Chorismate Mutase-Prephenate Dehydratase

PHENYLALANINE-INDUCED DIMERIZATION AND ITS RELATIONSHIP TO FEEDBACK INHIBITION*

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SUMMARY

The bifunctional enzyme chorismate mutase-prephenate dehydratase catalyzes the first two reactions specific for phenylalanine biosynthesis in Salmonella typhimurium. Both activities are subject to feedback inhibition by phenylalanine. In the presence of phenylalanine the sedimentation coefficient of the enzyme is increased from approximately 5.6 to 8.0 S and the molecular weight is increased from approximately 109,000 to 220,000. This apparent dimerization is dependent upon the concentrations of phenylalanine and enzyme. Chorismate mutase-prephenate dehydratase that has been rendered insensitive to feedback inhibition either chemically or by mutational alteration does not dimerize. Therefore, dimerization appears somehow related to end product control. However, dimerization is not obligatory for feedback inhibition of either activity. At low protein concentration some enzyme does not dimerize in the presence of phenylalanine. Such enzyme can still be inhibited by phenylalanine. It is therefore suggested that phenylalanine binds to the monomer (smallest active species) to produce an inhibited monomer which dimerizes at appropriate protein concentrations.

EXPERIMENTAL PROCEDURE

Materials

Substrates—Barium chorismate and chorismic acid were isolated from A. aerogenes strain 62-1 (29, 30) and were converted to the potassium salt before use. The concentration of potassium chorismate solutions was determined by enzymatic assay (31). Barium prephenate was obtained by the method of Gibson (29) and was converted to the potassium salt with a 1.5-fold excess of K₂SO₄. Prephenate was assayed by acid-catalyzed conversion to phenylpyruvate (2).

Phenylalanine-substituted Sepharose—Phenylalanine-substituted Sepharose was prepared according to the general method of Cuatrecasas, Wilchek, and Anfinsen (32) as previously described (3).

Bacterial Strains—The strains of S. typhimurium, derived from wild type strain LT2, were aro A48, aro A48 tyr 25, and SA 34. The first two strains were used as sources for native chorismate mutase-prephenate dehydratase while the last was the source for feedback-insensitive enzyme. These strains have been previously described (3).

Methods

Chorismate Mutase and Prephenate Dehydratase Assays—Chorismate mutase was assayed by measurement of the rate of conversion of chorismate to prephenate plus phenylpyruvate (2). Prephenate dehydratase was assayed by measurement of the rate of conversion of prephenate to phenylpyruvate (2). A molar extinction coefficient of 17,500 (1) was used to calculate the con-
centration of phenylpyruvate from its absorption at 320 nm in 1 n NaOH. One unit of activity corresponds to the conversion of 1 μmol of substrate to product in 10 min at 37°. Specific activity is defined as units per mg of protein. Protein was determined by the method of Lowry et al. as described by Layne (33) with bovine serum albumin as a standard or from absorbance measurements at 280 nm and 260 nm (33).

Growth of Bacteria—Strains aro A48 or aro A48 tpe 25 were grown in 1 liter of media in 2-liter flasks as described previously (3). Alternatively, these strains were grown in 5-liter batches in a 7-liter New Brunswick FS807 fermentor by the method of Chen and Segel (34). The media contained, per liter: 12 g of glucose, 7 g of ammonium chloride, 1 g of sodium sulfate, 70.5 g of dibasic potassium phosphate, 12.9 g of monobasic potassium phosphate, 2 ml of trace elements (34), 175 mg of L-tryptophan, 400 mg of l-tyrosine, and 15 mg of L-phenylalanine. The inoculum for each fermentor consisted of 50 ml of a culture grown overnight in nutrient broth. Several drops of silicone antifoam were added and the culture was stirred vigorously with high air flow. The pH was adjusted to 7.0 with 10 n NaOH at 1-hour intervals after the first 8 hours of incubation at 37°. About 30 ml of 10 n NaOH were added during the 15-hour incubation period. The cells were harvested in stationary phase after reaching a turbidity of about 3000 Klett units measured with a Klett colorimeter using a No. 42 filter. The yield was approximately 18 g of cell paste per liter. The cells were washed with 0.05 n potassium phosphate buffer, pH 7.4, and stored at -10° to -20°. The specific activity for prephenate dehydratase of cells grown in the fermentor was from 0.8 to 1.3 units per mg.

Strain SA 32 (the source of phenylalanine-insensitive chorismate mutase-prephenate dehydratase) was grown in 2-liter flasks as described for strain aro A48 (3).

Partial Purification of Chorismate Mutase-Prephenate Dehydratase—Partial purification to a specific activity of 28 to 66 for chorismate mutase and prephenate dehydratase activities was performed as described previously (2). Alternatively, enzyme of specific activity 130 to 250 was obtained by chromatography of a crude extract of either strain aro A48 or aro A48 tpe 25 on a column of phenylalanine-substituted Sepharose. A typical purification procedure using affinity chromatography was as follows. Crude extract (100 ml, 15 mg of protein per ml) prepared by some disruption (2) was passed through a column, 2.2 cm × 14 cm, of phenylalanine-substituted Sepharose. The column of phenylalanine-substituted Sepharose had been equilibrated with buffer solution containing 0.1 n potassium phosphate (pH 7.4), 0.4 n 1m dithiothreitol, and 0.2 n EDTA. More than 90% of the chorismate mutase-prephenate dehydratase activity was adsorbed. The column was then washed with 175 ml of buffer solution of the same composition as was used for equilibration. This was followed by 80 ml of buffer solution containing 10 mm potassium phosphate (pH 7.4), 0.4 n 1m dithiothreitol, and 0.2 n EDTA. The enzyme was eluted from the column with buffer solution containing 2 mm potassium phosphate (pH 7.4), 0.4 n 1m dithiothreitol, 0.2 n EDTA, and 1 mm phenylalanine. Fractions containing 9 ml were collected. The peak fraction was dialyzed for 2 hours against 15 volumes of buffer solution containing 0.1 n potassium phosphate (pH 7.4), 0.4 n 1m dithiothreitol, 0.2 n EDTA, and 1 mm phenylalanine. The yield based on chorismate mutase activity was approximately 25%.

Feedback-insensitive chorismate mutase-prephenate dehydratase was partially purified from strain SA 34 as described previously (3).

Sucrose Gradient Centrifugation—Linear 5 to 20% sucrose gradients (4.5 ml) were prepared at room temperature with buffer and other additions as indicated in the figure legends. An additional 0.5 ml of 20% sucrose solution was added to the bottom of tubes centrifuged at 60,000 rpm. Gradients were equilibrated for 3 to 6 hours at 2°. After centrifugation the tubes were punctured and 10-drop fractions were collected and assayed for enzyme activity. The s20,w value and approximate molecular weight of chorismate mutase-prephenate dehydratase were estimated (35) by comparison with yeast alcohol dehydrogenase. The s20,w value and molecular weight for yeast alcohol dehydrogenase were taken as 7.6 S and 141,000, respectively (36).

Gel Filtration—Gel filtration on Sephadex G-200 (37, 38) was used to estimate the molecular weight of chorismate mutase-prephenate dehydratase. Bovine liver catalase, molecular weight 232,000 (39), was assayed by measurement of the disappearance of H2O2 at 240 nm (40). Yeast alcohol dehydrogenase, molecular weight 141,000 (36), was assayed by measuring the reduction of NAD+ (41). Rabbit muscle lactate dehydrogenase, molecular weight 136,000 (42), was assayed by the method of Kornberg (43). Bovine serum albumin, molecular weight 65,000 (44), was determined by its absorbance at 280 nm. Horse-radish peroxidase, molecular weight 40,000 (45), was assayed by the method of Chance and Maehly (40).

RESULTS

Effect of Phenylalanine on Sedimentation Velocity and Apparent Molecular Weight of Chorismate Mutase-Prephenate Dehydratase—The sedimentation coefficient of chorismate mutase-prephenate dehydratase was estimated by sucrose gradient centrifugation. Typical data are shown in Fig. 1. In the absence of phenylalanine a sedimentation coefficient of 5.7 S was obtained for both chorismate mutase and prephenate dehydratase activities (Fig. 1A). The value for s20,w was increased to 7.7 S when centrifugation was conducted in the presence of 1 mm phenylalanine (Fig. 1B). An intermediate sedimentation coefficient of 6.6 S was obtained for centrifugation in 1 mm phenylalanine as shown in Fig. 1C. Under all conditions of centrifugation chorismate mutase and prephenate dehydratase activity profiles were always superimposable.

Both the slower and faster sedimenting forms of the enzyme were subject to inhibition by phenylalanine. Chorismate mutase activity from Fraction 21 (Fig. 1A) was inhibited 40% and that from Fraction 10 (Fig. 1B) was inhibited 52% when assayed with 0.2 nmm chorismate and 0.6 nmm phenylalanine. Prephenate dehydratase activity from these fractions was inhibited 80 and 91%, respectively, when assayed with 1 mm phenylalanine and 1 mm phenylalanine.

Phenylalanine caused an increase in the apparent molecular weight of the bifunctional enzyme as determined by gel filtration with Sephadex G-200 (Fig. 2). Gel filtration indicated that in the absence of phenylalanine (Fig. 2A) the enzyme was smaller than either yeast alcohol dehydrogenase or lactate dehydrogenase and had a molecular weight of approximately 118,000. In the presence of 1 mm phenylalanine, the elution position of the enzyme indicated a molecular weight of approximately 220,000, which is larger than either lactate dehydrogenase or alcohol dehydrogenase.

A summary of apparent molecular weights deter-
potassium phosphate buffer (pH 7.4), (B) buffer plus 1 mM phenyl-
6004
mutase-prephenate dehydratase are indicated by UT~OWS. In
for yeast alcohol dehydrogenase (~20,~ = 7.6 S) and chorismate
Fractions of 10 drops were collected. The positions of the peaks
same column was used for both determinations. Chorismate mu-
gradient was added 0.1 ml of enzyme solution containing about 1.0
activity containing the same concentration of phenylalanine as was
cluded in the gradient. Centrifugation was at 35,000 rpm for 14
ity of chorismate mutase (O-O-prephenate dehydratase
(0-O). Linear 5 to 20% sucrose gradients contained (A) 0.1
potassium phosphate buffer (pH 7.4), (B) buffer plus 1 mM phenyl-
alanine, and (C) buffer plus 1 μM phenylalanine. To each 4.5-ml
gradient was added 0.1 ml of enzyme solution containing about 1.0
unit of chorismate mutase activity (30 μg of chorismate mutase-
prephenate dehydratase) and 60 μg of yeast alcohol dehydroge-
ase. The enzyme solution had been dialyzed against buffer solu-
tion containing the same concentration of phenylalanine as was
cluded in the gradient. Centrifugation was at 35,000 rpm for 14
ours at 2° in the SW 30 rotor with a Spinco L2-65 B centrifuge.
ctions of 10 drops were collected. The positions of the peaks
for yeast alcohol dehydrogenase (s20,w = 7.6 S) and chorismate
mutase-prephenate dehydratase are indicated by arrows. In B
the position of yeast alcohol dehydrogenase was omitted from the
figure because of its close proximity to the peak of chorismate mu-
tase-prephenate dehydratase. PHE, phenylalanine.

Fig. 2 (right). The effect of phenylalanine on the apparent
molecular weight of chorismate mutase-prephenate dehydratase as
determined by gel filtration. A column, 1.4 X 35 em, of Sephadex
G-200 was equilibrated with 200 ml of 0.1 M potassium phosphate
(pH 7.4), 0.8 mM dithiothreitol, and 0.2 mM EDTA either in the
presence (bottom) or absence (top) of 1 mM phenylalanine. The
same column was used for both determinations. Chorismate mu-
tase-prephenate dehydratase (0.75 ml containing 20 units of chor-
ismate mutase activity) was dialyzed for 5 hours against the same
buffer solution that was used to equilibrate the column. To this
was added 0.15 mg of bovine liver catalase, 0.20 mg of yeast alcohol
dehydrogenase, 0.10 mg of rabbit muscle lactate dehydrogenase,
10 mg of bovine serum albumin, and 1 mg of homoceradish peroxi-
dase. Fractions containing 10 drops (~1 ml) were collected at 5°.
Enzyme assays were conducted as described under "Methods.
Position of the chorismate mutase activity is indicated by the
dashed lines. ADH, yeast alcohol dehydrogenase; LDH, lactate
dehydrogenase; BSA, bovine serum albumin.

mained by gel filtration and by sucrose gradient centrifugation is
given in Table I. The data show that phenylalanine caused an
approximate doubling of the apparent molecular weight using
both techniques. These results suggest that chorismate mutase-
prephenate dehydratase undergoes a phenylalanine-dependent
dimerization.

Effect of Different Concentrations of Phenylalanine and Other
Reagents on Sedimentation Velocity of Enzyme—The effect of
varying phenylalanine concentrations on the sedimentation co-
efficient and apparent molecular weight of chorismate mutase-
prephenate dehydratase was determined by sucrose gradient
centrifugation and is shown in Fig. 3. In the absence of phenyl-
alanine, an average value of 5.6 S corresponding to an approxi-
mate molecular weight of 88,000 was obtained from eight experi-
ments. Phenylalanine at a concentration of 1 μM caused a partial
shift to 6.9 S (mol wt approximately 120,000). A single sym-

| Additions         | Average | Range | Mol wt     |
|------------------|---------|-------|------------|
| None             | 5.6     | 5.0-6.2 | 88,000 (8) |
| Phenylalanine    | 8.0     | 7.7-8.3 | 152,000 (5) |
| D-β-Thienylalanine | 8.2   |         | 150,000    |
| Tryptophan       | 5.6     |         | 100,000 (2) |
| Phenylpyruvate   | 6.0     |         | 99,000     |
| Dithiothreitol   | 5.9     |         | 96,000     |
| Chorismate       | 5.4     |         | 85,000     |

* Number of determinations. In the absence of any designation,
a single determination was made.

Values from gel filtration. All other molecular weights were
determined by gel filtration. A single determination was
made.

Fig. 3. Effect of phenylalanine (○—○) and tyrosine
(▲—▲) concentration on the sedimentation coefficient and mo-
lar weight of chorismate mutase-prephenate dehydratase.
The sedimentation coefficient was estimated by sucrose gradient
centrifugation at the indicated concentrations of phenylalanine or
tyrosine. In cases in which multiple determinations were made
their number is indicated in parentheses and the range is indicated
by the vertical arrow. At least 1 unit of chorismate mutase-
prephenate dehydratase activity was used for each determination.
Centrifugation was at 35,000 rpm for 14 hours at 2° in an SW 30
or at 60,000 rpm for 8.5 hours at 2° in an SW 65 rotor with a
Spinco L2-65 B centrifuge.

Summary of estimated sedimentation coefficients and apparent
molecular weights for chorismate mutase-prephenate dehydratase

Sedimentation coefficients were estimated by sucrose gradient
centrifugation. Molecular weight was estimated from s20,w, as
described (35). Molecular weight was also estimated by gel
filtration with Sephadex G-200. All reagents were tested at a
concentration of 1 mM. At least 1 unit of chorismate mutase ac-
tivity was used for each sucrose gradient centrifugation experi-
iment. The conditions for gel filtration are given in Fig. 2.
metrical peak of enzyme activity was obtained (Fig. 1C), indicating a rapid association-dissociation (46) under these conditions. At 5 μM phenylalanine and higher, a “complete” shift to 8.0 S, molecular weight approximately 154,000, was observed.

Tyrosine caused an increase in apparent molecular weight for the enzyme but was not as efficient as phenylalanine (Fig. 3). At a tyrosine concentration of 100 μM a sedimentation coefficient similar to that obtained with 1 μM phenylalanine was found. With 1 mM tyrosine or 2 mM phenylalanine similar molecular weights were obtained. Thus, it is estimated that 100 to 500 times more tyrosine is required to evoke a sedimentation shift similar to that caused by phenylalanine. Neither 1 mM tyrosine nor 1 mM tryptophan, alone or in combination, modified the activity of chorismate mutase-prephenate dehydratase or alanine during preparation of the gradients.

10% of the chorismate mutase (Fig. 4A) and prephenate dehydratase activities, also caused aggregation (Table I). Tryptophan, chorismate, dithiothreitol, and phenylpyruvate, the product of prephenate dehydratase, did not affect the sedimentation velocity. The earlier report of aggregation in the presence of dithiothreitol (2) was an artifact caused by carryover of phenylalanine during preparation of the gradients.

Effect of Enzyme Concentration on Monomer-Dimer Equilibrium—Dimerization was dependent on enzyme concentration. At an enzyme concentration of 2.2 units per gradient more than 90% of the chorismate mutase (Fig. 4A) and prephenate dehydratase activities were dimer (7.8 S) in the presence of 5 μM phenylalanine. At 100-fold lower enzyme concentration (0.02 unit per gradient) both activities sedimented in broad activity profiles extending from the dimer region to the position where monomer would be expected (approximately 5 to 6 S). Yeast alcohol dehydrogenase was used as a standard at low enzyme concentrations because it was contaminated with a trace of prephenate dehydratase activity. The data in Fig. 4 show clearly that the sedimentation profile was dependent on the concentration of enzyme applied to the gradient. Hence, chorismate mutase-prephenate dehydratase undergoes a phenylalanine-induced concentration-dependent dimerization.

Effect of Desensitization to Phenylalanine Inhibition on Dimerization of Chorismate Mutase-Prephenate Dehydratase—Several methods have been reported (3, 4) to produce varying degrees of desensitization to feedback inhibition by phenylalanine. (a) Desensitization of chorismate mutase was achieved by treatment with bromopyruvate or p-mercuribenzoate. (b) Both activities from mutant strain SA 34 were completely insensitive to phenylalanine inhibition. (c) Desensitization of chorismate mutase activity was obtained at pH less than 6. The ability of the desensitized enzyme to undergo dimerization in the presence of phenylalanine was investigated in order to determine whether there is any correlation between feedback inhibition and dimerization.

It was shown previously that treatment of chorismate mutase-prephenate dehydratase with bromopyruvate resulted in loss of prephenate dehydratase activity (3). The remaining chorismate mutase activity was completely insensitive to inhibition by phenylalanine. The data in Fig. 5 show that a major fraction of bromopyruvate-desensitized chorismate mutase activity remained monomeric in the presence of 5 μM phenylalanine (equivalent to 5.0 S; mol wt approximately 74,000). A lesser fraction of the chorismate mutase activity was observed to associate. In a control experiment with untreated chorismate mutase-prephenate dehydratase, under identical conditions of centrifugation, a single peak of activity at 7.8 S (mol wt approximately 149,000) was obtained. Chorismate mutase activity from the peak fraction of the non-treated enzyme gave 66% inhibition when assayed with phenylalanine. Bromopyruvate-treated enzyme from fraction 24 of the 5.0 S peak was not inhibited by phenylalanine under identical conditions.

Similar experiments were performed with chorismate mutase-prephenate dehydratase from strain SA 34. It has been shown that both activities of the bifunctional enzyme from this strain...
are completely insensitive to inhibition by 4 mM phenylalanine (3). The enzyme activity profiles in Fig. 6 show that, whereas the wild type enzyme sedimented at 8.2 S in the presence of 1.0 mM phenylalanine, feedback-insensitive enzyme from strain SA 34 sedimented at 5.0 S whether phenylalanine was included in the gradient or not. The wild type enzyme in the absence of phenylalanine gave an intermediate sedimentation coefficient. This sedimentation coefficient was partially dependent on the extent of prior dialysis of the enzyme preparation to remove residual phenylalanine. Even after extensive dialysis, however, the sedimentation coefficient of the wild type enzyme in these experiments could not be decreased below 5.8 S (Fig. 6). The molecular weight of the feedback-insensitive enzyme in the presence of 1.0 mM phenylalanine was estimated by gel filtration to be 120,000 (data not shown) which is the same as that obtained for the wild type enzyme without phenylalanine (Fig. 2). Neither tyrosine nor tryptophan had any effect on the sedimentation coefficient of the feedback-insensitive enzyme. Thus, the feedback insensitive enzyme does not dimerize in the presence of phenylalanine.

At pH values below 6, chorismate mutase activity was relatively insensitive to inhibition by phenylalanine whereas prephenate dehydratase was fully inhibited (2). The ability of phenylalanine to cause dimerization of the enzyme was studied at different pH values. The data in Fig. 7 show that at pH 6.0 10 μM phenylalanine was required to promote maximal dimer formation while at pH 7.4, 5 μM phenylalanine was sufficient. At pH 5.0 the comparison was even more striking. At pH 5.0 maximal dimer formation required 100 times more phenylalanine than was needed at pH 7.4. In addition, even with 1 mM phenylalanine the estimated sedimentation coefficient was lower at pH 5.0 compared to that obtained at pH 7.4. These data show clearly that at low pH chorismate mutase-prephenate exhibits a decreased ability to dimerize in the presence of phenylalanine. The experiments of Figs. 5 to 7, taken together, show that treatments that desensitize chorismate mutase-prephenate dehydratase to feedback inhibition by phenylalanine also reduce or prevent enzyme dimerization in the presence of inhibitor. The phenylalanine regulatory sites are thus implicated in dimerization.
decreased tendency of the enzyme to associate (Fig. 7), we investigated the effect of low pH on the substrate saturation kinetics and inhibition of prephenate dehydratase by phenylalanine. Previous results (2) had indicated that prephenate dehydratase activity retained sensitivity to inhibition by phenylalanine at pH 6.0. As shown by the data in Fig. 8, prephenate dehydratase activity exhibited increased sensitivity to inhibition by phenylalanine at pH 6 relative to pH 7.4. At a phenylalanine concentration of 0.05 mM there was a 3-fold difference in sensitivity to feedback inhibition of prephenate dehydratase. Replots of the data in Fig. 8 according to the modified Hill equation (47) indicated a decreased interaction coefficient, n', from 1.8 at pH 7.4 to 1.5 at pH 6.0. This effect would be expected to be more pronounced at an even lower pH (Fig. 7) but such experiments could not be performed because of the instability of prephenate below pH 6.0. We therefore conclude that at pH 6, under conditions less favorable to enzyme dimerization, phenylalanine binding is retained but interaction between sites is somewhat decreased.

The effect of low pH on prephenate dehydratase activity is shown in Fig. 9. Lowering the pH decreased the \( \frac{V}{S} \) max from 4 enzyme units at pH 7.4 to 3 enzyme units at pH 6.0 but did not appreciably alter the \( K_m \) for prephenate. In both cases normal kinetics was obtained in the absence of phenylalanine. In the presence of 80 \( \mu \)M phenylalanine, cooperativity for prephenate is indicated by concave upward curvature in the double reciprocal plots. However, the cooperativity at pH 6.0 is less than that at pH 7.4. The interaction coefficient (a) for prephenate decreased from 2.3 at pH 7.4 to 1.6 at 6.0. Thus, the following changes occurred at pH 6.0. (a) Chorismate mutase activity was desensitized to inhibition by phenylalanine. (b) Dimerization of the enzyme in the presence of phenylalanine was hindered. (c) Interaction between phenylalanine regulatory sites and prephenate catalytic sites (in the presence of phenylalanine) was reduced.

Separation of Monomeric and Dimeric Forms of Chorismate Mutase-Prefenenate Dehydratase—Under special conditions multiple peaks of chorismate mutase-prephenate dehydratase activity corresponding to monomeric and dimeric species were obtained by sucrose gradient centrifugation. In order to obtain separation of monomeric and dimeric forms of the enzyme, low protein concentrations similar to those used for the experiments shown in Fig. 4 were required together with saturating concentrations of phenylalanine (100 \( \mu \)M or higher). It was also necessary to dialyze the enzyme prior to centrifugation in order to remove phenylalanine that was used to stabilize the enzyme during storage. After dialysis phenylalanine was added back to give a final concentration of 0.1 to 1.0 mM. The data in Fig. 10A show the activity profiles for chorismate mutase and prephenate dehydratase activities. Monomeric and dimeric forms contained both activities. The relative proportion between the two enzyme forms was influenced by the concentration of enzyme applied to the gradients. This is shown by a comparison of Fig. 10, 4 and B. For the experiment in Fig. 10A, 0.016 unit of chorismate mutase activity was applied in a volume of 0.05 ml to a gradient containing 100 \( \mu \)M phenylalanine. From the chorismate mutase activity profile it was calculated that approximately 60% of the enzyme was dimeric and 40% was monomeric. The same result was obtained with 1 mM phenylalanine. For the experiment in Fig. 10B the same number of enzyme units was applied in a volume of 0.2 ml to a similar gradient. Under these conditions the distribution was 25% dimer and 75% monomer.

The importance of prior dialysis to remove phenylalanine used for storage is shown by the experiment in Fig. 10C. In this experiment the stock enzyme solution containing 1 mM phenylalanine was diluted to 0.32 unit per ml and 100 \( \mu \)M phenylalanine. An aliquot (0.05 ml) containing 0.016 unit of chorismate mutase activity was applied to a sucrose gradient containing 100 \( \mu \)M phenylalanine. Under these conditions following centrifugation 92% of the activity remained dimeric.

Two distinct peaks of activity indicate that dimers and mono-
activities were strongly inhibited by phenylalanine (Fig. 10A). Inhibition of chorismate mutase activity by phenylalanine is not shown in Fig. 10A for reasons of clarity. The enzyme would not be expected to dimerize during the assay because it was diluted considerably during centrifugation and was diluted still further (1:5) in the assay mixture. Such dilution would favor dissociation rather than association. In control experiments, both enzyme activities were strongly inhibited by phenylalanine at 22° and at 2-4°. Furthermore, neither substrate promoted dimer formation. The enzyme should therefore remain monomeric during assay. It is thus concluded that the monomeric species is subject to inhibition by phenylalanine.

**DISCUSSION**

The data reported in this study confirm previous results (2) which show that the sedimentation coefficient of chorismate mutase-prephenate dehydratase is increased in the presence of phenylalanine. Sucrose gradient centrifugation in conjunction with gel filtration was used to distinguish between a large conformational change and an association-dissociation of the enzyme. The large increase in sedimentation coefficient of chorismate mutase-prephenate dehydratase caused by phenylalanine could be due either to association (i.e. increase in molecular weight) or to a large conformational change resulting in a smaller more compact molecule. Conformational changes of this magnitude have been reported (48). Gel filtration which separates proteins based on Stokes radius or size (37, 38) can be used to distinguish between these two possibilities. If the increase in sedimentation coefficient caused by phenylalanine is due to enzyme association, gel filtration should reveal an increased molecular weight. If the increase in sedimentation coefficient caused by phenylalanine is due to a conformational change, giving a more compact and therefore smaller molecule, the enzyme should be retarded during gel filtration and give a decreased estimated molecular weight. It is clear from the data in Fig. 2 that phenylalanine increased the apparent molecular weight of the enzyme as determined by Sephadex G-200 gel filtration. Therefore, phenylalanine caused an association of the enzyme. This is further supported by the observation that in the presence of phenylalanine the sedimentation coefficient was dependent on enzyme concentration (Fig. 4). The observed molecular weight change is consistent with a rapid monomer-dimer transition. Gel filtration experiments show that the estimated molecular weight is increased 2-fold in the presence of phenylalanine (Table I). Molecular weight estimations of native enzyme by sucrose gradient centrifugation indicate a 1.7-fold increase in the presence of phenylalanine. Although molecular weight estimation by sucrose gradient centrifugation can provide only an approximation of true molecular weight, this deviation from a doubling may be significant. It was consistently observed that the molecular weight of feedback-insensitive enzyme estimated by sucrose gradient centrifugation was lower than that of the native enzyme determined in the absence of phenylalanine (Fig. 6). One possibility is that the feedback-insensitive enzyme was completely monomeric because of inability to associate whereas a fraction of the native enzyme was in rapid association-dissociation perhaps due to tightly bound phenylalanine or a tendency to dimerize in the absence of inhibitor.

The reason for the large difference between molecular weights estimated from gel filtration and sucrose gradient centrifugation experiments is not fully understood. A contributing factor is that yeast alcohol dehydrogenase which was used as a reference
for sucrose gradient centrifugation did not fall on the straight line obtained from the other four standard proteins in gel filtration experiments (Fig. 2). However, this complication does not detract from the important conclusion that phenylalanine caused chorismate mutase-prephenate dehydratase to dimerize.

A higher concentration of phenylalanine was required to inhibit either chorismate mutase or prephenate dehydratase activity (2) than was needed for maximal association at relatively high enzyme concentration (Fig. 3). For 50% inhibition of chorismate mutase or prephenate dehydratase activities (assayed with 0.2 mM chorismate or 0.9 mM prephenate, respectively) 100 to 200 μM phenylalanine was required whereas maximal enzyme association, determined in the absence of substrates, was obtained with approximately 5 μM phenylalanine. It is possible that this discrepancy is due, at least in part, to the competitive relationship between phenylalanine and substrates.

A close correlation between dimerization and feedback inhibition is indicated by the observations that compounds which inhibited both activities caused the enzyme to dimerize (Table 1) and that desensitized enzyme did not dimerize (Figs. 5 and 6). Tyrosine, which does not inhibit either activity, may be a possible exception (Fig. 3). The concentration of tyrosine required to cause dimerization was 100 to 500 times higher than that required for a comparable effect with phenylalanine. It is possible that tyrosine is a weak analogue of phenylalanine and its effect on the enzyme, although not strong enough to cause inhibition in the presence of substrates, is sufficient to cause some dimerization in sucrose gradients. Enzyme desensitized to phenylalanine inhibition either by bromopyruvate treatment or by genetic modification did not dimerize in the presence of feedback inhibitor (Figs. 5 and 6). This suggests that binding of phenylalanine to the regulatory site(s) is responsible for both inhibition and dimerization.

The effect of low pH on association is complex. At low pH, relative to pH 7.4 considerably more phenylalanine is required to promote dimerization. This cannot result from a weaker affinity of phenylalanine for the enzyme because the sensitivity of prephenate dehydratase to feedback inhibition is maintained (Fig. 8). It is also unlikely that there are two classes of phenylalanine sites that participate in association or inhibition (or both) because association cannot occur in the feedback-insensitive enzyme from mutant strain SA 34. It therefore appears possible that at low pH a phenylalanine-induced change that leads to inhibition of prephenate dehydratase cannot be transmitted to the chorismate mutase site. These experiments show two main points: (a) concomitant alteration of feedback inhibition of one enzyme activity and phenylalanine-induced dimerization, and (b) decreased homotropic and heterotropic cooperative interactions under conditions of decreased enzyme association.

Although the results of several experiments indicate a relationship between feedback inhibition and dimerization, this effect is not obligatory for inhibition of either catalytic activity by phenylalanine. At sufficiently low protein concentration a major fraction of chorismate mutase-prephenate dehydratase remained monomeric and yet both activities were sensitive to inhibition by phenylalanine (Fig. 10A).

Any model proposed to explain association-dissociation of enzyme and the relationship between different forms with active and inactive states must accommodate four experimental observations. (a) A single apparently symmetrical peak of activity with intermediate sedimentation coefficient is obtained with relatively high enzyme concentration and less than saturating concentrations of phenylalanine. Therefore, under such conditions a rapid association-dissociation occurs. (b) A similar rapid association-dissociation occurs under conditions of relatively low enzyme concentration and less than saturating concentrations of phenylalanine. (c) Distinct monomer and dimer species are observed at relatively low enzyme and saturating phenylalanine concentrations. Therefore, under the latter conditions rapid association-dissociation does not occur. (d) Association-dissociation is phenylalanine- and protein concentration-dependent. A simple hypothetical scheme that accommodates these observations is shown in Fig. 11. All species except active dimer (AA) have been detected. It is proposed that active monomers (A) are converted into inhibited monomers (I) in the presence of phenylalanine in a reversible step. Inhibited monomers may dimerize (II) in a concentration-dependent step. Dissociation of inhibited dimer to active monomers may not occur readily. Upon dissociation of phenylalanine an active dimer may be formed which dissociates into active monomers. Such a cyclic scheme appears necessary in order to account for a rapid association-dissociation equilibrium at low phenylalanine concentration and a very slow association at low protein but saturating phenylalanine concentration. It is proposed that monomeric and dimeric species are observed under the latter conditions because low enzyme concentration ensures that association will be incomplete and high phenylalanine concentration prevents association of inhibited dimer to active monomers via active dimer.

In this report the smallest active species of molecular weight approximately 88,000 to 109,000 has been designated a monomer. Preliminary experiments by gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol (40) suggest that the monomer is composed of two polypeptide chains of similar size. The enzyme is the product of the phe A locus in S. typhimurium (50).

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