EFFECT OF PHENYLPYRUVATE AND HOMOGENTISATE ON THE FORMATION OF AROMATIC AMINOACYL-tRNAs

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1. Introduction

An excess of another aromatic amino acid inhibits the incorporation of phenylalanine or tyrosine into brain proteins in vivo [1] and in tissue homogenates [2,3] and cell-free preparations [4,5] in vitro. Also some other aromatic acids, viz. phenylpyruvate, phenyllactate and homogentisate, inhibit in vitro the incoporation of phenylalanine, tyrosine and tryptophan into brain proteins [6]. This latter study suggested that the formation of aminoacyl-tRNAs was the step in protein synthesis primarily affected. We have now further explored the matter by using yeast tRNA mixture or partially purified yeast tRNA specific for phenylalanine as the amino acid acceptor, and cell-free extract of the calf brain as aminoacyl-tRNA synthetase source.

2. Materials and methods

Newly removed calf brain was homogenized in 2 vol. cold 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M KCl and 0.012 M $MgCl_2$. The suspension was centrifuged for 2 h at 105 000 × *g*. The supernatant was applied to a Sephadex G-100 column and eluted with the above medium. The first large-molecular weight peak was collected and used as aminoacyl-tRNA synthetase source. Its protein content was determined by the method of Lowry et al. [7]. It was stored in small portions at −20°C before use. The mixed tRNA fraction used was Sigma (type III) No. R-7125 and the yeast tRNA specific for phenylalanine that of Sigma (type V) No. R-3001.

The standard incubation mixture was that of Chou et al. [8]. It contained in total 1.0 ml vol. 200 μmol Tris–HCl (pH 7.8), 20 μmol KCl, 20 μmol $MgCl_2$, 8 μmol ATP, 0.8 μmol CTP, 7 μmol 2-mercaptoethanol, 5 μmol creatine phosphate and 10 μg creatine kinase. It was supplemented with the indicated amounts of the above aminoacyl-tRNA synthetase solution, tRNA from yeast and with L-[G-^{3}H]phenylalanine, L-[G-^{3}H]tyrosine of L-[G-^{3}H]tryptophan (4 μCi, Amersham, spec. act. 1.0 kCi/mol, concentration ranges from 25–500 μM). The reaction mixtures were incubated at 37°C for 30 min during which time the rate of formation of aminoacyl-tRNAs was almost constant. Some incubations were performed with an excess (2 mM) of one of the other aromatic amino acids or with one of the following aromatic acids: homogentisic, phenylpyruvic, phenyllactic, phenylacetic, salicylic or benzoic acid. The reactions were stopped by adding 2 ml 67% (w/v) ethanol (at −20°C) containing 0.5 M NaCl and 0.1% (w/v) unlabelled

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phenylalanine, tyrosine or tryptophan, respectively. The aminoacyl-tRNA samples were prepared according to Fangman and Neidhardt [9]. All the samples were dissolved in 1.0 M NaOH and their radioactivity was determined as described by Lähdesmäki and Oja [6].

3. Results and discussion

Only phenylpyruvate and homogentisate significantly inhibited the incorporation of phenylalanine, tyrosine or tryptophan into yeast tRNA (table 1). When yeast tRNA specific for phenylalanine was used tyrosine also significantly inhibited the formation of phenylalanyl-tRNA. The inhibitory effects were quantitatively identical even when the experiments were carried out with a higher amount (50 g/l protein) of brain extract as aminoacyl-tRNA synthetase. Yeast tRNA can readily replace endogenous tRNA, since our previous results with endogenous tRNA synthetase fractions from the rat brain were qualitatively very similar [6]. Linear plots indicated that incorporation of aromatic amino acids into the respective tRNAs appeared to obey simple Briggs-Haldane kinetics within the concentration range studied, as shown in fig.1 for tRNA specific for phenylalanine, for example. The apparent K_m and V constants were determined from unweighted data in S/v versus S plots. In most cases both K_m and V were diminished in the presence of homogentisate or phenylpyruvate (table 2), suggesting an inhibition of the uncompetitive type. Only in the charging of tRNA specific for phenylalanine did phenylpyruvate elevate K_m and homogentisate reduce V, rendering competitive and mixed inhibition possible respec-

Table 1
Inhibition provoked by the excess of a second aromatic amino acid or some other aromatic acid in the incorporation of [^{3}H]phenylalanine, [^{3}H]tyrosine and [^{3}H]tryptophan into the respective tRNAs isolated from yeast

Inhibitor (2 mM)	Relative rate of incorporation into aminoacyl-tRNA			
	[^{3}H]Phenylalanine		[^{3}H]Tyrosine	[^{3}H]Tryptophan
	tRNA mixture (%)	tRNA specific for phenylalanine (%)	tRNA mixture (%)	tRNA mixture (%)
None	100.0 ± 5.9	100.0 ± 7.0	100.0 ± 8.1	100.0 ± 8.0
Phenylalanine			95.2 ± 8.7	95.6 ± 10.4
Tyrosine	83.8 ± 10.0	77.8 ± 6.6[b]		82.2 ± 9.2
Tryptophan	86.1 ± 9.9	88.3 ± 7.7	103.7 ± 4.7	
Homogentisate	49.2 ± 2.6[a]	28.1 ± 4.5[a]	51.8 ± 5.2[a]	61.5 ± 9.2[a]
Phenylpyruvate	61.6 ± 8.2[a]	75.3 ± 6.7[b]	71.0 ± 15.6[b]	62.6 ± 7.6[a]
Phenyllactate	82.7 ± 8.3	101.3 ± 7.9	84.2 ± 9.3	98.6 ± 11.6
Phenylacetate	92.2 ± 14.6		99.4 ± 4.8	100.4 ± 7.1
Salicylate	91.9 ± 8.5		101.9 ± 10.5	82.5 ± 9.6
Benzoate	106.4 ± 9.2		96.8 ± 9.3	97.8 ± 11.8
	(7)	(6)	(6)	(6)

Significant differences from control:
[a] $P < 0.01$
[b] $P < 0.05$

Number of experiments in parentheses

Standard incubation mixture, as described in Materials and methods, was incubated at 37°C for 30 min with 1 g/l yeast tRNA mixture, or 40 mg/l yeast tRNA specific for phenylalanine, cell-free extract of calf brain (7.6 g/l protein), 0.5 mM [^{3}H]phenylalanine, [^{3}H]tyrosine or [^{3}H]tryptophan (4 mCi/l) and the inhibitor under investigation. Results (means ± SEM) are given as percentages of the corresponding control incubations without the inhibitor

Table 2
Apparent K_m and V constants for the formation of [^{3}H]amino acyl-tRNAs

Inhibitor (2 mM)	Incorporation into amino acyl-tRNA							
	[^{3}H]Phenylalanine				[^{3}H]Tyrosine tRNA mixture		[^{3}H]Tryptophan tRNA mixture	
	tRNA mixture		tRNA specific for phenylalanine					
	K_m (μM)	V (nmol.s^{-1} kg prot.$^{-1}$)	K_m (μM)	V (nmol.s^{-1}.kg prot.$^{-1}$)	K_m (μM)	V (nmol.s^{-1}.kg prot.$^{-1}$)	K_m (μM)	V (nmol.s^{-1}.kg prot.$^{-1}$)
None	2249 ± 331	6.59 ± 0.91	272 ± 33	3.76 ± 0.48	733 ± 102	0.81 ± 0.10	1248 ± 219	1.41 ± 0.14
Phenylpyruvate	908 ± 117	1.92 ± 0.24[a]	509 ± 62[a]	3.63 ± 0.46	558 ± 94	0.56 ± 0.07	238 ± 44[a]	0.26 ± 0.03[a]
Homogentisate	723 ± 105[a]	1.51 ± 0.17[a]	260 ± 31	2.27 ± 0.26[b]	472 ± 84	0.43 ± 0.05[a]	461 ± 83[a]	0.34 ± 0.04[a]

Significant differences from control:
[a] $P < 0.01$
[b] $P < 0.05$

The constants were estimated from the S/v vs v. plots as in Fig.1. Mean values (± SEM) are given. Number of experiments in each case five or six

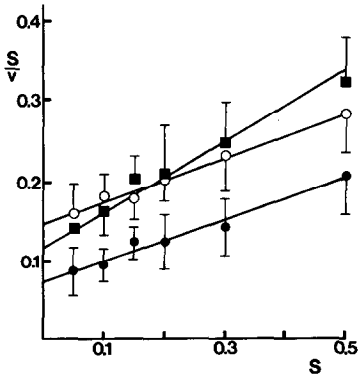

Fig.1. Inhibition of incorporation of [^{3}H]phenylalanine into yeast tRNA specific for phenylalanine by 2 mM homogentisate (■) and phenylpyruvate (○) as a function of phenylalanine concentration. Control incubations (●) were carried out without inhibitor. *S*, phenylalanine concentration (mM) and *v*, velocity (nmol s^{-1} kg $protein^{-1}$). Each point is the mean of five or six experiments. SEM are indicated by vertical bars.

tively. In general, homogentisate was a more potent inhibitor than phenylpyruvate.

The predominantly uncompetitive type of inhibition suggests that phenylpyruvate and homogentisate may reversibly combine with an aromatic amino acid–aminoacyl-tRNA synthetase complex and prevent its further reaction with tRNA. Inhibition of formation of aminoacyl-tRNAs may not, however, be the only mechanism by which phenylalanine and homogentisate bring about a strong inhibition of the whole process of protein synthesis [10]. The later translational steps are probably also affected [6]. An excess of phenylalanine – a greater concentration than tested in the present study – similarly affects both the formation of aminoacyl-tRNAs and the subsequent incorporation of amino acids into protein in the brain [11], even if in human leucocytes only the translational processes seem to be inhibited [12].

There may be up to 6 mM phenylalanine and about 0.1 mM phenylpyruvate in blood plasma of untreated patients suffering from phenylketonuria [13]. In alkaptonuria the concentration of homogentisate is about 0.2 mM [14]. The plasma levels do not reflect tissue concentrations correctly, however, since the metabolites of phenylalanine and tyrosine are effectively excreted in these diseases; the daily output of homogentisate in urine may amount to 50 mmol in alkaptonuria [14], and 30 mmol phenylpyruvate and 10 mmol phenyllactate may be excreted in phenylketonuria [13]. Owing to tubular reabsorption the daily loss of phenylalanine in urine in phenylketonuria is maximally only 5 mmol. It is thus not exactly known how much phenylalanine, phenylpyruvate, phenyllactate or homogentisate there is in brain tissue in human patients, but at least in animals the concentration of phenylalanine in the brain can be increased up to the mM range in experimental hyperphenylalaninaemia [1]. Then phenylalanine metabolites also accumulate in the brain [15], apparently mainly due to the action of brain phenylalanine aminotransferase (EC 2.6.1.5) [16]. In alkaptonuria there is obviously not any excess amino acid in the brain nor is the formation of homogentisate from tyrosine enhanced in situ. Consequently, in contrast to phenylketonuria, no major brain damage occurs in alkaptonuria.

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