THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 245, No. 15, Issue of August 10, pp. 3763-3770, 1970
Printed in U.S.A.

The Enzymology of Prephenate Dehydrogenase in *Bacillus subtilis**

(Received for publication, March 17, 1970)

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SUMMARY

Prephenate dehydrogenase (prephenate:NAD oxidoreductase (decarboxylating)) was examined in crude extracts of strains 168 and 23 of *Bacillus subtilis* by using a fluorometric assay procedure. Both strains had prephenate dehydrogenases with similar kinetic and allosteric characteristics. The enzyme had an absolute requirement for NAD as a cofactor. The intrinsic K_m for prephenate was 2 mM; for NAD the K_m equaled 1 mM. Optimal catalysis of the reaction was achieved at a temperature of 32°, pH 8.4.

L-Tyrosine, a competitive inhibitor of prephenate dehydrogenase activity, accentuated strongly the cooperativity of binding of prephenate molecules to the enzyme. L-Tyrosine also protected the enzyme from temperature inactivation. D-Tyrosine and L-phenylalanine were also competitive inhibitors of prephenate dehydrogenase. Inhibitions by L-tryptophan and *p*-hydroxyphenylpyruvate were mixed with respect to prephenate.

Further repression of the synthesis of prephenate dehydrogenase by L-tyrosine beyond the existing level of repression in minimal medium was small in both wild type strains. A 2-fold decrement in specific activity resulted when cells were cultured in broth or in minimal media containing L-tyrosine or combinations of aromatic amino acids.

Prephenate dehydrogenase from a regulatory mutant, insensitive to feedback inhibition by L-tyrosine, was also insensitive to inhibition by D-tyrosine, L-phenylalanine, and L-tryptophan.

In different microorganisms a multiplicity of control patterns have been shown to exist within the branched pathway for aromatic amino acid biosynthesis (1, 2). Tyrosine, phenylalanine, and tryptophan are the important controlling effectors of the enzymes in this pathway in many species (3); in others the branch point metabolites, prephenic acid and chorismic acid, result in feedback inhibition of the activity of the first enzyme in the pathway (4, 5). The existence of such regulatory alternatives means that the susceptibility of a particular enzyme in this pathway to feedback inhibition and repression by different metabolites is not necessarily predictable *a priori*.

Prephenate dehydrogenase (prephenate:NAD oxidoreductase (decarboxylating)) is the first enzyme in the aromatic amino acid pathway specific for the biosynthesis of tyrosine (6). This enzyme is feedback inhibited by tyrosine in *Bacillus subtilis* (7, 8) and other microorganisms (9–12). In addition, both phenylalanine and tryptophan have been shown to be inhibitors of this activity in *B. subtilis* (7). The existence of these unexpected cross inhibitions by other aromatic amino acids, and the important regulatory role of prephenic acid, the enzyme substrate (4), prompted a closer examination of the properties of this enzyme.

EXPERIMENTAL PROCEDURE

Bacterial Strains and Growth Media—The description of wild type strains of *B. subtilis* NP 40 and NP 93, derivatives of strains 168 and 23 (13), have been given previously (8). The isolation and characterization of mutant NP 164, a D-tyrosine-resistant excretor of L-tyrosine, has also been detailed (8).

The minimal salts medium of Spizizen (14), modified to contain a 4-fold greater concentration of sodium citrate, was used routinely. Glucose at 0.5% final concentration was added after separate sterilization. TSY broth was previously described (5).

Extract Preparation—Extracts for enzyme analysis were made from 200-ml cultures of cells grown to the late exponential phase of growth (8 to 12 hours) at 37°, with vigorous shaking. The cells were pelleted at 4° by centrifugation for 15 min at 3,000 × *g*, resuspended in 3.0 ml of potassium phosphate buffer (0.05 M, pH 7.8) containing 0.1 M KCl, and broken by 15 min of treatment with lysozyme (100 μg per ml) at 37°. DNase at a final concentration of 10 μg per ml was added to digest DNA. Cell debris was removed by centrifugation at 12,000 × *g* for 20 min at 4°, and 3 ml of the clear supernatant were immediately placed on a column (1.2 × 15 cm) of Sephadex G-25 (coarse). The leading 2.5-ml volume of protein eluate was collected for use in enzyme studies.

Substrate Preparation and Prephenate Dehydrogenase Assays—Barium prephenate was isolated and purified from culture supernatants of *Aerobacter aerogenes* 62-1 by a combination of the methods of Metzenberg and Mitchell (15) and Gibson (16). The substrate purity was greater than 95%, based on the acid conversion of prephenate to phenylpyruvate, by using an extinc-

* This investigation was supported by Public Health Service Training Grant 1-701-GM-01459-01 from the National Institute of General Medical Sciences and by Research Grant AM 13105 from the National Institute of Arthritis and Metabolic Diseases.

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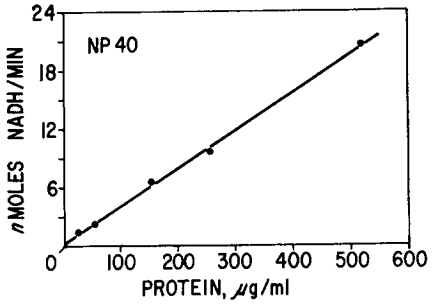

FIG. 1. Enzyme activity as a function of extract protein concentration. The slope defines the specific activity of the enzyme from NP 40, 38 nmoles of NADH per min per mg of protein.

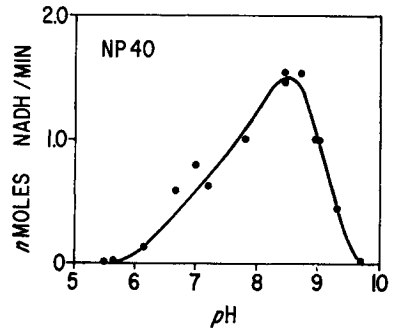

FIG. 2. Enzyme activity as a function of pH in NP 40. The incubation mixture (without enzyme) was adjusted to the pH indicated with 0.1 N HCl or 0.1 N KOH. Enzyme (40 μg of protein) was added to initiate the reaction, the velocity was measured, and the pH was redetermined at the end of the assay. No change in pH was found.

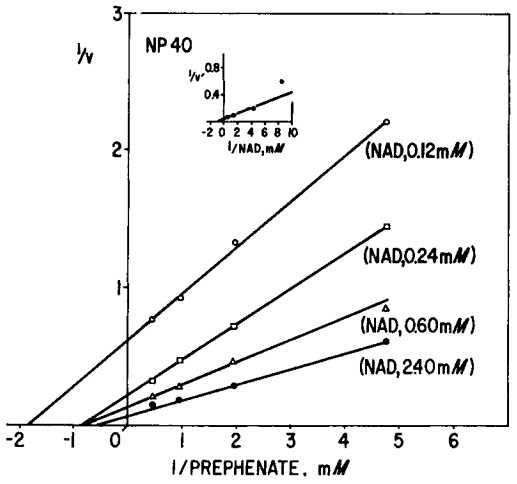

FIG. 3. Double reciprocal plot of initial velocity *versus* prephenate concentration for different fixed concentrations of NAD. *Inset*, secondary plot of reciprocal of apparent maximal velocity, v' (with variable prephenate), against reciprocal of NAD concentration. The extract made from NP 40 contained 205 μg of protein.

tion coefficient for phenylpyruvate of 1.75×10^4 liters mole^{-1} cm^{-1} at 320 nm in 1 N NaOH (9). All prephenate concentrations given have been corrected for purity. Stock solutions of prephenate at 2.0 mg per ml and NAD at 4.0 mg per ml were made in 0.05 M Tris·HCl buffer, pH 8.1.

Assays for prephenate dehydrogenase activity were conducted by monitoring the increase in fluorescence of NADH formed in

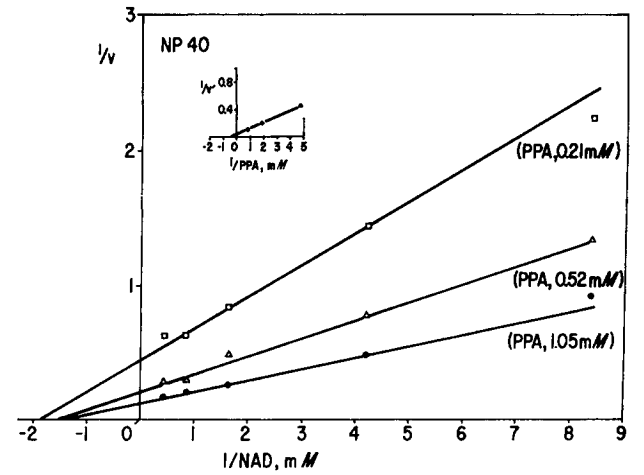

FIG. 4. Double reciprocal plot of initial velocity *versus* NAD concentration for different fixed concentrations of prephenate (*PPA*). *Inset*, secondary plot of reciprocal of apparent maximal velocity, v' (with variable NAD), against reciprocal of prephenate concentration. The extract made from NP 40 contained 205 μg of protein.

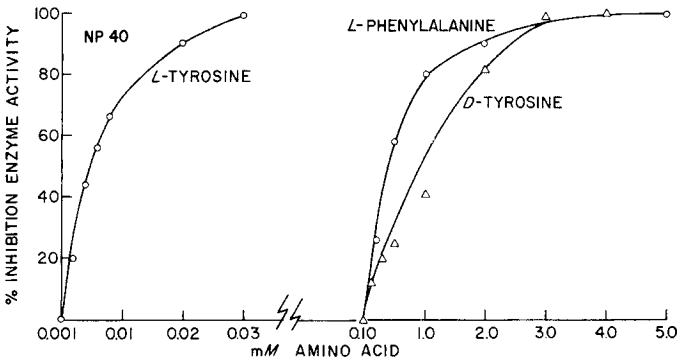

FIG. 5. Inhibition curves for activity of prephenate dehydrogenase in the presence of L-tyrosine, D-tyrosine, or L-phenylalanine in NP 40. Percentage of inhibition equals $v_o - v_i/v_o$, where v_o = velocity in absence of inhibitor and v_i = velocity in the presence of inhibitor.

the reaction (9) with an Aminco-Bowman spectrophotofluorometer. The excitation wave length was 340 nm; the emission wave length was 460 nm (uncorrected). The reaction mixture contained 30 μmoles of potassium phosphate, pH 7.8, 20 μmoles of Tris·HCl, pH 8.1, 60 μmoles of KCl, 1.2 μmoles of NAD, 1.0 μmole of barium prephenate, and 0.01 to 0.10 ml of extract in a total reaction volume of 1.0 ml. The enzyme and NAD were previously incubated at 37° for 2 to 3 min and prephenate was added to initiate the reaction. Initial velocities, determined from the slopes of lines traced on a recorder, were linear for at least 5 min. No increase in fluorescence occurred in the absence of either substrate, and the reaction began without a lag upon the addition of prephenate. A circulating water bath was used to maintain constant temperatures in the cuvette chamber of the spectrophotometer. The convenience, sensitivity, accuracy, and specificity of this assay contrast markedly with the more tedious and less precise Millon assay for phenols (6).

When the Millon assay was used, the reaction volume was scaled down to 0.5 ml and enzyme incubations were conducted in test tubes (13 × 100 mm) for 20 min in a reciprocal shaker at 37° (7). Comparable results were obtained with either assay

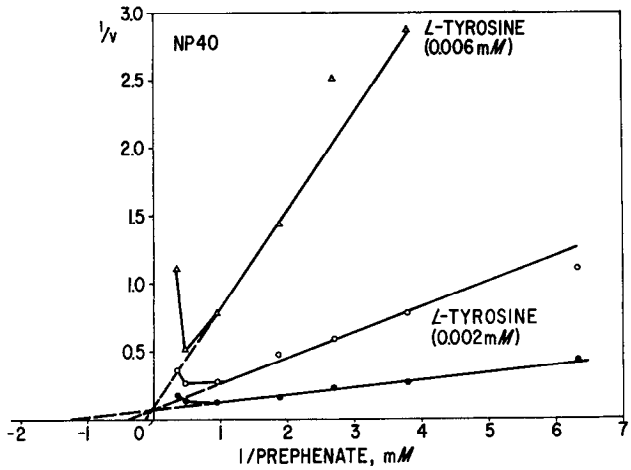

FIG. 6. Double reciprocal plot for inhibition of prephenate dehydrogenase activity by L-tyrosine in NP 40. The activity in the presence of 0.002 (○——○) or 0.006 (△——△) mM L-tyrosine is compared with the control activity assayed in the absence of L-tyrosine (●——●). The reaction mixture contained 276 μg of protein. Substrate inhibition occurs at high substrate concentrations, *i.e. points to the left.*

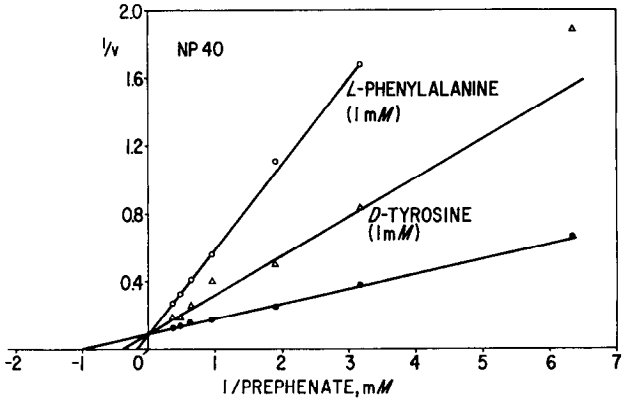

FIG. 7. Double reciprocal plot for inhibition of prephenate dehydrogenase activity by D-tyrosine and L-phenylalanine in NP 40. The activity in the presence of 1.0 mM D-tyrosine (△——△) or 1.0 mM L-phenylalanine (○——○) is compared with the control activity assayed in the absence of either amino acid (●——●). The reaction mixture contained 215 μg of protein.

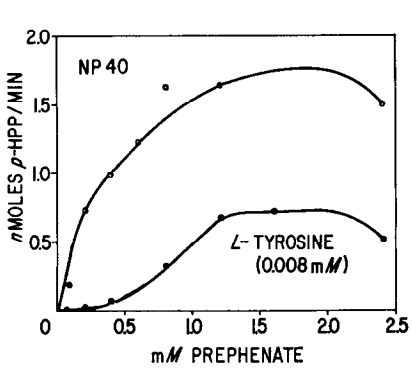

FIG. 8. Substrate saturation curve for the inhibition of prephenate dehydrogenase activity by L-tyrosine in NP 40. *p*-Hydroxyphenylpyruvate (*p-HPP*) formation was assayed by the Millon assay (6). The activity in the presence of 0.008 mM L-tyrosine (●——●) is compared to the control activity assayed in the absence of L-tyrosine (○——○). The NAD concentration was 1.0 mM. The reaction mixture contained 83 μg of protein.

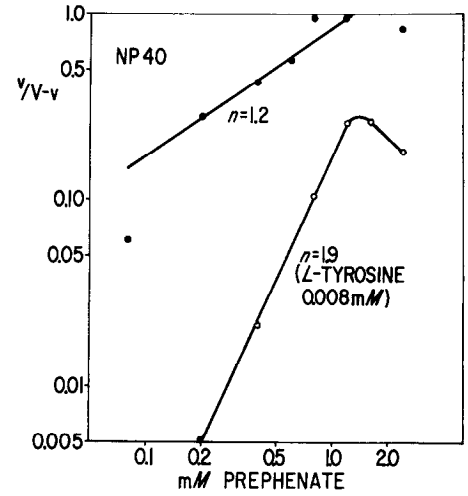

FIG. 9. Hill plot of prephenate dehydrogenase activity in NP 40 in the presence and absence of L-tyrosine. The data from Fig. 8 are plotted as log $v/V_{max} - v$ *versus* log of prephenate concentration, mM. The V_{max} was determined from a double reciprocal plot (not shown). The slopes express the values of interaction coefficients (n) exerted between substrate binding sites with and without 0.008 mM L-tyrosine.

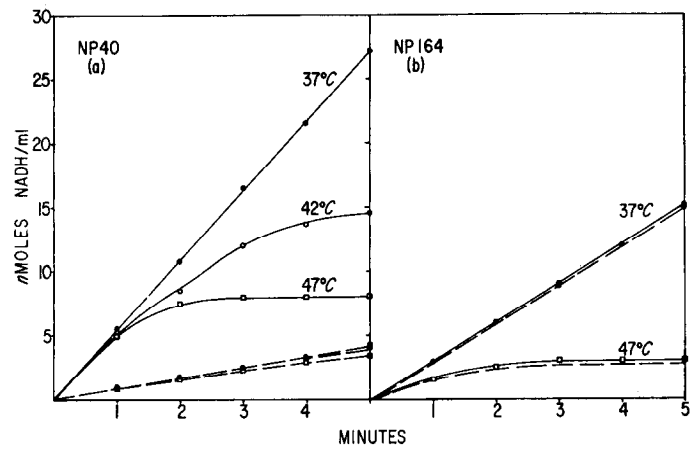

FIG. 10. Activity of prephenate dehydrogenase at different temperatures and protection by L-tyrosine in NP 40. *a*: ——, enzyme from NP 40 incubated at 37°, 42°, or 47° for the time duration indicated; – – –, as above in presence of 0.01 mM L-tyrosine. The reaction mixtures contained 0.43 mg of protein. *b*: ——, enzyme from NP 164 incubated at 37° or 47° for time durations indicated; – – –, as above in presence of 1.0 mM L-tyrosine. The reaction mixtures contained 0.78 mg of protein.

procedure and equivalent amounts of *p*-hydroxyphenylpyruvate and NADH were formed in the reaction mixtures. Specific activities are expressed as nanomoles of NADH (or *p*-hydroxyphenylpyruvate) formed per min per mg of protein.

Protein concentrations were estimated by the procedure of Lowry *et al.* (17) by using crystalline bovine serum albumin as a standard.

Inhibition and Repression—Compounds to be tested as inhibitors of prephenate dehydrogenase activity were prepared as stock solutions at 10 mM in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 M KCl. Inhibitors were diluted 10-fold in the incubation mixture containing the enzyme and NAD (1.2 mM) and were incubated at 37° for 3 to 5 min before the addition of prephenate (1.0 mM).

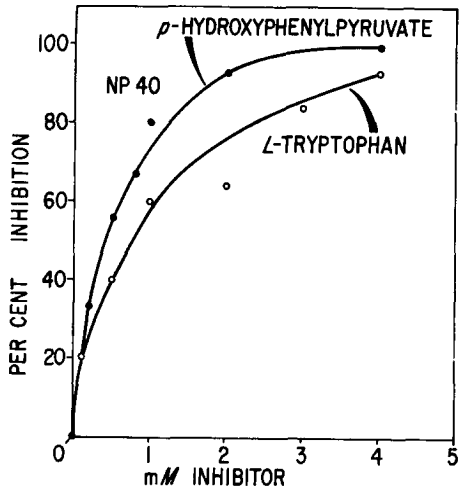

FIG. 11. Inhibition curves for activity of prephenate dehydrogenase in the presence of L-tryptophan or *p*-hydroxyphenylpyruvate in NP 40. Percentage of inhibition equals $v_o - v_i/v_o$.

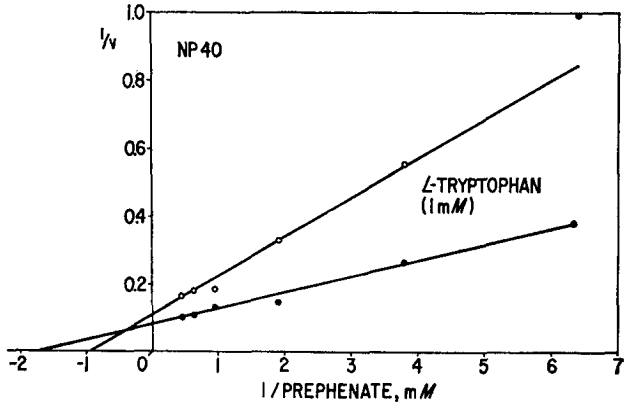

FIG. 12. Substrate saturation data plotted in double reciprocal form for inhibition of prephenate dehydrogenase activity by L-tryptophan in NP 40. The activity in the presence of 1.0 mM L-tryptophan (○——○) is compared to the control activity, assayed in the absence of L-tryptophan (●——●). The reaction mixtures contained 258 μg of protein.

Repression of prephenate dehydrogenase synthesis was examined by growing cells in minimal medium supplemented with different aromatic amino acids, usually at concentration of 100 μg per ml. Enzyme levels in cells grown in TSY broth were also assayed. Enzyme activity was assayed under conditions of substrate saturation by using several dilutions of each extract. The specific activity of prephenate dehydrogenase in each extract was compared to the specific activity of extracts from cells grown in minimal medium without supplementation.

Chemicals—Lysozyme (crystallized three times), DNase, bovine serum albumin, NAD, NADP, NADH, and *p*-hydroxyphenylpyruvate were the products of Sigma. Amino acids were purchased from Calbiochem. Other chemicals were of the highest reagent quality that was commercially available.

RESULTS

Properties of Enzyme—Prephenate dehydrogenase was initially studied in extracts of *B. subtilis* NP 40. Gentle lysis, effected by the use of lysozyme as well as 0.1 M KCl in the buffer, was found to be important in preserving the activity of the enzyme. $MgCl_2$ at 1.0 mM, β-mercaptoethanol at 1.0 mM, and EDTA at 0.1 mM

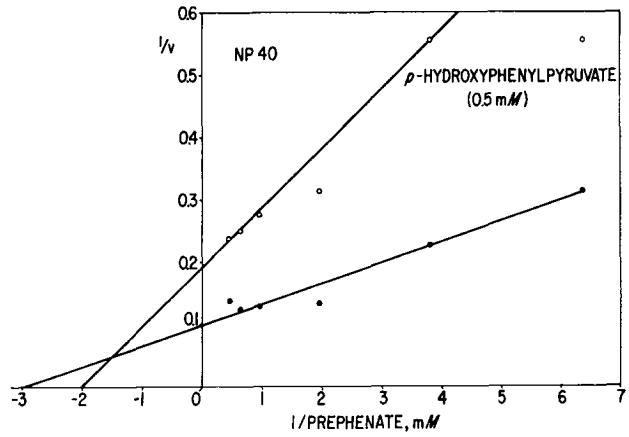

FIG. 13. Substrate saturation data plotted in double reciprocal form for inhibition of prephenate dehydrogenase activity by *p*-hydroxyphenylpyruvate in NP 40. The activity in the presence of 0.5 mM *p*-hydroxyphenylpyruvate (○——○) is compared to the control activity assayed in the absence of this compound (●——●). The reaction mixtures contained 303 μg of protein.

neither enhanced the activity nor prolonged the stability of the enzyme.

The enzyme reaction required NAD. NADP at equivalent concentrations did not substitute for NAD in the reaction. NADP did not inhibit the reaction in the presence of NAD.

The stoichiometry of the two reaction products was examined by incubating the enzyme with saturating concentrations of substrates for 30 min at 37°. The increase in fluorescence at 460 nm corresponded to the production of 46 nmoles of NADH per ml of reaction mixture. This mixture was divided into two parts and duplicate determinations of *p*-hydroxyphenylpyruvate (as Millon-positive material) were made. From this assay it was determined that 45 nmoles of *p*-hydroxyphenylpyruvate per ml of incubation mixture were formed.

The specific activity of the enzyme did not differ significantly in extracts prepared from cells harvested at different phases of the growth cycle.

The reaction velocity was a linear function of protein concentration from 25 to 500 μg of protein per ml (Fig. 1). The slope of this line is the specific activity of the enzyme, 38 nmoles of NADH per min per mg of protein.

The pH optimum for enzyme activity was 8.4 (Fig. 2). This pH is also very likely the range of over-all substrate stability, since prephenate is very acid-labile (16) and NAD is destroyed by mild alkali treatment (18). Although the temperature optimum for enzyme activity was found to be 32° (19), most assays were conducted at 37°.

By independently varying each substrate, a series of substrate saturation curves were obtained, relating enzyme velocity to variable concentrations of one substrate, at fixed concentrations of the second. These data, expressed as Lineweaver-Burk plots (20), are presented in Figs. 3 and 4. With prephenate as the variable substrate, a series of intersecting lines were obtained, which extrapolate to give a value of $s_{0.5}$ (21) for prephenate of 1.0 mM. *Insets* shown in these figures are secondary plots of the reciprocal of the apparent maximal velocity of the variable substrate against the reciprocal of the concentration of the fixed substrate. These latter plots permit extrapolation to intrinsic kinetic constants for the enzyme. The intrinsic K_m for prephenate from the *inset* of Fig. 4 is 2.0 mM. The intrinsic K_m for

TABLE I

Compounds tested as inhibitors of prephenate dehydrogenase from NP 40

All concentrations were 1 mM.

Compound	Relative activity[a]
D-Phenylalanine	1.00
L-Histidine	1.00
Phenylpyruvate	0.98
Barium chorismate	0.55
Shikimate	0.86
p-Aminobenzoate	0.80
p-Hydroxybenzoate	1.00
Phenol	1.00
Benzene	1.00

[a] Activity in the absence of inhibitor was taken as 1.00; the control activity was 4.0 nmoles of NADH per min. The reaction mixture contained 255 μg of protein.

TABLE II

Enzyme intrinsic kinetic constants for prephenate dehydrogenase in NP 40

Substrate	K_m	V_{max}
	mM	*nmoles/min*
PPA	2.0	20
NAD	1.0	20

NAD is 1.0 mM. For each substrate, the intrinsic maximum velocity is 20 nmoles per min. For the amount of protein used (205 μg), this gives an intrinsic specific activity for the enzyme in crude cell extracts of 100 nmoles per min per mg of protein. Under the assay conditions normally used, about 35 to 40% of this activity was observed.

Inhibition Analysis—The inhibition curves in Fig. 5 show the influence of L-tyrosine, D-tyrosine, and L-phenylalanine on the activity of prephenate dehydrogenase. L-Tyrosine was a potent feedback inhibitor of this enzyme. The K_i for L-tyrosine was 0.003 mM as determined from Dixon plots of reciprocal velocity against inhibitor concentration (22). D-Tyrosine and L-phenylalanine also inhibited this activity, although concentrations about 100 times greater than that of L-tyrosine were required. The K_i for L-phenylalanine was 0.3 mM; the K_i for D-tyrosine was 1.3 mM.

Substrate saturation data plotted in double reciprocal form are given in Figs. 6 and 7. Inhibition by L-tyrosine was competitive with respect to prephenate (Fig. 6). Substrate inhibition by prephenate occurred in the absence of L-tyrosine and was even more apparent in the presence of this inhibitor. L-Phenylalanine and D-tyrosine were also competitive inhibitors of prephenate dehydrogenase activity (Fig. 7).

The effect of L-tyrosine upon the cooperative interactions between substrate molecules (23) is illustrated in Fig. 8. It is readily apparent that L-tyrosine converts a slightly sigmoid curve to a very pronounced sigmoid curve. These data are replotted in Fig. 9 according to the Hill equation: $\log v/V_{max} - v = n \cdot \log (S) - \log K$ (24, 25). The interaction coefficient, n, in the absence of L-tyrosine, equaled 1.2. In the presence of 0.008 mM L-tyrosine, n equaled 1.9.

The enzyme velocity decreased rapidly with time at temperatures above 37° (Fig. 10*a*). L-Tyrosine at 0.01 mM, while substantially inhibiting the activity, allowed a linear velocity for much longer periods of time at elevated temperatures. This protective influence of L-tyrosine on the enzyme activity is substantiated by the data in Fig. 10*b*, which show that L-tyrosine had no protective effect on the enzyme from NP 164, a mutant insensitive to the growth inhibitory effects of D-tyrosine (8). This altered enzyme, which was not inhibited by L-tyrosine, also was not protected by it, presumably because the enzyme no longer bound tyrosine.

L-Tryptophan and the reaction product, *p*-hydroxyphenylpyruvate, were also inhibitors of the enzyme activity. The inhibition curves for these compounds are given in Fig. 11. From Dixon plots the K_i values were 0.7 mM for L-tryptophan and 0.4 mM for *p*-hydroxyphenylpyruvate. Both inhibitions were mixed with respect to prephenate, as shown by Figs. 12 and 13.

A number of other compounds were tested as possible inhibitors of the activity of prephenate dehydrogenase. These results are summarized in Table I. Of the amino acids, aromatic intermediates, vitamins, and related compounds tested, only chorismate, the immediate precursor of prephenate, showed any strong inhibition at 1.0 mM. It may function as a substrate analogue, by virtue of its structural similarity to prephenate (16).

The intrinsic kinetic constants for the enzyme, derived from Figs. 3 and 4, are listed in Table II. A summary of the data from the inhibition analyses is presented in Table III.

Prephenate Dehydrogenase in Other Strains of B. subtilis—Prephenate dehydrogenase from mutant NP 164 was insensitive to feedback inhibition by L-tyrosine (Fig. 10*b*). As detailed in

TABLE III

Enzyme inhibition analysis for prephenate dehydrogenase in NP 40

Effector	Type	K_i	n
		mM	
None			1.2
L-Tyrosine	Competitive	0.003	1.9
D-Tyrosine	Competitive	1.30	
L-Phenylalanine	Competitive	0.30	
L-Tryptophan	Mixed	0.70	
p-Hydroxyphenylpyruvate	Mixed	0.40	

TABLE IV

Effect of inhibitors on prephenate dehydrogenase from NP 164

The concentration of each inhibitor was 1 mM.

Inhibitor	Relative activity at various prephenate concentrations[a]					
	2.62 mM	1.58 mM	0.53 mM	0.16 mM	1.05 mM	0.26 mM
L-Tyrosine	1.00	0.92	0.83	0.70		
D-Tyrosine	1.00	0.85	0.83	0.50		
L-Phenylalanine	1.00	0.92	0.83	0.70		
L-Tryptophan					1.00	1.00
p-Hydroxyphenylpyruvate					0.50	0.20

[a] Activity in the absence of each inhibitor is taken as 1.00; the control activity is equivalent to the reciprocal of velocity indicated on Fig. 14 for each prephanate concentration. The reaction mixtures contained 228 and 190 μg of protein, respectively.

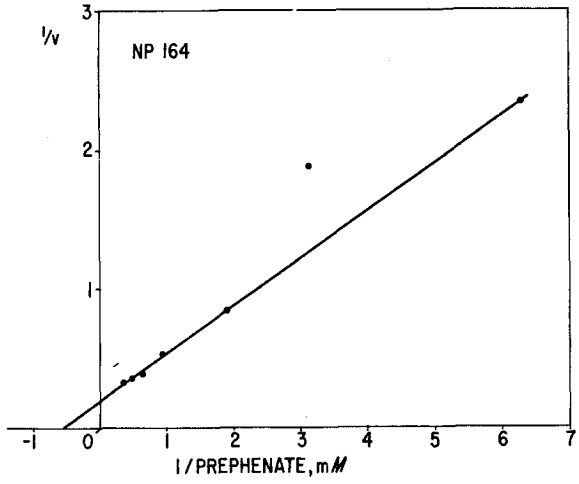

FIG. 14. Substrate saturation data plotted in double reciprocal form for prephenate dehydrogenase activity from mutant NP 164. The reaction mixtures contained 228 μg of protein.

TABLE V

Repression of prephenate dehydrogenase

Strain	Growth medium	k^a	Activity	Protein	Specific activity	Repression
			nmoles/min	*μg*	*nmoles/min/mg*	*-fold*
NP 40	Minimal	0.55	7.4	240	35	0
NP 40	L-Tyrosine	0.55	3.0	193	18	2.0
NP 40	Group III[b]	0.55	3.8	240	18	2.0
NP 40	TSY broth	0.92	3.8	220	18	2.0
NP 164	Minimal	0.49	2.0	255	7.8	4.5
NP 93	Minimal	0.69	6.4	61	105	0
NP 93	L-Tyrosine	0.69	3.0	44	64	1.65

[a] Specific growth rate per hour (26).

[b] L-Tyrosine + L-phenylalanine + L-tryptophan, each at 100 μg per ml.

Table IV, L-tyrosine at 1.0 mM was only slightly inhibitory at all substrate concentrations tested. The altered enzyme in NP 164 was also insensitive to inhibition by L-phenylalanine, D-tyrosine, and L-tryptophan (Table IV). Of the compounds affecting the enzyme of NP 40, only *p*-hydroxyphenylpyruvate was significantly inhibitory in NP 164.

Substrate saturation data obtained with the enzyme from NP 164 are shown in double reciprocal form in Fig. 14. The $s_{0.5}$ for prephenate was 2.0 mM and the apparent maximum velocity was 5.55 nmoles per min. The specific activity of the enzyme of this strain was reduced about 4-fold, due to the repressive effects of the tyrosine overproduced (Table V).

NP 93 is a second wild type strain of *B. subtilis* (8). For prephenate the $s_{0.5}$ was determined to be 0.5 mM; for NAD $s_{0.5}$ was 0.25 mM. L-Tyrosine, D-tyrosine, and L-phenylalanine competitively inhibited the activity in this strain, as they did in NP 40 (Fig. 15). The specific activity of the enzyme from NP 93 was 110 nmoles per min per mg, about 3 times as great as that of NP 40, comparing extracts from cultures grown in minimal medium.

Repression of Enzyme Synthesis—Table V is a compilation of the data relevant to the repression of the synthesis of prephenate dehydrogenase in strains NP 40, NP 164, and NP 93. The *last column* of Table V indicates the diminution in specific activity of cells grown in supplemented medium compared with cells grown in minimal (unsupplemented) medium.

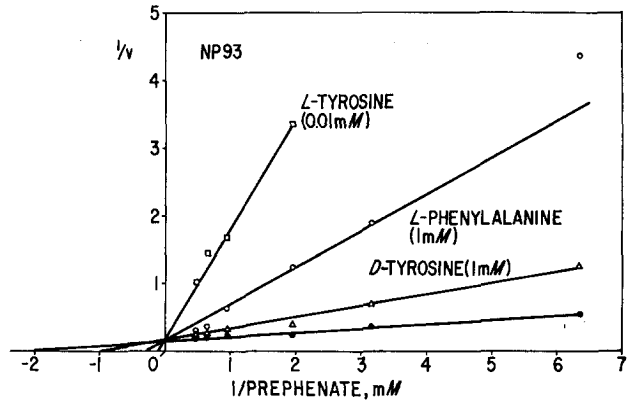

FIG. 15. Substrate saturation data plotted in double reciprocal form for inhibition of prephenate dehydrogenase activity by L-tyrosine, D-tyrosine, or L-phenylalanine in strain NP 93. The activity in the presence of 0.01 mM L-tyrosine, 1.0 mM D-tyrosine, or 1.0 mM L-phenylalanine is compared to the control activity assayed in the absence of each amino acid (●——●). The reaction mixtures contained 61 μg of protein.

L-Tyrosine at 100 μg per ml repressed the synthesis of the enzyme in NP 40 2-fold, compared to the specific activity of the cells cultured in minimal medium. The addition of L-phenylalanine and L-tryptophan at 100 μg per ml or growth in a complete medium (TSY) caused no further repression of this enzyme. In NP 93, the absolute values of the specific activities were about 3-fold greater than in NP 40, but the relative repression observed in the presence of L-tyrosine was identical.

Maximal repression was observed in strain NP 164 even when grown in minimal medium because of the repressive effects of the L-tyrosine overproduced by this mutant. Supplementation with aromatic amino acids during growth did not decrease this level of activity further.

DISCUSSION

Prephenate dehydrogenase in *B. subtilis* is an important regulatory enzyme in the aromatic amino acid pathway, as expected of an enzyme located at a metabolic branch point. Its feedback control by tyrosine and the cross-inhibition of its activity by phenylalanine and tryptophan have been studied (7, 8). Repression control by tyrosine over the synthesis of this enzyme is also important (27). Influential but unexplained effects of histidine on the activity and synthesis of prephenate dehydrogenase have been described (28). Mutational loss of the specificity for control of this enzyme by feedback inhibition results in tyrosine excretion (8, 19, 27). The sensitivity of prephenate dehydrogenase to false feedback inhibition by D-tyrosine plays an important role in the mechanism of the action of this antimetabolite as a metabolic inhibitor (8).

Prephenate dehydrogenase, as assayed in crude extracts, was characterized by a requirement for KCl for optimal activity, a relatively high pH optimum (8.4), and an absolute requirement for NAD. The stimulation of activity by KCl is similar to the stimulation of tryptophan synthetase activity in *B. subtilis* by this salt (29). A high pH optimum was also characteristic of the enzyme isolated from *Escherichia coli* (6) and from *A. aerogenes* (10). In the latter two microorganisms, NADP could

partially substitute for NAD, in contrast to the result obtained in *B. subtilis*.

The kinetic analysis was compatible with the theoretical result expected for an enzyme displaying an independent binding of two substrates in a random order (30). Either substrate initiated the reaction without a detectable lag. Verification of this mechanism, of course, must await similar kinetic studies with purified enzyme preparations.

Feedback inhibition of prephenate dehydrogenase provides the major control in the pathway of tyrosine biosynthesis. Although the transamination reaction between *p*-hydroxyphenylpyruvate and tyrosine has not been studied in *B. subtilis*, it is assumed that the transamination is not the limiting reaction of tyrosine biosynthesis. L-Tyrosine was a potent competitive inhibitor of prephenate dehydrogenase activity with respect to prephenate and increased the cooperative binding of prephenate to the enzyme. L-Tyrosine may also have a function *in vivo* of stabilizing the enzyme against temperature inactivation. Prephenate dehydrogenase was inhibited by L-tyrosine in *A. aerogenes* and *E. coli* (10), in *Saccharomyces cerevisiae* (2), and in *Neurospora crassa* (12).

The activity of prephenate dehydrogenase was also affected by L-phenylalanine and D-tyrosine. Both of these amino acids were competitive inhibitors with prephenate; neither compound was as effective an inhibitor as L-tyrosine. The control of this enzyme *in vivo* by phenylalanine may be important in the partitioning of prephenic acid between prephenate dehydrogenase and prephenate dehydratase (7). False feedback inhibition by D-tyrosine contributes to the growth-inhibitory effects of this compound (8). Neither amino acid is significantly inhibitory to the enzymes of *A. aerogenes* or *E. coli* (10). L-Phenylalanine is stimulatory to prephenate dehydrogenase activity in *S. cerevisiae* (2) and in *N. crassa* (12). Neither D-phenylalanine nor phenylpyruvate influenced the activity of the enzyme in *B. subtilis*. The reported inhibition of prephenate dehydrogenase activity in *B. subtilis* by L-histidine (28) was not observed in this study. Since the method of extract preparation was stated to be important in preserving sensitivity to histidine inhibition, this explanation could account for the difference in results.

L-Tryptophan and the reaction product, *p*-hydroxyphenylpyruvate, were both mixed type inhibitors of the enzyme activity. Tryptophan inhibition of this activity was also demonstrated by Nester and Jensen (7). Its physiological significance is unknown. Product inhibition by *p*-hydroxyphenylpyruvate could be important in controlling the amount of substrate available for transamination to tyrosine *in vivo*. *p*-Hydroxyphenylpyruvate is also an inhibitor of prephenate dehydrogenase in *A. aerogenes* (10).

Strain NP 93, a poorly transformable wild type strain of *B. subtilis* (31), was as sensitive as wild type NP 40 to inhibition by L-tyrosine, L-phenylalanine, and D-tyrosine. The specific activity of prephenate dehydrogenase in this strain was 3-fold greater than the comparable activity in NP 40. The D-tyrosine-resistant mutant, NP 164, was insensitive to feedback inhibition by L-tyrosine. In addition, this altered enzyme had a decreased affinity for prephenate as well as a concomitant loss of sensitivity to inhibition by L-phenylalanine, L-tryptophan, and D-tyrosine. Only the product, *p*-hydroxyphenylpyruvate, produced a significant inhibition at each substrate concentration tested. Insensitivity of mutant NP 164 to inhibition by tyrosine correlated with an increased excretion of tyrosine (19), and an increased temperature lability of the enzyme, as well as decreased specific activity, the result of repression by tyrosine.

Mutant NP 164 possesses a prephenate dehydrogenase which has lost its specificity for feedback control by tyrosine. Even though a compensatory and maximal repression of enzyme synthesis occurs, tyrosine is still overproduced. Hence, repression of prephenate dehydrogenase synthesis by L-tyrosine alone is obviously not sufficient to control tyrosine biosynthesis in *B. subtilis*. These results contrast with those in *Salmonella typhimurium*, in which tyrosine supplementation can result in a total inhibition of prephenate dehydrogenase biosynthesis (32).

The regulatory enzymes of the other two branches of the aromatic pathway in *B. subtilis* are known to interact with unexpected allosteric metabolites, both *in vivo* as well as *in vitro*. Anthranilate synthetase is activated by histidine (33) and prephenate dehydratase is activated by methionine and leucine and inhibited by tryptophan (34). The physiological significance of these examples of "metabolic interlock" have been discussed (35). It will be interesting to examine prephenate dehydrogenase in this context, since it also has several unexpected allosteric specificities.

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