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GRAMICIDIN S-SYNTHETASE

A FURTHER CHARACTERIZATION OF PHENYLALANINE RACEMASE, THE LIGHT ENZYME OF GRAMICIDIN S-SYNTHETASE

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Summary

1. Chromatography on hydroxyapatite and on aminohexyl-Sepharose as well as isoelectric focusing were introduced as new effective purification procedures for phenylalanine racemase (EC 5.1.1.11). The enzyme preparations obtained were essentially homogeneous, as demonstrated by specific activity measurements and polyacrylamide gel electrophoresis.

2. The enzyme is not dissociable by sodium dodecyl sulfate.

3. Phenylalanine racemase is an acidic protein with an isoelectric point of approx. 4.6 (isoelectric focusing).

4. The Michaelis constants of L-Phe and D-Phe in the aminoacyl adenylate activation are 0.06 and 0.13 mM, respectively.

5. From our studies with structural analogues of phenylalanine we infer that the amino group of this amino acid is essential for its binding to the aminoacyl adenylate reaction center. The carboxyl group is not at all or only weakly bound. The benzene ring of phenylalanine which determines substrate recognition also seems to be of minor importance for substrate binding.

Introduction

Phenylalanine racemase (EC 5.1.1.11) is the light component of the multienzyme complex that catalyzes the biosynthesis of gramicidin S. This enzyme racemizes phenylalanine, activating it first to aminoacyl adenylate and in a second step to the thioester. From this stage the amino acid is transferred to the heavy enzyme thus initiating biosynthesis of antibiotic [1—4].

Abbreviations: TES, tris-(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid.

Though phenylalanine racemase was highly purified by Kurahashi and his colleagues [5,6], and characterized by several authors [5—12], only a small amount of information is available concerning its molecular properties, the architecture of its active sites and the mechanism of its action.

In this paper we report new purification procedures for this enzyme and discuss the results of our experiments further characterizing phenylalanine racemase. Preliminary results were published on the 10th FEBS congress at Paris 1975 [13].

Materials and Methods

Materials

L-phenylalanine, L-tyrosine, hydrocinnamic acid and the calibration proteins used for the molecular weight determination of phenylalanine racemase were purchased from Merck/Schuchardt.

D-phenylalanine as well as 1- and 2-phenylethylamine were products from Fluka, and *p*-fluoro-D,L-phenylalanine from Bachem.

Triethanolamine-hydrochloride, AMP and ATP were obtained from Boehringer, TES from Serva and dithiothreitol from RSA Corp.

Column materials were DE-52 from Whatman, Sepharose 6B and aminohexyl-Sepharose 4B from Pharmacia and hydroxyapatite "Hypatite C" from Clarkson Chem. Comp.

L-[U-^{14}C]phenylalanine was purchased from ICN and tetrasodium[^{32}P]-pyrophosphate from Amersham.

Methods

1. Growth of organism. Bacillus brevis ATCC 9999 was cultured as described in the preceding paper [14].

2. Assays for the reactions of phenylalanine racemase. The activation of phenylalanine by phenylalanine racemase was measured by the ATP-PP_i exchange reaction according to Kleinkauf et al. [15,16]. For the measurement of phenylalanine racemization, a modified procedure of Yamada et al. was used [7,17]. All samples were incubated at 37°C for 30 min.

Gramicidin S synthesis was estimated by the millipore filter assay of Gevers et al. [18], as described in the preceding paper [14].

3. Protein determination. In the first steps of purification of phenylalanine racemase up till the gel filtration on Sepharose 6B, the protein concentration was determined spectrophotometrically by the method of Lowry et al. [19]. At higher purification stages a slightly modified method of Schaffner and Weissmann [20] was used.

4. Polyacrylamide gel electrophoresis. Gels containing 8% acrylamide and 0.18% *N,N'*-methylenebisacrylamide were run either in 0.1 M Tris · HCl pH 8.5 at 4°C or in 0.1 M phosphate pH 7.2 at room temperature. All buffers contained 0.1% 2-mercaptoethanol. Samples up to 0.1 ml in 20 mM triethanolamine buffer or 0.01—0.1 M phosphate buffer with addition of sucrose were layered directly onto the Tris or phosphate gels respectively.

Electrophoresis was performed at 1—2 mA/tube for the Tris gels and at 10 mA/tube for the phosphate gels. The gels were stained 15 min at 80°C in a solu-

tion of 0.01% Coomassie brilliant blue R 250 in 12.5% trichloroacetic acid. For sodium dodecyl sulfate/polyacrylamide gel electrophoresis buffers contained 0.1% sodium dodecyl sulfate. Samples were treated with 0.1% sodium dodecyl sulfate at 50°C for 15 min. The gels were stained according to the method of Weber and Osborn [21], and destained in a solution of 5% methanol and 7.5% acetic acid.

5. *Isoelectric focusing.* A linear sucrose gradient containing approx. 1% ampholine (pH 3—10 or pH 4—6)was built up in a 110 ml LKB column and a pH gradient was preformed over night at 4°C. Samples of 1 ml protein solution with addition of 0.35 g sucrose were carefully inserted into the upper third of the column. Electrophoresis was performed at 400 V for at least 15 h at 4°C.

6. *Measurements of absorbance.* The measurements of absorbance were performed with a Zeiss spectrophotometer PMQ 3 or a Gilford spectrophotometer model 2400 S. Polyacrylamide gels were scanned at 590 nm with a gel scanning accessory to the Gilford instrument.

7. *Purification of the enzyme. Step 1*: preparation of the crude cell extract. *Step 2*: ammonium sulfate fractionation and desalting on Sephadex G 50 medium. *Step 3*: DEAE-cellulose chromatography. *Step 4*: gelfiltration on Sepharose 6B. These purification steps were described in detail in the preceding paper [14]. The two enzymes of gramicidin synthetase are separated on the Sepharose 6B column according to their molecular weight. *Step 5*: hydroxyapatite chromatography. The fractions from the Sepharose 6B gelfiltration containing phenylalanine racemase localized by filter tests for gramicidin S biosynthesis were applied to a hydroxyapatite column (2 × 12 cm) which was equilibrated with 0.01 M sodium phosphate buffer, pH 7.5. The enzyme was eluted with a linear gradient of 0.01—0.2 M phosphate buffer (2 × 100 ml) at a flow rate of 80 ml/h. Both equilibration and elution buffers contained 2 mM $MgCl_2$; 0.25

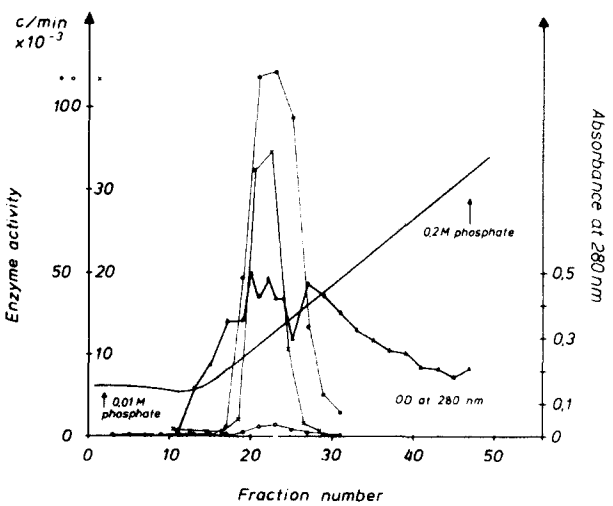

Fig. 1. Hydroxyapatite chromatography of phenylalanine racemase. This chromatography was performed as described in the text. The fractions obtained were tested for gramicidin S biosynthesis (x); D-Phe-dependent ATP-PP_i exchange (●); ATP-PP_i exchange without the addition of amino acid (○) and absorbance at 280 nm (▲).

mM EDTA and 2.5 mM dithiothreitol (buffer P). Fractions of approx. 4 ml were collected and tested for enzyme activity. A characteristic elution pattern of a hydroxyapatite chromatography of phenylalanine racemase is shown in Fig. 1. In Fig. 2—1a and b the distribution of protein in the active fractions 19—25 of the experiment in Fig. 1 is demonstrated by polyacrylamide gel electrophoresis. *Step 6*: chromatography on aminohexyl-Sepharose 4B. The active fractions from the hydroxyapatite column were layered onto a aminohexyl-Sepharose 4B column (1 × 10 cm), previously equilibrated with 20 mM triethanolamine/HCl buffer pH 7.8. The enzyme was eluted with a linear gradient of 0.1—1.0 M KCl in the same buffer (2 × 100 ml) at a flow rate of 80 ml/h. Both equilibration buffer and gradient solutions contained 10 mM $MgCl_2$; 0.25 mM EDTA

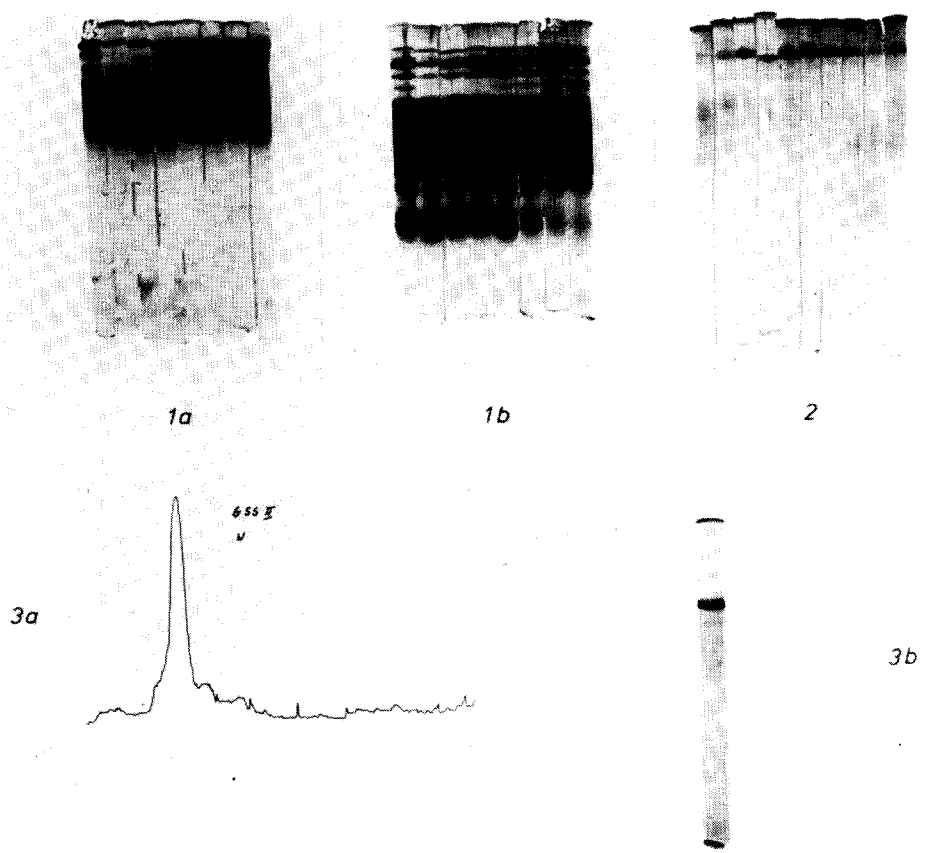

Fig. 2. Polyacrylamide gel patterns of the active fractions of the last three steps of phenylalanine racemase purification. 1a, Normal 8% polyacrylamide gels run in 0.1 M phosphate, pH 7.2, of the fractions 19—25 of the hydroxyapatite chromatography shown in Fig. 1. A sample of the pooled fractions was tested in the first gel on the left side of this figure. The following 7 gels refer to the single fractions 19—25. 1b, 8% polyacrylamide-sodium dodecyl sulfate gels of the same samples. 2, 8% polyacrylamide-sodium dodecyl sulfate gels run in 0.1 M Tris · HCl at pH 8.5 of the active fractions of the following aminohexyl/Sepharose chromatography of phenylalanine racemase. In the last gel on the right side a sample of the pooled fractions was tested. 3a, Normal 8% polyacrylamide gel of the pooled active fractions of the final sucrose gradient. The gel was scanned at 590 nm. 3b, polyacrylamide/sodium dodecyl sulfate gel of the same material. In this case the sample was treated with a concentration of 3.3% sodium dodecyl sulfate prior to electrophoresis instead of 0.1% sodium dodecyl sulfate normally used.

and 2.5 mM dithiothreitol (buffer A). Fractions of approx. 4 ml were collected and tested for enzyme activity. An example of an elution pattern of an aminohexyl Sepharose chromatography of phenylalanine racemase is shown in Fig. 3.

A sodium dodecyl sulfate/polyacrylamide gel pattern indicating the stage of purity obtained after this purification step is demonstrated in Fig. 2—2.

The active fractions were collected and concentrated by $(NH_4)_2SO_4$ precipitation (60% saturation). The precipitate was dissolved in 1 ml 0.01 M buffer P and dialyzed overnight against 500 ml of the same buffer. *Step 7*: Sucrose gradient centrifugation. The concentrated protein sample obtained from step 6 was layered on 5—20% (w/v) sucrose gradients in 0.01 M buffer P (total volume 17.5 ml). Centrifugation was performed in a SW 27-rotor for 30 h at 0°C and 27 000 rev./min. Fractions of 0.8 ml were collected and tested for enzyme activity. The active fractions were pooled. The high purity of the enzyme preparation is demonstrated in Fig. 2—3a and b by polyacrylamide gel electrophoresis.

All these purification procedures were carried out at 4°C.

A purification scheme for phenylalanine racemase is presented in Table I. The specific activity was measured on the basis of the D-Phe-dependent ATP-PP_i exchange catalyzed by the enzyme, which is more stable and reproducible than the activity of these preparations in promoting gramicidin S biosynthesis. In addition to the specific ATP-PP_i exchange activity presented in Table I, we also measured the specific racemase activity of the enzyme. A racemisation rate of 0.6 μmol phenylalanine per mg protein and per min is representative for our highly purified enzyme preparations.

The yield was calculated on the basis of the protein solution obtained after

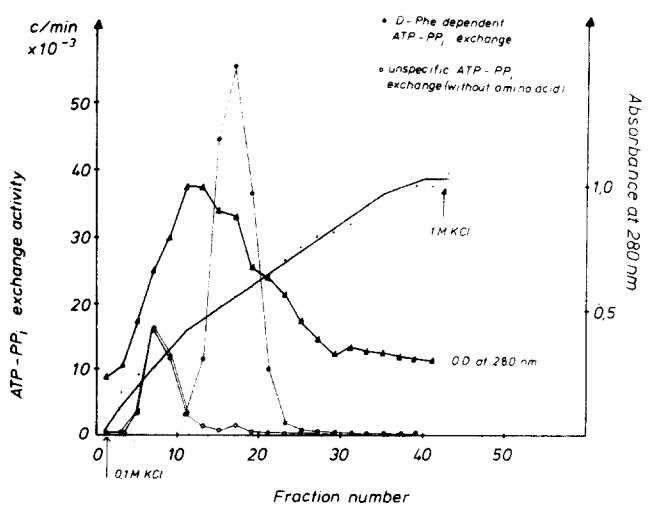

Fig. 3. Aminohexyl/Sepharose chromatography of phenylalanine racemase. This chromatography was performed as described in the text. The fractions obtained were tested for D-Phe-dependent ATP-PP_i exchange (●); ATP-PP_i exchange without the addition of amino acid (○) and the absorbance at 280 nm (▲).

TABLE I

PURIFICATION OF PHENYLALANINE RACEMASE FROM *B. BREVIS*

Total D-Phe-dependent ATP-PP_i exchange activity was expressed in μmol PP_i exchanged per total amount of protein and per min and specific activity in μmol PP_i exchanged per mg protein and per min. Protein content and ATP-PP_i exchange activity were determined as described in Materials and Methods.

Purification steps	Protein [mg]	Total D-Phe-dependent ATP-PP_i exchange	Specific activity	Yield (%)
1 40 000 × *g* supernatant	4700	1.2	$2.5 \cdot 10^{-4}$	
2 Ammonium sulfate (55% saturation)	750	2.8	$3.6 \cdot 10^{-3}$	100
3 DEAE-cellulose	129	2.3	$1.8 \cdot 10^{-2}$	82
4 Sepharose 6B	32	1.4	$4.3 \cdot 10^{-2}$	50
5 Hydroxyapatite	6.6	1.2	$1.8 \cdot 10^{-1}$	43
6 Aminohexyl/Sepharose	0.42	0.3	$6.7 \cdot 10^{-1}$	11
7 Sucrose gradient	0.35	0.15	$4.0 \cdot 10^{-1}$	5

the ammonium sulfate fractionation, because of the abnormally low specific enzyme activity in the crude extract. Use of buffer A instead of TES and phosplate buffer up to step 4 and of a 0—0.6 M KCl gradient for elution of protein from the DEAE column gave enzyme preparations of comparable activity and final purity.

Results

1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of phenylalanine racemase, molecular weight determination

Fig. 2—3b shows that phenylalanine racemase appears as one single band on 8% sodium dodecyl sulfate/polyacrylamide gels. We found that the enzyme cannot be dissociated into subunits by sodium dodecyl sulfate. The highest concentration of sodium dodecyl sulfate in the incubation mixture was 3.3%. We used sodium dodecyl sulfate/polyacrylamide gel electrophoresis for a molecular weight determination of phenylalanine racemase. The migration of this enzyme and of a series of calibration proteins (pepsin, molecular weight 36 000; aldolase subunit, monomer and dimer, 39 500 and 79 000; ovalbumin, monomer and dimer, 45 000 and 90 000; bovine serum albumin, 67 000; and phosphorylase, 96 000) was measured relative to the migration of the marker bromophenol blue on sodium dodecyl sulfate/polyacrylamide gels. The molecular weight of phenylalanine racemase was estimated from a plot of the logarithm of the molecular weight against the relative mobilities of these proteins. From several experiments with our highly purified enzyme preparations we found an average molecular weight of approx. 95 000.

2. Isoelectric focusing of phenylalanine racemase

We performed focusing of phenylalanine racemase at different stages of purification. From such experiments with highly purified material we derived an isoelectric point of approx. 4.6. For the experiment of Fig. 4 we replaced the final sucrose gradient centrifugation by isoelectric focusing using a pH gradient from pH 3 to 10. Fig. 5 shows the polyacrylamide gels of the frac-

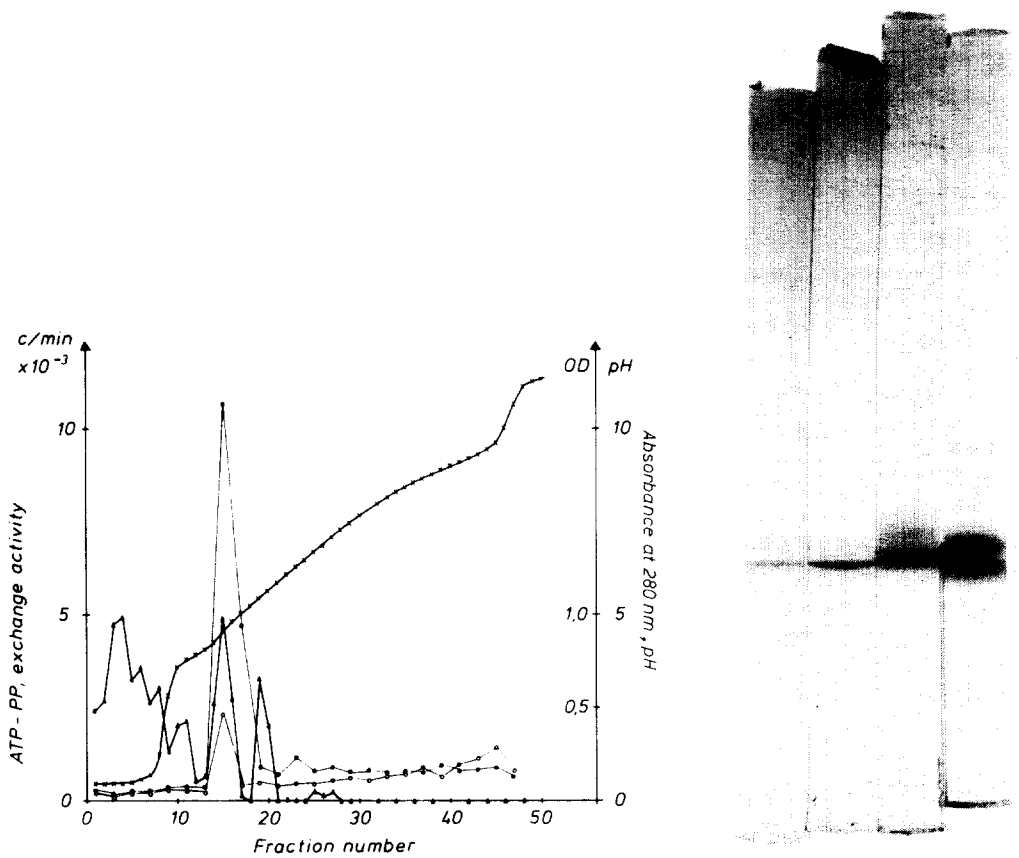

Fig. 4. Isoelectric focusing of phenylalanine racemase. Isoelectric focusing of the enzyme was performed as described in Materials and Methods. For this experiment a pH gradient from pH 3 to 10 was used. Sample: 1 ml of the pooled fractions of an aminohexyl-sepharose chromatography dialyzed against 0.01 M buffer P with addition of 0.35 g sucrose. Electrophoresis was carried out at 400 V and 4°C for 17 h. The fractions obtained were tested for D-Phe-dependent ATP-PP_i exchange (●); ATP-PP_i exchange without addition of amino acid (○) and absorbance at 280 nm (▲).

Fig. 5. Polyacrylamide gel pattern of the active fractions of an isoelectric focusing of phenylalanine racemase. Aliquots of the fractions 14—17 of the focusing experiment shown in Fig. 4 were dialyzed against 0.1 M Tris · HCl buffer pH 8.5 with addition of 0.1% 2-mercaptoethanol. 100 µl of each of the dialyzed samples are tested on normal 8% polyacrylamide gels run in the same buffer.

tions 14—17 (left to right) which are active in D-Phe-dependent ATP-PP_i exchange. This pattern demonstrates that the enzyme in fraction 16 and 17 of Fig. 4 is present in a very pure form.

3. Some kinetic properties and substrate specificity of phenylalanine racemase

The Michaelis constants for the activation of L- and D-phenylalanine, as well as those of the substrate analogues L-tyrosine and *p*-fluoro-D,L-phenylalanine were derived on the basis of ATP-PP_i exchange experiments from Lineweaver-Burk plots. The results are summarized in Table II. Furthermore we found that L-tryptophane was not a substrate for phenylalanine racemase.

TABLE II

MICHAELIS-MENTEN CONSTANTS AND NEGATIVE FREE ENERGIES OF BINDING FOR SUBSTRATES AND INHIBITORS OF PHENYLALANINE RACEMASE

All constants K_m and K_i were derived from Lineweaver-Burk plots.
D-Phenylalanine was used as the substrate in the determination of the inhibition constants K_i.

Substrate resp. inhibitor	ATP-PP_i exchange measurements		
	K_m (mM)	K_i (mM)	ΔG (kcal/mol)
L-phenylalanine	0.06	—	5.9
D-Phenylalanine	0.13	—	5.5
p-Fluoro-DL-phenylalanine	1.4	—	4.0
L-Tyrosine	2.1	—	3.8
1-Phenylethylamine	—	0.26	5.1
2-Phenylethylamine	—	0.15	5.4
Hydrocinnamic acid	—	11.7	2.7

4. Studies with structural analogues of phenylalanine

We investigated the effect of structural analogues of phenylalanine lacking either its carboxyl or its amino group, on the D-Phe-dependent ATP-PP_i exchange. From the double reciprocal plot shown in Fig. 6 it is apparent that 1-phenylethylamine acts as a competitive inhibitor for this reaction. We found a similar result also for 2-phenylethylamine.

On the other hand results with hydrocinnamic acid show that it inhibits by a noncompetitive mechanism (Fig. 7).

The inhibition constants for these Phe-analogues are listed in Table II.

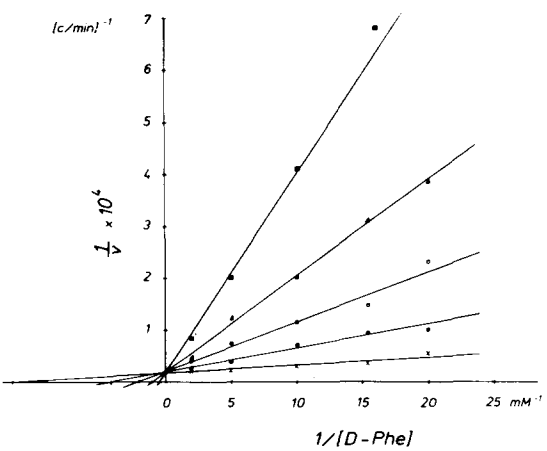

Fig. 6. Double-reciprocal plot for the inhibition of the D-Phe dependent ATP-PP_i exchange of phenylalanine racemase by 1-phenylethylamine. The concentration of D-Phe was varied at definite concentrations of the inhibitor. The concentration of ATP and PP_i was kept constant at 1.25 mM. Concentrations of 1-phenylethylamine: 0.5 mM (●); 1 mM (○); 2.5 mM (▲) and 5 mM (■); absence of inhibitor (X).

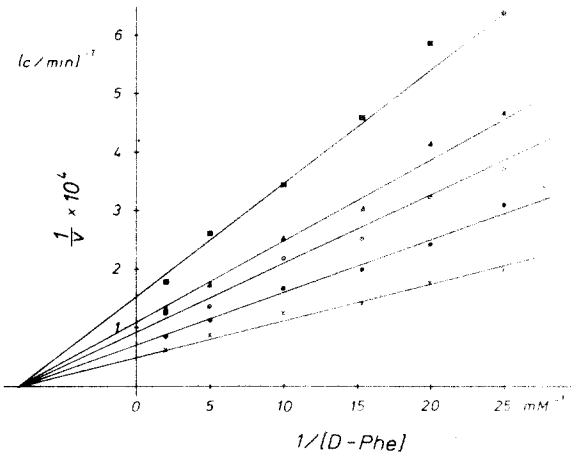

Fig. 7. Double- reciprocal plot for the inhibition of the D-Phe dependent ATP-PP_i exchange of phenylalanine racemase by hydrocinnamic acid. The concentration of D-Phe was varied at definite concentrations of the inhibitor. The concentration of ATP and PP_i was kept constant at 1.25 mM. Concentrations of hydrocinnamic acid: 5 mM (●); 10 mM (○); 15 mM (▲) and 25 mM (■); absence of the inhibitor (X).

Discussion

Phenylalanine racemase can be highly purified by the procedure outlined under Methods, as demonstrated by specific activity measurements and polyacrylamide gel electrophoresis. Two new effective purification steps are introduced. After the gel filtration of gramicidin S-synthetase on Sepharose 6B, phenylalanine racemase was chromatographed on hydroxyapatite under mild conditions. The advantage of the following aminohexyl-Sepharose chromatography is its ability to separate this enzyme quantitatively from other proteins, often abundant in *B. brevis* cells, that mediate high unspecific ATP-PP_i exchange rates. Thus, this step is especially useful for measurements of aminoacyl adenylate formation and ATP-PP_i exchange studies with phenylalanine racemase. The high effectivity of this chromatography for purification of this enzyme is demonstrated in Fig. 2. A serious disadvantage of this purification step is that the activity promoting biosynthesis of gramicidin S declines rapidly due to the relatively high salt concentration needed for the elution of the enzyme. The specific ATP-PP_i exchange activity of our highly purified enzyme is similar to the data reported by Yamada et al. [5]. On the other hand we found much higher specific racemization rates than these authors [5].

Phenylalanine racemase is an acidic protein with an isoelectric point of approx. 4.6, as measured by isoelectric focusing. This technique also provides an effective procedure for final purification of the enzyme (see Figs. 4 and 5).

It is demonstrated that phenylalanine racemase cannot be dissociated by sodium dodecyl sulfate. Thus it may consist of only one polypeptide chain. This conclusion needs to be substantiated by further splitting experiments and by end group analysis. Applying sodium dodecyl sulfate/polyacrylamide gel electrophoresis, we estimated a molecular weight of approx. 95 000 for phenylalanine racemase, which agrees well with previous results from other authors [5,15].

From ATP-PP_i exchange measurements we derived the Michaelis constants and the free energies of binding for aminoacyl adenylate activation of L- and D-phenylalanine (Table II). These K_M-values are 2—3 times higher than those reported in [5,9]. In contrast to the results of Kristensen et al. [9], we find different Michaelis constants for the activation of L- and D-phenylalanine.

Studies with substrate analogues provide information on the architecture of the active centers of enzymes and on the intermolecular forces which determine substrate recognition and binding. From our studies with analogues of phenylalanine we observed the following characteristic features for the aminoacyl adenylate binding site of phenylalanine racemase (see Table II):

a) If the benzene ring of phenylalanine is substituted by polar groups, as in the case of L-tyrosine and *p*-fluoro-D,L-phenylalanine, the Michaelis constants for the aminoacyl adenylate formation increase by at least one order of magnitude, indicating a loss of binding enery of approx. 2 kcal/mol.

b) 2-Phenylethylamine, the amine analogue of phenylalanine, acts as a competitive inhibitor of the D-Phe dependent ATP-PP_i exchange. The corresponding inhibition constant derived from a Lineweaver-Burk diagram is only slightly different from the K_M-value for D-phenylalanine. The K_i-value for 1-phenylethylamine is also of the same order of magnitude.

c) In contrast to the amine analogues hydrocinnamic acid inhibits the D-Phe-dependent ATP-PP_i exchange non-competitively. Obviously the specificity of phenylalanine racemase for the binding of phenylalanine analogues to the active center is lost when the amino group is lacking.

All these inhibitors bind to the enzyme in 1 : 1 stoichiometry, as indicated by the Lineweaver-Burk plots in Figs. 6 and 7, and in the Hill diagrams for the inhibition of the D-Phe-dependent ATP-PP_i exchange [22,23].

From these results, we infer that the amino group of phenylalanine is essential for its binding to the aminoacyl adenylate activation site of phenylalanine racemase. The carboxyl group seems not at all or only weakly bound to the enzyme. Our previous results [24], that esters of appreciable size are efficient substrates of phenylalanine racemase, support this conclusion. The experiment using hydrocinnamic acid as inhibitor of this enzyme further indicates that the benzene ring moiety of phenylalanine appears necessary for substrate recognition rather than for binding. We obtained similar results for the heavy enzyme of gramicidin synthetase (Vater, J., unpublished).

Analogous characteristics are also known for the aminoacyl adenylate activation of amino acids by tRNA synthetases in protein biosynthesis [25—27]. In contrast to gramicidin S-synthetase, however, a free carboxyl group is needed for substrate activity.

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