper No. 3491, Purdue Agricultural Experiment Station.

† Recipient of a David Ross Grant, Purdue Research Foundation.

(Pittard and Wallace, 1966) and *S. typhimurium* (Nishioka *et al.*, 1967) chromosome.

The purpose of this report is to describe the partial purification of prephenate dehydratase activity and its associated chorismate mutase activity from *S. typhimurium* and to describe some properties of this enzyme or enzyme complex. The enzyme or enzyme complex is designated chorismate mutase–prephenate dehydratase.

Experimental Procedure

Materials and Methods. Barium chorismate was prepared according to the method of Gibson (1964). The concentration of barium chorismate was determined by enzymatic conversion into anthranilate using partially purified anthranilate synthetase from *S. typhimurium*. The reaction mixture for this assay contained 5 mM glutamine, 5 mM $MgCl_2$, 50 mM potassium phosphate (pH 7.0), partially purified anthranilate synthetase, and 0.1–5.0 mμmoles of barium chorismate in a final volume of 1.0 ml. Anthranilate was measured directly in an Aminco-Bowman spectrophotofluorimeter as described previously (Zalkin and Kling, 1968). Quantitation of fluorescence was by reference to a calibration curve. Barium prephenate was prepared by mildly heating solutions of barium chorismate and was isolated according to Gibson (1964). Barium prephenate was assayed by conversion into phenylpyruvate. Barium prephenate (0.02–0.1 μmole) was incubated in 0.4 ml of 0.5 N HCl for 10 min at 37° and then 1.6 ml of 1.0 N NaOH was added. The concentration of phenylpyruvate was determined from the absorbance at 320 mμ and a molar extinction coefficient of 17,500 (Cotton and Gibson, 1965). The barium salts of chorismate and prephenate were 70–90% pure according to the assays described. Barium chorismate and barium prephenate were converted into the potassium salts with a twofold molar excess of potassium sulfate. All other chemicals and reagents were commercial preparations of the highest purity available and were used without further purification.

S. typhimurium aro D80, an aromatic amino acid bradytroph, was grown under conditions that gave derepression of chorismate mutase and prephenate dehydratase activities. Cells were grown in 2-l. flasks with 1 l. of medium containing 0.4% glucose, salts (Vogel and Bonner, 1956), 40 mg/l. of L-tyrosine, 20 mg/l. of L-tryptophan, and 2.5 mg/l. of L-phenylalanine. Flasks were inoculated with 5 ml of an overnight culture, grown in the same medium, and incubated at 37° with vigorous shaking for 20 hr. Cells were harvested by centrifugation, were washed once with 0.05 M potassium phosphate (pH 7.4), and were then frozen at −20° until used.

Chorismate Mutase and Prephenate Dehydratase Assays. Chorismate mutase activity was assayed by measurement of the rate of formation of prephenate (plus phenylpyruvate) or the rate of disappearance of chorismate. For the former method, reaction mixtures contained 1 mM potassium chorismate, 0.1 M potassium phosphate (pH 7.4), 0.1 mg/ml of bovine serum albumin, and a limiting amount of enzyme in a final volume of 0.5 ml. In order to study inhibition of chorismate mutase activity by phenylalanine, the potassium chorismate concentration was reduced to 0.2 mM and phenylalanine was included. Appropriate control reaction mixtures without enzyme were always included. Reaction mixtures were usually incubated for 10 min at 37°. Deviations from this procedure are specified where applicable. Reactions were terminated with 0.2 ml of 1 N HCl and the mixtures were then incubated at 37° for 10 min to convert prephenate into phenylpyruvate. If necessary, denatured protein was removed by centrifugation in a clinical centrifuge. Aliquots were taken for spectrophotometric determination of phenylpyruvate in 1.0 N NaOH at 320 mμ. Correction for the nonenzymatic reaction was always made. For assay of chorismate mutase activity by measurement of the initial rate of chorismate utilization (Figure 8), reaction mixtures contained variable amounts of potassium chorismate, 0.1 M potassium phosphate (pH 7.4), potassium prephenate as indicated, 0.1 mg/ml of bovine serum albumin, and a limiting amount of enzyme. The rate of chorismate disappearance was measured in a 1-ml cuvet at 274 mμ and 37° in a Cary 15 spectrophotometer. The reference cuvet contained a similar reaction mixture from which enzyme was omitted. A molar extinction coefficient of 2630 (Edwards and Jackman, 1965) was used to calculate the concentration of chorismic acid that was utilized.

Prephenate dehydratase activity was assayed in 0.25-ml reaction mixtures containing 1 mM potassium prephenate, 0.1 M potassium phosphate (pH 7.4), 0.1 mg/ml of bovine serum albumin, and a limiting amount of enzyme. A control reaction mixture lacking enzyme was always included. After incubation for 5 min at 37° the reaction was terminated with 0.75 ml of 1.33 N NaOH and phenylpyruvate was determined by its absorbance at 320 mμ. Deviations from this procedure are specified where applicable.

A unit of activity corresponds to the conversion of 1 μmole of substrate in 10 min. Specific activity is defined as units per milligram of protein. Protein was determined by the biuret method or from the absorbance at 280 and 260 mμ (Layne, 1957).

Sucrose Gradient Centrifugation. Linear 5–20% sucrose gradients were prepared with: (A) 0.1 M potassium phosphate (pH 7.4), (B) 0.1 M potassium phosphate (pH 7.4) and 1 mM phenylalanine, and (C) 0.1 M potassium phosphate (pH 7.4) and 0.1 mM dithiothreitol. The gradients were equilibrated for 6 hr at 2°. Chorismate mutase–prephenate dehydratase and yeast alcohol dehydrogenase were prepared by dialyzing for 2 hr against the same buffer solution used in the different gradients. To each 4.5-ml gradient was added 0.1 ml of the appropriately dialyzed enzymes. The tubes were centrifuged at 35,000 rpm in an SW 39 rotor of a Spinco Model L centrifuge for 12 hr at 2°, then punctured, and about 33 10-drop fractions were collected and assayed for chorismate mutase, prephenate dehydratase, and alcohol dehydrogenase activities. The $s_{20,w}$ value of chorismate mutase–prephenate dehydratase was estimated by comparison with yeast alcohol dehydrogenase (Martin and Ames, 1961). The $s_{20,w}$ value for yeast alcohol dehydrogenase was taken as 6.7 S (Hayes and Velick, 1954).

TABLE I: Summary of Purification of Chorismate Mutase–Prephenate Dehydratase.[a]

Fraction	Vol (ml)	Protein Concn (mg/ml)	Enzyme	Act. (units)	Sp Act. (units/mg)	Mutase Dehydratase	Yield (%)[b]
I. Extract	216	15.7	Mutase	2440	0.72	0.85	
			Dehydratase	2880	0.85		
II. Protamine sulfate	221	12.7	Mutase	2320	0.83	0.86	93
			Dehydratase	2690	0.96		
III. Ammonium sulfate (0–40%)	42	19.2	Mutase	895	1.11	1.0	30
			Dehydratase	854	1.06		
IV. Sephadex G-100	54	2.6	Mutase	1020	7.28	1.1	32
			Dehydratase	916	6.55		
V. DEAE-cellulose	44	0.30	Mutase	648	49.2	0.99	23
			Dehydratase	657	49.8		

[a] See Experimental Procedure for details. [b] Based on prephenate dehydrastase activity.

Results

Partial Purification of Chorismate Mutase–Prephenate Dehydratase Activities. Cells grown as described in the Experimental Procedure were routinely 8–10-fold derepressed, relative to wild-type cells, for chorismate mutase and prephenate dehydratase activities. For a typical purification, cells obtained from 12 l. of culture medium (52 g wet weight) were suspended in 200 ml of 0.05 M potassium phosphate (pH 7.4) and were broken by sonic disruption with a Branson S-125 Sonifier. Particulate material was sedimented by centrifugation at 39,000*g* for 45 min at 1° and discarded. The protein concentration of the extract was about 15 mg/ml. All succeeding steps were performed at 0–5°.

A freshly prepared 2% solution of protamine sulfate in 0.1 M potassium phosphate (pH 7.0) was added slowly with gentle stirring to a final concentration of 0.1 mg of protamine sulfate/mg of protein. Stirring was continued for 20 min after the last addition of protamine sulfate. The solution was centrifuged at 39,000*g* for 10 min and the sediment was discarded. A saturated solution of $(NH_4)_2SO_4$ at 2°, adjusted to approximately pH 7 with NH_4OH, was added slowly with stirring (66 ml of $(NH_4)_2SO_4$/100 ml of extract). Stirring was continued for 60 min after the last addition of $(NH_4)_2SO_4$ and the solution was then centrifuged at 39,000*g* for 10 min. The sediment containing about 800 mg of protein was dissolved in 25 ml of 0.05 M potassium phosphate (pH 7.4) plus 1 mM phenylalanine and was dialyzed against 2 l. of the same buffer solution for 3 hr. The dialyzed solution was applied to a 4.2 × 64 cm column of Sephadex G-100. Elution was with 0.05 M potassium phosphate (pH 7.4) containing 1 mM phenylalanine. Fractions containing the highest specific activity were pooled and applied to a 1.8 × 25 cm column of DEAE-cellulose. Elution was with a linear KCl gradient using 250 ml of 0.05 M potassium phosphate (pH 7.4) containing 1 mM phenylalanine in the mixing chamber and the same buffer solution plus 0.5 M KCl in the reservoir. Fractions with the highest specific activity were pooled. The partially purified enzyme was distributed into small aliquots and stored at −20°. A summary of a typical purification is shown in Table I.

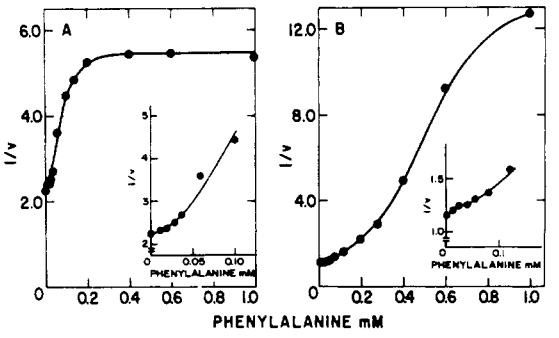


FIGURE 1: Effect of phenylalanine concentration on enzyme activity. (A) Chorismate mutase activity. (B) Prephenate dehydratase activity. Enzyme activities were assayed as described in Experimental Procedure. The inserts show expanded plots of the data for low phenylalanine concentration.

The small apparent purification by $(NH_4)_2SO_4$ precipitation (fraction III) may be due to inhibition of enzyme activity by residual $(NH_4)_2SO_4$. In more recent experiments, a three- to fourfold purification has been obtained at step III by desalting with a Sephadex G-25 column instead of employing dialysis.

The ratio of chorismate mutase activity to prephenate dehydratase activity has been found to remain relatively constant throughout a 50- to 70-fold purification. In extracts the ratio of these two activities has been variable, ranging from 0.85 to 1.20. Examination of the partially purified enzyme (fraction V) by disc gel electrophoresis (Davis, 1964) indicated heterogeneity. The inability to separate chorismate mutase activity from prephenate dehydratase activity suggests that the two activities are associated with one protein or with separate but tightly associated proteins.

End-Product Inhibition by Phenylalanine. Both activ-

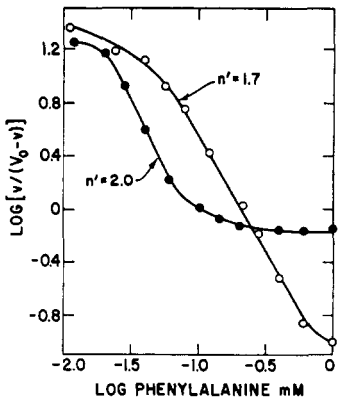


FIGURE 2: Hill plots for effect of phenylalanine concentration on chorismate mutase and prephenate dehydratase activities. The data from Figure 1A,B are replotted as log $[v/(V_0 - v)]$ with respect to log phenylalanine concentration. (●—●) Chorismate mutase and (○—○) prephenate dehydratase.

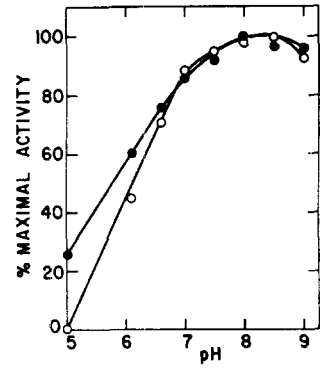


FIGURE 3: Effect of pH on chorismate mutase and prephenate dehydratase activities. Reaction mixtures are as described in the Experimental Procedure except that the buffer was a solution of 0.048 M K_2HPO_4, 0.048 M citric acid, and 0.048 M boric acid that was adjusted to the appropriate pH with HCl or KOH. Enzyme was Sephadex G-100 eluate (fraction IV). All points were corrected for nonenzymatic reaction. The symbols are as described in Figure 2.

ities, chorismate mutase and prephenate dehydratase, are inhibited by phenylalanine. Inhibition by phenylalanine is shown by plots of $1/v$ against phenylalanine concentration in Figure 1A for chorismate mutase activity and in Figure 1B for prephenate dehydratase activity. The inserts in each figure show an expansion of the data at low inhibitor concentration. From the data in Figure 1A it is deduced that inhibition of chorismate mutase activity by phenylalanine is only partial, since the curve reaches a maximum at 0.3 mM phenylalanine. Under these conditions, maximal inhibition approaches 60%. A finite initial slope of the curve is observed as the phenylalanine concentration approaches zero (Figure 1A, insert). The concave upward curvature is consistent with cooperative interactions of phenylalanine. The data of Figure 1A are plotted in Figure 2 in terms of the Hill equation (Changeux, 1963): $\log [v/(V_0 - v)] = \log K' - n' \log I$, where v is the velocity in the presence of inhibitor, V_0 the velocity in the absence of inhibitor, K' a constant, n' an interaction coefficient, and I the inhibitor concentration. In the ideal case (total cooperativity), plots of $\log [v/(V_0 - v)]$ against $\log I$ result in a straight line of slope n'. Thus, n' has been interpreted as an indication of the order of the reaction (Atkinson *et al.*, 1965) and as a measure of cooperativity (interaction coefficient; Monod *et al.*, 1965). The Hill plot for chorismate mutase (Figure 2) is linear for phenylalanine concentrations of 0.02–0.06 mM corresponding to 6–38% inhibition. The value of n' for this linear region is 2.0 which suggests cooperative interaction of phenylalanine sites in this concentration range.

Inhibition of prephenate dehydratase activity by phenylalanine is also partial. Under the conditions used for the experiment shown in Figure 1B, maximum inhibition is 91%. Similar to the case for chorismate mutase activity, the data in the insert to Figure 1B show a finite slope as the phenylalanine concentration approaches zero. Cooperativity for phenylalanine binding is suggested by the upward concavity shown in Figure 1B and the value of n' of 1.7 (Figure 2). The Hill plot for prephenate dehydratase activity is linear for the concentration range 0.06–0.6 mM phenylalanine which corresponds to 10–87% inhibition.

Phenylalanine increases the substrate concentration required for half-maximal velocity, $(S)_{0.5}$, but does not change the maximal velocity for either enzyme activity (Figure 6). Tyrosine and tryptophan are without effect on chorismate mutase and prephenate dehydratase activities. They do not modify the inhibition by phenylalanine.

Effect of pH on Chorismate Mutase and Prephenate Dehydratase Activities and on Inhibition by Phenylalanine. Chorismate mutase and prephenate dehydratase activities show similar response to pH over the range pH 5–9 (Figure 3). The small deviation between the two activities that occurs between pH 5 and 6.5 may reflect the chemical instability of prephenic acid at these pH values. It is well known that the nonenzymatic conversion of chorismate into prephenate is catalyzed by base and the nonenzymatic conversion of prephenate into

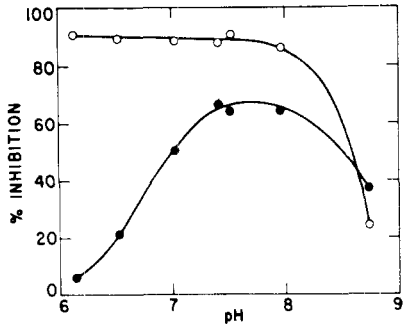


FIGURE 4: Effect of pH on inhibition of chorismate mutase and prephenate dehydratase activities by phenylalanine. Enzyme assays are described in Experimental Procedure. The phenylalanine concentration was 0.4 mM for inhibition of chorismate mutase activity and 0.6 mM for inhibition of prephenate dehydratase activity. The buffers were 0.1 M potassium phosphate from pH 6.1 to 7.4 and 0.1 M Tris-Cl from pH 7.5 to 8.7. The symbols are as described in Figure 2.

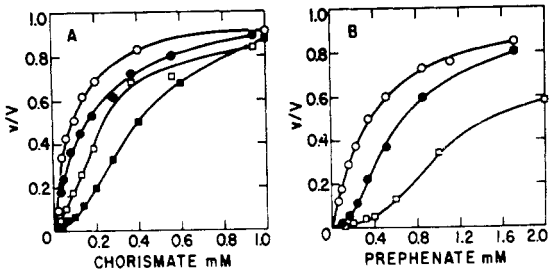


FIGURE 5: Effect of substrate concentration on enzyme activity. (A) Chorismate mutase activity. The conditions of assay were as described in the Experimental Procedure except that the potassium chorismate concentration was varied and phenylalanine was included as indicated. (○—○) No phenylalanine, (●—●) 0.04 mM phenylalanine, (□—□) 0.08 mM phenylananine, and (■—■) 0.24 mM phenylalanine. Data from two experiments were combined. (B) Prephenate dehydratase activity. The conditions of assay were as described in the Experimental Procedure except that the potassium prephenate concentration was varied and phenylalanine was included as indicated. (○—○) No phenylalanine, (●—●) 0.12 mM phenylalanine, and (□—□) 0.24 mM phenylanine. Data from two experiments were combined.

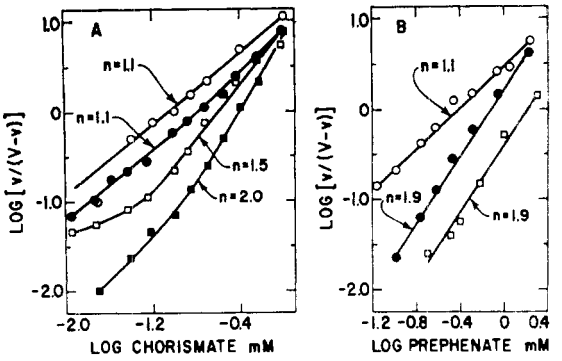


FIGURE 7: Hill plots of the data in Figure 5. (A) Chorismate mutase. Symbols as in Figure 5A. (B) Prephenate dehydratase. Symbols as in Figure 5B.

phenylpyruvate is acid catalyzed (Metzenberg and Mitchell, 1956; Gibson, 1964).

Inhibition of chorismate mutase activity and prephenate dehydratase activity by phenylalanine as a function of pH is shown in Figure 4. The two enzyme activities have different pH profiles for inhibition by phenylalanine.

Initial Velocity Patterns for Saturation by Substrates. Saturation of chorismate mutase activity and prephenate dehydratase activity by chorismate and prephenate, respectively, in the presence and absence of feedback inhibitor is shown in Figure 5. Data from two different experiments have been normalized and combined by expressing the observed initial velocity relative to the maximal velocity (v/V). In the absence of phenylalanine an apparent Michaelis hyperbolic substrate saturation curve is obtained for each activity. Substrate saturation in the presence of the feedback inhibitor, phenylalanine, yields sigmoidal curves. The data in Figure 5A,B show that the sigmoidicity of the saturation curves is increased with increasing phenylalanine concentration.

Reciprocal plots of the data from Figure 5 are shown in Figure 6. In the absence of phenylalanine the reciprocal plot for chorismate mutase activity (Figure 6A) is linear and yields a K_m of 8.0×10^{-5} M for chorismate. Curves that bend concave upward are obtained for chorismate saturation in the presence of feedback inhibitor. There is qualitative correspondence between sigmoidicity of the substrate saturation curves in Figure 5A and concavity of the curves in Figure 6A. These data are indicative of cooperative interactions for chorismate. Reciprocal plots for prephenate dehydratase activity are shown in Figure 6B. The curve obtained for saturation with prephenate, in the absence of feedback inhibitor, has slight upward curvature. Similar to the case for chorismate mutase, addition of phenylalanine causes increased sigmoidicity in the plots of v/V against prephenate concentration (Figure 5B) and increased curvature in the reciprocal plots (Figure 6B). Owing to curvature in the reciprocal plot for the data obtained in the absence of phenylalanine, a K_m cannot be calculated. For these data the substate concentration at the half-maximal rate, $(S)_{0.5}$, is 3.5×10^{-4} M. Intensification of sigmoidicity (Figure 5B) and upward curvature (Figure 6B) caused by phenylalanine is consistent with cooperative interactions for prephenate.

Figure 7 shows the substrate saturation data plotted according to the Hill equation (Changeux, 1963): log

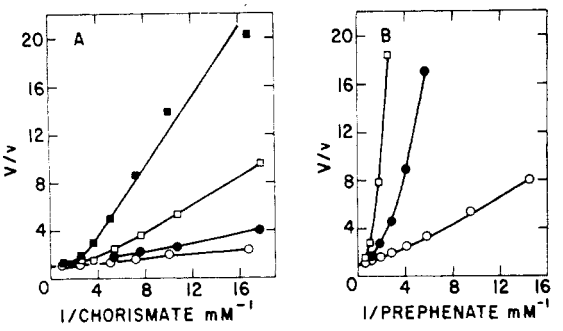


FIGURE 6: Lineweaver–Burk plots for substrate saturation. (A) Chorismate mutase activity. The data were obtained from Figure 5A. The symbols are described in Figure 5A. (B) Prephenate dehydratase activity. The data were obtained from Figure 5B. The symbols are described in Figure 5B.

TABLE II: Summary of Some Kinetic Measurements for Chorismate Mutase and Prephenate Dehydratase Activities.

Phenylalanine Concn (mM)	Chorismate Mutase			Prephenate Dehydratase	
	K_m (mM)	$(S)_{0.5}$ (mM)	n	$(S)_{0.5}$ (mM)	n
0	0.080	0.080	1.1	0.35	1.1
0.04		0.16	1.1		
0.08		0.23	1.5		
0.12				0.68	1.9
0.24		0.39	2.0	1.5	1.9

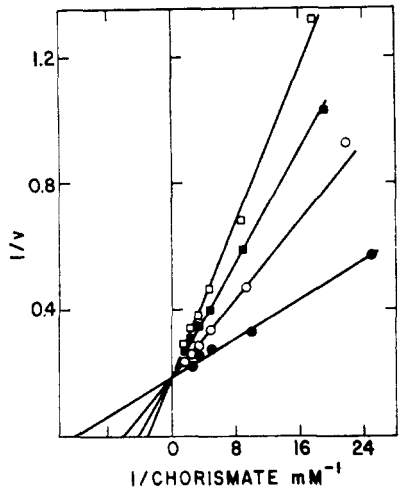


FIGURE 8: The effect of potassium prephenate concentration on chorismate mutase activity. Chorismate mutase activity was assayed by measuring the rate of potassium chorismate utilization in the presence of varying amounts of potassium prephenate. (●—●) No potassium prephenate, (○—○) 0.1 mM potassium prephenate, (■—■) 0.2 mM potassium prephenate, and (□—□) 0.3 mM potassium prephenate.

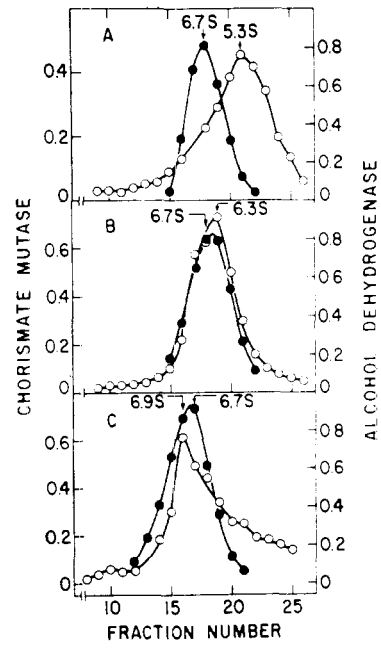


FIGURE 9: Sedimentation of chorismate mutase–prephenate dehydratase and yeast alcohol dehydrogenase in sucrose gradients. (A) Effect of 0.1 M potassium phosphate (pH 7.4). (B) Effect of 0.1 M potassium phosphate (pH 7.4) and 1 mM phenylalanine. (C) Effect of 0.1 M potassium phosphate (pH 7.4) and 0.1 mM dithiothreitol. The data in C were obtained in a separate experiment thus accounting for the shift in the peak for yeast alcohol dehydrogeanse. Chorismate mutase activity (○—○) is expressed as absorbance at 320 mμ/20 min. Yeast alcohol dehydrogenase activity (●—●) is expressed as absorbance at 340 mμ/min.

$[v/(V - v)] = n \log S - \log K$ where v, V, n, S, and K represent the observed velocity, maximal velocity, interaction coefficient, substrate concentration, and a constant, respectively. In the absence of phenylalanine $n = 1.1$ for both activities. This value of n suggests the lack of cooperativity for substrate binding in the absence of phenylalanine. The slight curvature observed for saturation of prephenate dehydratase activity in the absence of phenylalanine (Figure 6B) is not reflected in the value of n (Figure 7B). In the presence of phenylalanine, n values, taken from the maximal slope, approach 2 and thus suggest cooperativity. Values for n, $(S)_{0.5}$, and K_m, where applicable, are tabulated in Table II.

Product Inhibition of Chorismate Mutase Activity. Prephenate inhibits chorismate mutase activity and inhibition is competitive with chorismate (Figure 8). For this experiment chorismate mutase activity was assayed by measuring the initial rate of chorismate utilization spectrophotometrically. Under these conditions the K_m for chorismate is 0.084 mM and the K_i for prephenate is 0.096 mM. Because prephenate was used at concentrations below the $(S)_{0.5}$, conversion into phenylpyruvate was minimal during the time required to measure the initial rate of chorismate utilization. Attempts to study inhibition of chorismate mutase and prephenate dehydratase activities by phenylpyruvate have been unsuccessful.

Sucrose Gradient Centrifugation. A broad peak of enzyme activity corresponding to an $s_{20,w}$ value (relative to yeast alcohol dehydrogenase, 6.7 S) of approximately 5.3 S was obtained when chorismate mutase–prephenate dehydratase was sedimented in a sucrose gradient (Figure 9A). Addition of 1 mM phenylalanine to the enzyme solution and to the sucrose gradient causes a sharpening of the peak of enzyme activity and a change in sedimentation to 6.3 S (Figure 9B). Dithiothreitol also causes a change in sedimentation as shown in Figure 9C. In the presence of 0.1 mM dithiothreitol, a shift to 6.9 S was observed although there was considerable trailing into the region of the gradient corresponding to lower $s_{20,w}$ values. The position of the reference yeast alcohol dehydrogenase marker is slightly displaced because these latter data were obtained in a separate experiment. The profile of chorismate mutase obtained in the presence of phenylalanine plus dithiothreitol was identical with that shown in Figure 9B. These data suggest that phenylalanine and dithiothreitol cause changes in the conformation and/or state of aggregation of chorismate mutase–prephenate dehydratase.

The data in Figure 9 show only the profiles of chorismate mutase activity. The profiles for prephenate dehydratase activity were exactly coincident with the profiles for chorismate mutase activity in every experiment.

Formation of Free Prephenate in the Over-All Conversion of Chorismate into Phenylpyruvate. Chorismate mutase–prephenate dehydratase catalyzes the over-all conversion of chorismate into prephenate. The question arises whether prephenate is an enzyme-bound or dissociable intermediate in the over-all synthesis of phenylpyruvate from chorismate. In order to investigate this question, the rate of synthesis of prephenate and phenylpyruvate from chorismate was measured simultaneously. In the experiment described in Figure 10 chorismate was converted into prephenate without a lag, whereas synthesis of phenylpyruvate ensued only after

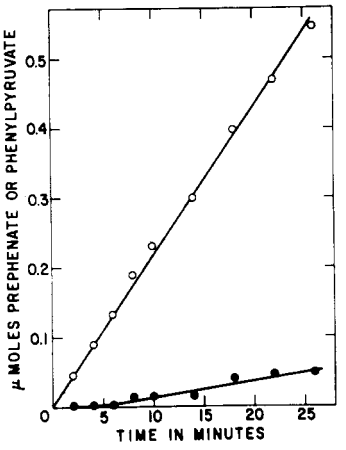


FIGURE 10: Comparison of the rate of synthesis of prephenate and phenylpyruvate from chorismate. The reaction mixture contained 2 mM potassium chorismate, 0.1 M potassium phosphate (pH 7.4) (0.2 mg of bovine serum albumin), and enzyme (0.23 unit of chorismate mutase activity) in a final volume of 2.0 ml. Aliquots were removed for determination of prephenate and phenylpyruvate. The data have been corrected for nonenzymatic reaction. The symbols are: (O—O) prephenate, (●—●) phenylpyruvate.

a lag of 6 min. Furthermore the rate of phenylpyruvate synthesis was only 10% of the rate of prephenate synthesis. These results indicate that under the conditions employed, direct synthesis of phenylpyruvate from chorismate does not occur. Rather, chorismate is converted into prephenate which dissociates from the enzyme; When sufficient prephenate accumulates, enzymatic conversion into phenylpyruvate ensues.

Discussion

In accord with the suggestion that a chorismate mutase activity and prephenate dehydratase activity are associated with a single enzyme or enzyme complex in *E. coli* and *A. aerogenes* (Cotton and Gibson, 1965), an approximately 60-fold purification of prephenate dehydratase activity from derepressed extracts of *S. typhimurium* has failed to separate the two activities (Table I). Since the crude extracts were prepared from cells that were approximately 8–10-fold derepressed for prephenate dehydratase activity, relative to the wild-type strain, the partially purified prephenate dehydratase activity was 480–600 times higher than the enzyme activity of an extract from wild-type cells. The partially purified enzyme (fraction V, Table I) is heterogeneous by the criterion of disc gel electrophoresis. Recent experiments (J. C. Schmit and H. Zalkin, unpublished data) indicate that chorismate mutase and prephenate dehydratase activities can be further purified to near homogeneity without appreciable change in the ratio of the two activities from fraction V. It is therefore concluded that chorismate mutase and prephenate dehydratase activities are tightly associated. If the two activities are separable, the native enzyme could be considered a complex. If the two activities are inseparable, the native enzyme could be considered a single oligomeric protein. Precedents for the former (Ito and Yanofsky, 1966) and latter (Plaut, 1963) possibilities are available. Genetic experiments (Nishioka *et al.*, 1967), thus far, indicate one complementation group and therefore favor a single oligomeric protein. It seems appropriate at this time to refer to the native enzyme or enzyme complex as chorismate mutase–prephenate dehydratase. Enzymes or enzyme complexes with inseparable multiple activities that participate in the biosynthesis of amino acids have recently been obtained. An enzyme or enzyme complex having threonine-sensitive homoserine dehydrogenase and aspartokinase obtained from *E. coli* K 12 (Trauffa-Bachi *et al.*, 1968) and an enzyme or enzyme complex having chorismate mutase and prephenate dehydrogenase activities from *A. aerogenes* (Cotton and Gibson, 1968) and *S. typhimurium* (Dayan and Sprinson, 1968) may have some similarity to chorismate mutase–prephenate dehydratase.

Chorismate mutase–prephenate dehydratase is sensitive to end-product inhibition by phenylalanine. Phenylalanine is a partially competitive inhibitor for both activities as shown by the data in Figures 1 and 6. For this type of inhibition, the inhibitor affects the affinity of the enzyme for the substrate, although the inhibitor and substrate combine with different groups. Finite slope on plots of $1/v$ against phenylalanine concentration (Figure 1), as the phenylalanine concentration approaches zero, may suggest that inhibition is caused by combination of a single inhibitor molecule per enzyme molecule. Combination of additional phenylalanine enhances the effect; inhibition is cooperative. Upward concavity in plots of $1/v$ against inhibitor concentration (Figure 1) corresponds to sigmoidicity in plots of v against inhibitor concentration and has been interpreted in terms of cooperative ligand binding (Monod *et al.*, 1965; Koshland *et al.*, 1966). The extent of such interactions has been analyzed by means of the Hill equation (Changeux, 1963; Atkinson *et al.*, 1965). Maximal values for n' of 2.0 and 1.7 for the effect of phenylalanine on chorismate mutase and prephenate dehydratase activities, respectively (Figure 2), are consistent with cooperative interaction of at least two phenylalanine sites; phenylalanine exhibits homotropic cooperative interactions. The relationship of the phenylalanine binding sites to the catalytic site(s) is under investigation.

The kinetics for substrate saturation of the two activities suggest cooperative interaction of substrate sites in the presence of phenylalanine; chorismate and prephenate exhibit homotropic cooperative interactions and phenylalanine exhibits heterotropic interactions. Maximal values for n of 2.0 and 1.9 for saturation of chorismate mutase and prephenate dehydratase activities in the presence of 0.24 mM phenylalanine suggest at least two binding sites for substrate. The question of whether chorismate and prephenate binding sites are identical or separate will be discussed below. For saturation of chorismate mutase in the absence of phenylalanine the double-reciprocal plot is linear (Figure 6A), indicating classical Michaelis behavior, while slight deviation from linearity is observed in the analogous plot for saturation of prephenate dehydratase activity (Figure 6B). Since Hill plots for these data yield

values for n of 1.1 for both activities, it is suspected that there is little or no cooperativity in the absence of inhibitor. The kinetics of chorismate mutase–prephenate dehydratase may be accommodated by the models of Monod *et al.* (1965) for "K systems" or Koshland *et al.* (1966). It must be emphasized, however, that there are alternative interpretations to sigmoidal kinetics (Ferdinand, 1966; Rabin, 1967). In terms of the model of Monod *et al.*, the enzyme exists largely in the "relaxed" form in the absence of feedback inhibitor. Addition of phenylalanine shifts the "relaxed–tight" equilibrium ($R \rightleftharpoons T$) to the right and results in a conformation having a reduced affinity for the binding of substrate. The data in Figure 8 show that phenylalanine changes the apparent $s_{20,w}$ value of the enzyme. This result suggests a change in conformation or state of aggregation caused by phenylalanine. The parallel displacement of both chorismate mutase and prephenate dehydratase activities to larger sedimentation values by phenylalanine and dithiothreitol is a further indication that the two activities are tightly associated.

It is presently unclear if the chorismate and prephenate active sites are identical or separate. It may be noteworthy that prephenate, the product of the chorismate mutase reaction, dissociates from the enzyme and accumulates in the reaction mixture prior to conversion to phenylpyruvate (Figure 10). Dissociation of intermediate prephenate is consistent with the value of $(S)_{0.5}$ for prephenate in the prephenate dehydratase reaction which is fourfold higher than the K_m of chorismate mutase for chorismate. Whereas the above evidence may favor a separate site model, the similarity between the K_m for chorismate and the K_i for prephenate with respect to chorismate mutase may favor a common site model. Further evidence is required in order to clarify the relationship between the chorismate mutase and prephenate dehydratase active sites.

Acknowledgments

We are grateful to Dr. W. W. Cleland for his suggestions, interpretations, and analysis of the kinetic data.

References

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965), *J. Biol. Chem. 240*, 2682.

Bauerle, R. H., and Margolin, P. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 203.

Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Cotton, R. G. H., and Gibson, F. (1965), *Biochim. Biophys. Acta 100*, 76.

Cotton, R. G. H., and Gibson, F. (1967), *Biochim. Biophys. Acta 147*, 222.

Cotton, R. G. H., and Gibson, F. (1968), *Biochim. Biophys. Acta 160*, 188.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dayan, J., and Sprinson, D. B. (1968), *Fed. Proc. 27*, 290.

Edwards, J. M., and Jackman, L. M. (1965), *Australian J. Chem. 18*, 1227.

Egan, A. F., and Gibson, F. (1966), *Biochim. Biophys. Acta 130*, 276.

Ferdinand, W. (1966), *Biochem. J. 98*, 278.

Gibson, F. (1964), *Biochem. J. 90*, 256.

Hayes, J. E., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 225.

Ito, J., and Yanofsky, C. (1966), *J. Biol. Chem. 241*, 4112.

Koshland, D. E., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

Layne, E. (1957), *Methods Enzymol. 3*, 447.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Metzenberg, R. L., and Mitchell, H. K. (1956), *Arch. Biochem. Biophys. 64*, 51.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Nishioka, Y., Demerec, M., and Eisenstark, A. (1967), *Genetics 56*, 341.

Pittard, J., and Wallace, B. J. (1966), *J. Bacteriol. 91*, 1494.

Plaut, G. W. E. (1963), *Enzymes 7*, 105.

Rabin, B. R. (1967), *Biochem. J. 102*, 226.

Trauffa-Bachi, P., Van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *European J. Biochem. 5*, 73.

Vogel, H. J., and Bonner, D. M. (1956), *J. Biol. Chem. 218*, 97.

Zalkin, H., and Kling, D. (1968), *Biochemistry 7*, 3566.