Chorismate Mutase-Prephenate Dehydratase from *Escherichia coli* K-12

II. KINETIC PROPERTIES

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**Theo. A. A. Dopheide,** Pauline Chewther,§ and Barrie E. Davidson

*From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia*

**SUMMARY**

The results of kinetic investigations with pure chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12 were as follows: a pH optimum of 7.3 for the mutase activity, a $K_m$ of 0.045 mM for chorismate, and a $K_m$ of 1.0 mM for prephenate. L-Phenylalanine inhibited both the mutase and the dehydratase activities. In each case the relationship between activity and concentration of L-phenylalanine was sigmoidal and analysis of the results by the Hill equation gave values of $n' = 2.3$. D-Phenylalanine and L-tryptophan did not inhibit either activity whilst varying degrees of inhibition were observed with α-, m-, and p-monosubstituted fluoro-, chloro-, and hydroxyphenylalanines. Investigations with other phenylalanine analogues indicated that the α-NH$_2$ group is not essential for inhibition, although it is required for maximal effect. There is an absolute requirement for an unmodified α-COOH group.

Thiol compounds such as β-mercaptoethanol and dithioerythritol have little effect on the mutase activity but stimulate the dehydratase activity by approximately 150%.

The recent purification of chorismate mutase-prephenate dehydratase (3, 4) led us to investigate in detail some of its kinetic properties, including the feedback inhibition by phenylalanine. The results of these investigations are reported in this paper. Concurrently with this study Koch *et al.* have investigated the kinetics of pure chorismate mutase-prephenate dehydrogenase (5) so that the comparative abilities of these two enzymes to utilize chorismate can now be assessed.

**EXPERIMENTAL PROCEDURE**

Materials—Chorismic acid, barium prephenate, and chorismate mutase-prephenate dehydratase were prepared and assayed as described previously (3). Pure enzyme from *E. coli* K-12 strain JP492 (26 dehydratase units per mg, see Step VII of Table I in Reference 3) was used for all work described in this report.

Phenylalanine and its analogues were purchased as follows: L-phenylalanine, L-tyrosine, 2-phenylethylamine, DL-β-phenylactic acid, DL-p-aminophenylalanine, and DL-α-, m-, and p-fluoro-phenylalanines from the Sigma Chemical Co.; n-phenylalanine, 2-phenylpropionic acid, 3-phenylpropionic acid, phenylpropionic acid, DL-α-, m-, and p-chlorophenylalanines, and DL-α- and m-hydroxyphenylalanines from Koch-Light Laboratories; L-phenylalanine ethyl ester, N-carbobenzyloxy-L-phenylalanine, L-tryp- tophan, and L-phenylalanine amide HCl from Mann Research Laboratories, Inc.; L-alanine from L. Light and Co., Buckinghamshire, England; and DL-β-phenylserine from Nutritional Biochemicals Corp.

Other analogues were donated as follows: L-Phe-Gly-Gly and L-Phe-Gly by Dr. F. H. C. Stewart; Gly-L-Phe by Dr. D. E. Rivett; p-methoxyphenylalanine by Dr. B. R. Milligan; and 2-phenylethanol by Dr. W. G. Crewther. DL-α-Methylphenylalanine was synthesized by the method of Stein *et al.* (6).

All of the above compounds were found to be chromatographically or electrophoretically pure except for L-phenylalanineamide HCl, from which about 5% L-phenylalanine was removed by chromatography on Dowex AG1-X2. Solutions of L-phenylalanine ethyl ester and purified L-phenylalanineamide were prepared immediately before use. The d-phenylalanine contained approximately 99% of the l-isomer (determined by measuring the uptake of O$_2$ with an oscillating Pt electrode when L-amino acid oxidase was added to a solution of the compound). Pure d-phen-

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† Present address, CSIRO Division of Protein Chemistry, 363 Royal Parade, Parkville, Victoria 3052, Australia.

‡ Present address, Walter and Eliza Hall Institute of Medical Research, Royal Parade, Parkville, Victoria 3052, Australia.

§ Queen Elizabeth II Fellow, to whom requests for reprints should be addressed.
ylalanine was obtained by incubating a solution of the impure material with L-amino acid oxidase for 2 hours at 37°.

Chorismate Mutase Assay—Chorismate mutase activity was assayed in 0.8-ml reaction mixtures containing chorismate, 100 mM Tris-Cl, pH 7.8, 0.5 mM EDTA, 0.01% bovine serum albumin, 20 mM mercaptoethanol, and enzyme. After incubation for 5 min at 37°, the enzyme reactions were terminated by the addition of 0.1 ml of 4.5 M HCl. After a further incubation at 37° for 10 min to convert prephenate into phenylpyruvate, 0.1 ml of 12 M NaOH was added and the absorbance at 320 nm was measured to determine phenylpyruvate. Blanks, to which enzyme was added after the addition of NaOH, were included since the substrate contributes a significant absorbance. A unit of enzyme was defined as the quantity of enzyme that catalyzed the conversion of 1.0 μmole of chorismate to prephenate in 1 min under the assay conditions.

Prephenate Dehydratase Assay—Prephenate dehydratase activity was assayed in 0.4-ml reaction mixtures containing barium prephenate, 20 mM Tris-Cl (pH 8.2), 0.5 mM EDTA, 0.01% bovine serum albumin, 20 mM mercaptoethanol, and enzyme. After 5 min incubation at 37° the reaction was terminated by the addition of 0.8 ml of 1.0 M NaOH and the absorbance at 320 nm was measured. Blanks were included as for the mutase assay. A unit of enzyme was defined as the quantity of enzyme that catalyzed the conversion of 1.0 μmole of prephenate to phenylpyruvate in 1 min under the assay conditions.

RESULTS

Effects of Bovine Serum Albumin and Thiol Compounds on Enzyme Activity—Bovine serum albumin activates the purified enzyme, having a greater effect on the mutase activity than on the dehydratase activity (Table I).

Table I

Differential effects of bovine serum albumin and thiol compounds on chorismate mutase and prephenate dehydratase activities

| Addition                  | Concentration | Enzyme activity | Dehydratase | Mutase |
|---------------------------|---------------|-----------------|-------------|--------|
| None                      |               |                 |             |        |
| Bovine serum albumin      | 1.0 μg/ml     | 100             | 100         |        |
|                           | 10 μg/ml      | 116             | 139         |        |
|                           | 50 μg/ml      | 128             | 181         |        |
|                           | 100 μg/ml     | 152             | 270         |        |
|                           | 500 μg/ml     | 188             | 285         |        |
| Mercaptoethanol           | 5 mm          | 225             | 111         |        |
|                           | 20 mm         | 240             | 124         |        |
|                           | 50 mm         | 253             | 111         |        |
| Dithioerythritol          | 0.5 mm        | 162             |             |        |
|                           | 5 mm          | 238             |             |        |
|                           | 10 mm         | 248             |             |        |

Thiol compounds stimulate the dehydratase activity of the purified enzyme an additional 150% but have little effect on the mutase activity (Table I).

Effect of pH on Enzyme Activity—The mutase activity shows a bell shaped curve over the pH range from 5.5 to 9.5 with a well defined maximum at pH 7.3. The pH dependence of the dehydratase activity possesses some unusual features that we cannot explain at present. In some experiments a single maximum of activity was observed at pH 6.5, whereas on other occasions a bi-lobed curve was found with maxima at pH 6.5 and 8.5. This difference in pH activity relationship could not be attributed to specific buffer ions, since it was observed with a variety of different buffers. The only variable between experiments appeared to be the age of the enzyme preparation. However, until the phenomenon is examined in more detail we prefer not to comment about its possible causes, apart from noting the related observation (see below) that aging affects the extent and type of inhibition by phenylalanine.

Little effect of pH on the value of K₉₀ for prephenate was noted over the range from pH 6.5 to pH 9.0.

Substrate Saturation Curves for Dehydratase and Mutase Activities—The effect on the reaction rate of varying the concentration of substrate is shown in Figs. 1A and 2A (curve labeled No Phe). Plots of 1/v against 1/s were linear for both substrates and thus the kinetics are in accord with the model of Michaelis and Menten.

![Fig. 1. Effect on prephenate dehydratase activity of varying prephenate concentration at fixed concentrations of L-phenylalanine. For assay details see “Experimental Procedure”; 0.07 μg of pure enzyme was used in each assay. The same symbols are used in A and B.](http://www.jbc.org/)

![Fig. 2. Effect on chorismate mutase activity of varying chorismate concentration at fixed concentrations of L-phenylalanine. For assay details see “Experimental Procedure”; 0.045 μg of pure enzyme was used in each assay. In B, chorismate concentrations are as follows: O--O, 1.0 mM; △-△, 0.25 mM; ×-×, 0.175 mM; ●-●, 0.10 mM; ■-■, 0.075 mM; ▲-▲, 0.050 mM; ○-○, 0.025 mM.](http://www.jbc.org/)
or that derived from steady state theory for the system \( E + S \rightleftharpoons ES \rightleftharpoons E + P \). The values obtained for \( K_n \) were 0.045 mM for chorismate and 1.0 mM for prephenate. Hill plots of these data were linear over the concentration ranges explored and had slopes of 0.9 and 1.1 for mutase and dehydratase, respectively. These results demonstrate the absence of homotropic co-operative effects for either of the substrates.

**Inhibition by L-Phenylalanine**—Both activities are inhibited by L-phenylalanine, although the dehydratase activity is generally more sensitive than the mutase. Plots of activity versus inhibitor concentration are sigmoidal for both activities (Figs. 3 and 4).

A Hill plot of the inhibition of mutase activity by L-phenylalanine is linear between inhibitor concentrations of 0.02 mM and 0.40 mM, with a slope of 2.3 (Fig. 4, inset). Similarly for the inhibition of the dehydratase activity by L-phenylalanine, the Hill plot has a linear portion with a slope of 2.3 between inhibitor concentrations of 0.025 mM and 0.10 mM (Fig. 3, inset). Thus the effector, L-phenylalanine, exhibits co-operative homotropic interactions in its inhibition of both activities.

The inhibition of both mutase and dehydratase activities by L-phenylalanine showed little dependence on pH over the pH range of 6 to 9.

The effect of aging of the enzyme on its sensitivity to L-phenylalanine is quite marked. Thus one batch of enzyme stored in dilute solution at 4° for 2 weeks exhibited hyperbolic inhibition kinetics for the dehydratase activity and a Hill plot with slope of 1.0 (Fig. 3), although the maximal percentage of inhibition was unaffected. Another sample, stored in dilute solution at -20° for 3 weeks lost all sensitivity of mutase activity to inhibition (at 0.5 mM L-phenylalanine), and the dehydratase activity was inhibited only 65% by 1 mM L-phenylalanine. The extent of this desensitization towards L-phenylalanine is quite variable and has not, as yet, been linked to a definite manipulation of the enzyme preparation apart from aging. We also observed that desensitization occurs during the course of a 30-min enzyme assay. This makes it desirable that all assays investigating the kinetics of inhibition by L-phenylalanine measure initial rates. In the case of the experimental results presented above, a close approximation to this has been obtained by using freshly prepared enzyme in 5-min assays (except for the effect of pH on the inhibition when longer assay times were used).

**Effect of L-Phenylalanine on Substrate Saturation Curve for Dehydratase Activity**—The data in Fig. 1A show that the plot of \( v \) versus \( s \) for dehydratase activity, which is hyperbolic in the absence of phenylalanine, becomes sigmoidal in its presence. The

### Table II

| Inhibitor                          | Inhibition |
|-----------------------------------|------------|
| None                              | 0          |
| L-Phenylalanine                   | 94         |
| p-Phenylnalanine                  | 0          |
| Alanine                           | 3          |
| o-Fluorophenylalanine             | 43         |
| m-Fluorophenylalanine             | 92         |
| p-Fluorophenylalanine             | 85         |
| o-Chlorophenylalanine             | 16         |
| m-Chlorophenylalanine             | 52         |
| p-Chlorophenylalanine             | 20         |
| o-Hydroxyphenylalanine            | 57         |
| Tyrosine                          | 0          |
| p-Methoxyphenylalanine            | 1          |
| p-Aminophenylalanine              | 1          |
| Tryptophan                        | 0          |
| β-Phenylserine                    | 0          |
| 2-Phenylethanol                   | 2          |
| Phenylactic acid                  | 2          |
| α-Methylphenylalanine             | 3          |
| 2-Phenylpropionic acid            | 6          |
| 3-Phenylpropionic acid            | 10         |
| Gly-Phe                           | 3          |
| Phenylpropionic acid              | 8          |
| N-Carbobenzoxyphenylalanine       | 29         |
| Phenylalanine amide               | 0          |
| Phenylalanine ethyl ester         | 0          |
| Phe-Gly                           | 2          |
| Phe-Gly-Gly                       | 2          |
| β-Phenylethylamine                | 1          |
effects also apply to chorismate-prephenate dehydratase from wild type E. coli. The activity in strain JP171 showed a decreased sensitivity to inhibition by nitro-N-nitrosoguanidine (9). The prephenate dehydratase activity was derived directly from a strain (JP171) which had been isolated on the basis of resistance to the growth-inhibitory effect of 1.0 mM N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid).3 Further clarification of this aspect awaits sequence studies.

Effect of Thiol Compounds—It is tempting to implicate a thiol group in the catalysis of the dehydratase activity since maximal dehydratase activity in pure enzyme preparations is dependent upon the addition of thiols such as β-mercaptoethanol or dithioerythritol. Schmit et al. (10, 11) have demonstrated that the dehydratase activity of partially purified chorismate mutase-prephenate dehydratase from S. typhimurium is inhibited by 1.0 mM N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid).3 Further clarification of this aspect awaits sequence studies.

Kinetic Properties—The kinetics of both activities is consistent with the model of Michaelis and Menten, and it is of interest to compare the value of Km for chorismate of 0.045 mM determined in this investigation with those of 0.39 mM for chorismate mutase-prephenate dehydrogenase (5) and 0.0012 mM for anthranilate synthetase (12). These three enzymes compete for chorismate, and the comparison indicates that when chorismate is present at low concentrations in vivo most of it could be directed towards the biosynthesis of tryptophan. This may explain the earlier observation of Davis (13) that strains of E. coli containing leaky mutations in the common pathway may show a preferential requirement for phenylalanine and tyrosine before tryptophan.

The Km for prephenate of 1.0 mM is somewhat higher than that of 0.37 mM found for chorismate mutase-prephenate dehydrogenase by Koch et al. (5).

Inhibition by L-Phenylalanine—L-Phenylalanine, which is the end product of the pathway, acts as a feedback inhibitor of the enzyme. At a fixed substrate concentration the inhibition of both activities increases in a sigmoidal manner as the concentration of phenylalanine is increased, and Hill plots have slopes in excess of two, i.e. a homotropic co-operative effect is observed. Inhibition is mainly directed towards the dehydratase activity, although some inhibition of the mutase activity also occurs. In contrast, inhibition of the analogous enzyme chorismate mutase-prephenate dehydrogenase by the end product of its pathway, tyrosine, is directed solely towards the dehydratase activity, and there is no inhibition of the mutase activity (5).

### DISCUSSION

**Effect of Thiol Compounds**

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1. Phe A specifies the structural gene for chorismate mutase-prephenate dehydratase (8).
2. M. J. Gething, T. A. A. Dopheide, and B. E. Davidson, unpublished results.
The kinetics in general suggests that L-phenylalanine binds at a site on the enzyme separate from the active site. This conclusion is reinforced by the observation that on aging the enzyme becomes desensitized to phenylalanine. A similar conclusion has been reached after studies with the enzyme from *S. typhimurium* (11).

**Effect of L-Phenylalanine on Substrate Saturation Curves of Chorismate Mutase-Prephenate Dehydratase-L-Phenylalanine induces homotropic co-operative effects in the enzyme with respect to prephenate. That is, in the presence of fixed concentrations of L-phenylalanine the substrate saturation curves of the dehydratase activity become sigmoidal. In this respect the behavior of the enzyme is similar to that of the same enzyme from *S. typhimurium* (14). This behavior could be explained by either the model of Monod et al. (15) or that of Koeshlund (16), although qualitative examination of the data does not discriminate against either theory. Since there is experimental evidence that the enzyme is capable of self-interaction (3), the possibility that a theory involving polymerization (17) could provide the correct explanation of the sigmoidal kinetics should not be excluded.

In contrast to its effect on the dehydratase activity, L-phenylalanine does not induce co-operative effects for chorismate in the mutase activity. In this respect the enzyme from *E. coli* is different from the enzyme from *S. typhimurium*, which was found by Schmit and Zalkin (14) to give sigmoidal substrate saturation curves for the mutase activity in the presence of L-phenylalanine. The kinetics observed by us for the inhibition of the *E. coli* enzyme is of a partial competitive nature (7). The physical interpretation of this type of inhibition is that L-phenylalanine, in binding to a regulatory site on the enzyme, decreases the affinity of the enzyme for the substrate but has no effect on the rate of conversion of the enzyme-substrate complex to products. If the sole function of the enzyme in the catalysis of the mutase reaction is to bind chorismate exclusively in the conformation necessary for a spontaneous *S*-1' rearrangement to prephenate (18), then the absence of any effect of an inhibitor on the rate of breakdown of the enzyme-substrate complex is to be expected.

**Nature of Phenylalanine Binding Site**—The pattern of the inhibitions obtained with a wide range of phenylalanine analogues enables a number of conclusions to be drawn. These conclusions are valid for the enzyme from strain JP492, but whether they can be extrapolated to the wild type enzyme remains to be investigated.

1. The L-isomer only is active.
2. The ring is required for any significant degree of inhibition.
3. There is a restriction on the size of ring substituents that can be accommodated, the degree of restriction depending on the position of the ring substitution.
4. There is a restriction on the size of substituents that can be accommodated on the β and α carbon atoms since β-phenylserine and α-methylphenylalanine do not inhibit. The observation that phenylpropionic acid inhibits as much as 3-phenylpropionic acid suggests that the α and β hydrogen atoms do not have an active role in the inhibition.
5. The α-NH₂ group is not essential for inhibition, although it is required for a maximal effect.
6. There is an absolute requirement for an unmodified α-COOH group since β-phenylethylamine and phenylalanine amide do not inhibit.

Failure of an analogue to cause inhibition can be due either to its failure to bind satisfactorily to the regulatory site or to its inability, once bound, to induce an appropriate conformational change. In the absence of binding data it is impossible to decide which of these alternatives provides the better explanation for any of the above effects.

**Comparison of Chorismate Mutase-Prephenate Dehydratases from *E. coli* and *S. typhimurium*—**A comparison of the results reported in this and the accompanying paper (3) with those reported for *E. coli* are from this paper and those for *S. typhimurium* are from Reference 14.

| Property                        | *E. coli* | *S. typhimurium* |
|--------------------------------|-----------|------------------|
| Degree of purity               | 95-100% (3) | Partially purified |
| Purification factor, compared with wild type | 2,400-fold (3) | 600-fold |
|                                 |           | 1,500-3,000-fold (19) |
| Specific activity              |           |                  |
| Mutase                         | 64 units/mg (3) | 4.0 units/mg⁻¹ |
| Prephenate                     | 28 units/mg (3) | 5.0 units/mg⁻¹ |
| Mutase to dehydratase          | 2.3 (3) | 1.0 |
| Estimated molecular weight     | 85,000 (3) | 88,000-100,000 (10) |
| Estimated subunit molecular weight | 40,000 (3) |                  |
| Kₐ                             |           |                  |
| Chorismate                     | 0.045 mM | 0.080 mM |
| Prephenate                     | 1.0 mM | 0.35 mM |
| pH optimum                     |           | pH 8.0-8.5 |
| Mutase                         | pH 7.3 | pH 8.0-8.5 |
| Dehydratase                    | Complex |                  |
| Kinetics of phenylalanine inhibition | Partial competitive | Sigmoidal |
| Mutase                         | Sigmoidal |                  |
| Dehydratase                    | Sigmoidal |                  |
| Extent of phenylalanine inhibition | 55%⁻¹ | 55%⁻¹ |
| Mutase                         | 85%⁻¹ | 91%⁻¹ |
| Dehydratase                    |                  |                  |
| Hill coefficient for           |           |                  |
| Mutase                         | 2.0 (0.24 mM PhE) | 2.0 (0.24 mM PhE) |
| No Phe                         | 0.9 | 1.1 |
| Plus Phe                       | 0.8 (0.60 mM PhE) | 2.0 (0.24 mM PhE) |
| Dehydratase                    | 1.1 | 1.1 |
| No Phe                         | 1.1 |                  |
| Plus Phe                       | 2.5 (0.20 mM PhE) | 1.9 (0.24 mM PhE) |
| Phenylalanine inhibition       |                  |                  |
| Mutase                         | 2.3⁴ | 2.6⁴ |
| Dehydratase                    | 9.3⁴ | 1.7⁴ |

* The definition of a unit of enzyme activity given in Reference 14 has been altered to correspond to the conversion of 1 μmol of substrate per min instead of per 10 min.

⁺ The value given is the prephenate concentration at V/2.

⁻ 0.2 mM substrate.

⁻ 1.0 mM substrate.

* 0.5 mM substrate.
obtained by Schmit and Zalkin (14, 19) using partially purified preparations of chorismate mutase-prephenate dehydratase from *S. typhimurium* is given in Table III. Comments have been made on a number of these comparisons in the preceding text and at this stage it suffices to note that, as may be expected when comparing the same enzyme from closely related genera, there are many similarities and few differences in properties.

In addition to the tabulated similarities there is an indication that the polymerization phenomenon observed with the *S. typhimurium* enzyme (19) is also a property of the *E. coli* enzyme (3). The details of this polymerization and its possible relationship to the inhibition by phenylalanine must await further investigations with the pure enzyme.

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Chorismate Mutase-Prephenate Dehydratase from *Escherichia coli* K-12: II. KINETIC PROPERTIES
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Additions and Corrections

Vol. 247 (1972) 7157–7162

In Kredich, Nicholas M., Bruce S. Keenan, and Linda J. Foote. The Purification and Subunit Structure of Cysteine Desulphydrase from Salmonella typhimurium.

The final paragraph of the section “Assay for Cysteine Desulphydrase” under “Experimental Procedure,” Equation 7 should read:

Rearrangement of Equation 6 to:

\[ Q = 2K_aV_i(K_a + A)/K_aQ - 2K_a(K_a + A)/K_a \quad (7) \]

yields an equation which shows that, using a constant amount of enzyme and measuring \( Q \) at various times, \( t \), a plot of \( Q \) versus \( t/Q \) will give a straight line with an intercept on the \( y \) axis equal to the negative reciprocal of \( K_a/2K_a(K_a + A) \). Under the conditions of our standard assay such a plot is in fact linear with an intercept of \(-0.2 \text{ mM}\). Substituting a value of \( 5 \text{ mM}^{-1} \) as the value for \( K_a/2K_a(K_a + A) \) in Equation 6, a plot of \( V_i \) versus enzyme concentration is linear to a sulfide concentration as high as \( 0.08 \text{ mM} \). All enzyme activities reported here have been calculated using Equation 6.

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In Dopheide, Theo. A. A., Pauline Crewther, and Barrie E. Davidson. Chorismate Mutase-Prephenate Dehydratase from Escherichia coli K-12. II. Kinetic Properties.

The caption along the right-hand horizontal axis of Fig. 1 should read:

\[ \log [\text{Pre}] \]

The caption along the vertical axis of Fig. 5 should read:

% Dehydratase Activity

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.