USE OF MUTANT ENZYMES TO DEMONSTRATE THE PRESENCE OF TWO ACTIVE SITES ON PHENYLALANYL-tRNA SYNTHETASE FROM *ESCHERICHIA COLI*

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1. Introduction

Phenylalanyl-tRNA synthetase (PRS) from *Escherichia coli* is a tetramer with a molecular weight of 267 000 and the apparent subunit composition $\alpha_2\beta_2$ where α and β have molecular weights of 39 000 and 94 000, respectively [1,2]. Consistent with the quarternary symmetry of this enzyme is the recent finding, that there are two binding sites for the substrates phenylalanine, ATP and $tRNA^{Phe}$ [3,4]. Using physico–chemical methods Bartmann et al. [3] have provided evidence that the two molecules of substrates bound to PRS can be converted by the enzyme into two molecules of reaction products which indicates that two active sites are functioning on the enzyme. In this paper we demonstrate that biochemical and genetic experiments with the help of mutant enzymes are leading to the same conclusion.

The basis for this study was provided by the findings (i) that the α subunits in the PRS seem to carry the phenylalanine binding sites [5] and (ii) that two different types of PRS mutants are available which have an altered α subunit [5,6] clearly distinguished by their unique substrate specificities. The principle of our experimental approach was to construct a hybrid mutant PRS which should contain these two distinct α subunits in one molecule. The question of whether or not two active sites are present can then be answered in the following manner: if the unique substrate specificity of only one of the two mutant α subunits is measurable, the presence of only one active site seems to be indicated; if, however, the two distinct enzymatic activities of both α subunits in the hybrid enzyme are expressed, the presence of two active sites can be inferred. The results presented in this paper are in favour of the second possibility.

2. Materials and methods

The preparations of PRS, of the α and β subunits, and of the crude tRNA were as described previously [5,7]. The PRS-activity was assayed by aminoacylation of tRNA with L-[^{14}C]phenylalanine [7]. The *p*-fluorophenylalanine-resistant (or sensitive) character of the enzyme was measured using *p*-fluoro-D,L-[^{14}C]phenylalanine as substrate [5]. Crude cell extracts (S100) were prepared as described [6]. In vitro complementation experiments were performed according to Comer and Böck [6] using the conditions for reconstitution as reported in [5]. For sucrose gradient centrifugation of hybrid enzymes, 10 ml gradients (5–20% sucrose) were applied [6]. They were centrifuged at 40 000 rev./min for 22 h in a Beckman SW41 rotor. Sephadex G-200 gel filtration was performed on a 2.5 × 95 cm column, equilibrated in 20 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, 0.2 mM dithioerythritol, and 0.1 M KCl.

The bacterial strains used together with their genotype relevant for this work and their source or derivation are listed in table 1. Media and growth conditions were as described [6]. Selection for streptomycin resistance (Str^r) and for rifampicin resistance (Rif^r) was on plates containing 100 μg/ml of the respective antibiotic [8]. Selection for *p*-fluorophenylalanine resistance ($pFphe^r$) was on minimal plates containing the D,L mixture of this analogue at a concentration of 200 μg/ml [5]. The presence of the

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Table 1
E.coli strains used

Strain	Relevant genotype[a]	Source or derivation
KLF48/KL159	F', F148 his$^+$, aroD$^+$/his-4, aroD5	K. B. Low [10] via B. J. Bachmann
NP37	Hfr, pheS5^b	F. C. Neidhardt [11]
MC1699	Hfr, recA1	M. M. Comer (t.s.$^+$ derivative of KL1699 [12])
AB1360	F$^-$, his-4, aroD6	A. L. Taylor [13] via B. J. Bachmann
AB1360-12	F$^-$, his-4, aroD6, pheS12^c	Reference [5]
MC101	F$^-$, his-4, aroD6, pheS12^c, rpsL	M. M. Comer [6]
HH1	F$^-$, his-4, pheS5^b	Transduction: P1/NP37⟶AB1360; selection for Aro$^+$
HH11	F$^-$, his-4, pheS5^b, rpoB	Spontaneous Rif^r mutant of HH1
HH111	F$^-$, his-4, pheS5^b, rpoB, thyA	Spontaneous Thy$^-$ mutant of HH11 selected with trimethoprim
HH12	F$^-$, his-4, pheS5^b, rpoB, recA	Mating: MC1699 × H111; selection for Thy$^+$ and Rif^r
F148-12/MC101	F', F148 pheS12^c, his$^+$, aroD$^+$/his-4, aroD6, pheS12^c, rpsL	Episome transfer: KLF48/KL159⟶MC101; selection for His$^+$, Aro$^+$, Str^r, and pFphe^r
F148-12/HH12	F', F148 pheS12^c, his$^+$, aroD$^+$/his-4, pheS5^b, rpoB, recA	Episome transfer: F148-12/MC101⟶HH12; selection for His$^+$, Rif^r at 37°C.

[a]Gene symbols were adopted from Bachmann et al. [9]
[b]Temperature-sensitive PRS
[c]*p*-Fluorophenylalanine-resistant PRS

pheS5 allele was indicated by failure to grow on rich medium plates incubated at 42°C. Thymine requiring strains were selected on minimal plates containing 10 μg/ml of trimethoprim and 25 μg/ml each of thymine and thymidine [8]. Selection for aroD$^+$ was on minimal plates lacking the aromatic amino acids. Presence of the recA allele was tested by ultraviolet light irradiation of cells for 20 s with 70 erg/s/cm^2 immediately after streaking them on rich medium plates. Transductions with phage P1, matings, episome transfers, and the homogenization procedure were performed as described by Miller [8].

3. Results and discussion

3.1. *In vitro and in vivo formation of the hybrid PRS*

Two different *E.coli* strains possessing a mutant PRS with an altered α subunit were available for this study: (i) strain AB1360-12 is *p*-fluorophenylalanine-resistant (p.f.r.). It possesses a mutant enzyme unable to use this amino acid analogue as a substrate for activation and attachment to tRNA^{Phe}, whereas phenylalanine incorporation is only partly affected [5]. (ii) Strain NP37 is temperature-sensitive (t.s.) and its PRS-activity can only be detected when certain precautions are followed during extract preparation and activity determination [11,14]. Under such conditions then, NP37 PRS is able to use *p*-fluorophenylalanine like the wild-type enzyme. Consequently, strain NP37 is *p*-fluorophenylalanine-sensitive in vivo. With these two mutants the formation of hybrid enzymes was achieved by both a biochemical and a genetic approach.

The first method consisted of in vitro complementation of NP37 crude extracts [6] either with purified

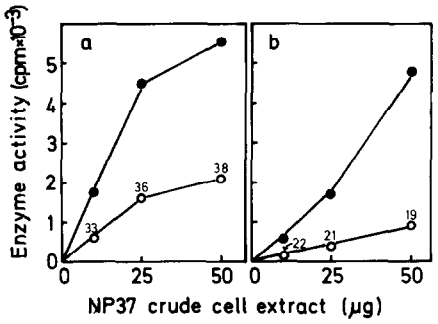

Fig.1. In vitro complementation of NP37-phenylalanyl-tRNA synthetase with purified α subunits. 0.2 μg Purified wild-type α subunit (a) and 0.2 μg purified *p*-fluorophenylalanine-resistant α subunit (b) were added to the indicated amounts of NP37 crude cell extract in a volume of 0.15 ml. The samples were incubated for 30 min at 28°C, and then 0.1 ml of assay mixture was added containing either L-[^{14}C]phenylalanine (•) or *p*-fluoro-D,L-[^{14}C]phenylalanine (○) and the other substrates [7]. The test tubes were incubated for 10 min at 28°C. The numbers in the graph give the *p*-fluorophenylalanine attachment to tRNA as a percentage of phenylalanine incorporation.

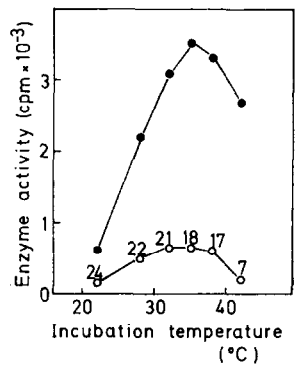

Fig.2. Temperature dependence of the PRS-activity of the active complementation product. 25 μg of crude extract from NP37 were mixed with 0.2 μg of purified analogue-resistant α subunit and incubated as described in fig.1. The activity of the reconstituted enzyme was determined in the aminoacylation assay at the indicated temperatures. The acylation of tRNA with phenylalanine (•) and with *p*-fluorophenylalanine (○) was measured. Numbers in the graph give the *p*-fluoro-phenylalanine attachment to tRNA as a percentage of phenylalanine incorporation.

wild-type (w.t.) α or with p.f.r. α subunits. Figure 1 shows the activity of the hybrid enzyme species thus generated. With w.t. α the attachment of *p*-fluorophenylalanine to tRNA is about 35% of that obtained with phenylalanine (fig.1a). This value is somewhat less than the 50% found in the native w.t. enzyme [5] or in NP37 PRS as control (not shown). The addition of the p.f.r. α however, yields reconstituted enzyme material which is nevertheless able to use *p*-fluorophenylalanine as a substrate, with about 20% of phenylalanine incorporation (fig.1b). This value is about one half of that in the experiment from fig.1a. Figure 2 shows the activity of that hybrid enzyme material generated upon complementation of NP37 crude extract with p.f.r. α subunit as a function of the incubation temperature. Whereas at 42°C neither the w.t. nor the native p.f.r. PRS are temperature-sensitive in vitro (not shown), the reconstituted mutant enzyme exhibits intermediate temperature sensitivity: above 35°C the activity decreases, and it is particularly the activity to attach *p*-fluoro-phenylalanine to tRNA that is affected at higher temperatures. Thus, the resulting active enzyme behaves as if it contains both the t.s. and the p.f.r. α subunits.

The same type of enzyme could be also constructed by in vivo assembly, using a merodiploid *E.coli* strain with the t.s. mutation in the α gene on the bacterial chromosome and the p.f.r. mutation in the α gene on an episome. For this purpose the p.f.r. marker of strain AB1360-12 was incorporated into the episome F148 by homogenotization. The resulting episome F148-12 was then transferred into strain HH12 which carries the NP37 mutation in the AB1360 background (see table 1). The PRS-activity of the resulting heterogenote F148-12/HH12 as well as from the control strains MC101 and F148-12/MC101 was assayed in the aminoacylation test at different temperatures using phenylalanine or *p*-fluorophenylalanine as substrates (fig.3). The enzymes from MC101 and from F148-12/MC101 are completely p.f.r. (fig.3a,b). In strain F148-12/MC101 there is a double amount of PRS-activity (fig.3b) due to the gene-dosis effect [15]. At low temperatures strain F148-12/HH12 has also double the amount of PRS-activity, but not at 42°C (fig.3c). The PRS of this strain is able to attach *p*-fluorophenylalanine to tRNA at low temperatures, but becomes more p.f.r. above 37°C. Thus, there is a striking similarity between the in vitro and the in vivo made hybrid enzymes (compare

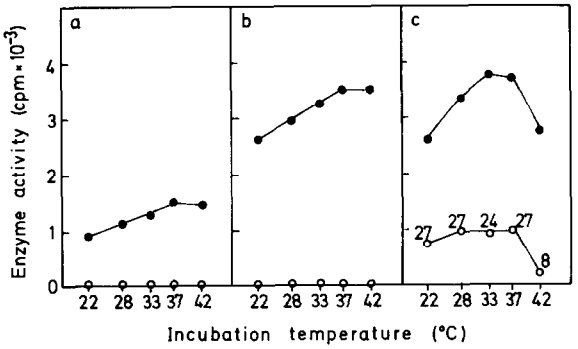

Fig.3. Temperature dependence of the PRS-activity from different *E.coli* strains. The activity of 4 μg crude extract protein was measured in the standard aminoacylation assay with phenylalanine (•) and *p*-fluorophenylalanine (○) as substrates. (a) Strain MC101, (b) strain F148-12/MC101, (c) strain F148-12/HH12.

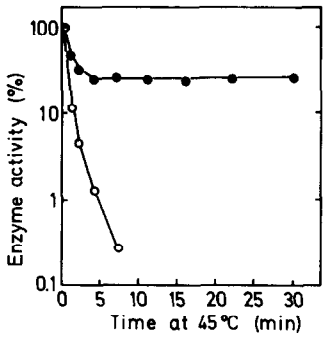

Fig.4. Heat inactivation kinetics of the PRS-activity from strain F148-12/HH12. Crude extracts of this strain with a protein concentration of 1 mg/ml were incubated at 45°C. At the indicated times samples were taken to assay the residual PRS-activity in the aminoacylation test at 28°C with phenylalanine (•) and with *p*-fluorophenylalanine (○) as substrates.

fig.2 and fig.3c). In a very similar manner the growth of the merodiploid strain F148-12/HH12 was dependent on the temperature: above 39°C the growth rates drop significantly, and the strain becomes strictly p.f.r. (data not shown).

In this heterogenote strain the following enzyme species are theoretically formed in vivo under the assumption of random assembly:

type I (25%)	type II (50%)	type III (25%)
$\alpha^{t.s.}\alpha^{t.s.}\beta\beta$	$\alpha^{t.s.}\alpha^{p.f.r.}\beta\beta$	$\alpha^{p.f.r.}\alpha^{p.f.r.}\beta\beta$

The type I enzyme is inactive in vitro under our test conditions and with the crude extract concentrations employed [11,14]; type III enzyme is active with phenylalanine as substrate, but completely p.f.r. [5]. Therefore only type II enzyme can account for the partial use of *p*-fluorophenylalanine and for the partial thermolability. Since enzyme I is inactive in vitro the type III enzyme theoretically represents one third and enzyme II two thirds of the total PRS-activity in the crude extract of strain F148-12/HH12. A temperature inactivation experiment supports this assumption (fig.4). When the PRS of strain F148-12/HH12 was inactivated at 45°C, the ability to use *p*-fluorophenylalanine as substrate is rapidly lost, whereas about 25–30% of the phenylalanine attachment activity remains thermostable. Only the type III enzyme can be responsible for the residual thermostable PRS-acitivity. The loss of *p*-fluorophenylalanine attachment to tRNA corresponds to the inactivation of 70–75% thermolabile enzyme.

Although one cannot rule out completely the possibility of an unspecific inductive effect of the t.s. α subunit on the p.f.r. α subunit the results presented favour the conclusion that the t.s. α subunit in the hybrid enzyme regains its activity in the presence of the p.f.r. α subunit. The restored activity of the t.s. α subunit is manifested by its ability to use *p*-fluorophenylalanine as a substrate and by the thermolability of this activity. Thus, the p.f.r. α subunit seems to exert a quarternary constraint on the t.s. α subunit resulting in a more correct tertiary folding of the latter which is necessary for catalytic activity. This phenomenon is not unique for the hybrid PRS: a similar quarternary constraint was also observed, for example, in the subunit interactions of a hybrid aspartate transcarbamylase containing both w.t. and mutant catalytic subunits [16]. From the fact that the catalytic activities of both types of mutant α subunits are expressed in the hybrid enzyme we conclude that two active sites on the α subunits are functioning: both of them being able to participate in the acylation of $tRNA^{Phe}$ with phenylalanine but only one of them being responsible for the *p*-fluorophenylalanine attachment to tRNA. From our results one cannot conclude, however, whether these two

active sites are functioning simultaneously side by side or alternatively, as one should presume from the anticooperative nature of substrate binding on phenylalanyl-tRNA synthetase [3]. Our hypothesis of the existence of two active sites on the PRS molecule agrees well with the recent results of Bartmann et al. [3].

3.2. *Investigation of the structure of the hybrid enzyme*

Two different methods were employed to demonstrate whether or not the hybrid mutant enzyme is a tetrameric molecule of the type $\alpha^{t.s.}\alpha^{p.f.r.}\beta\beta$:

(i) Crude extracts of the merodiploid strain F148-12/HH12 were chromatographed on a calibrated Sephadex G 200 column. It was found that active PRS material eluted at a position corresponding to the mol. wt 267 000 [1,2] of the native PRS. Quantitative analysis of the recovery, however, revealed that this material represented only about 20% of the PRS-activity applied to the column. Furthermore, this enzyme was completely p.f.r. Therefore this enzyme is obviously the type III PRS species ($\alpha^{p.f.r.}\alpha^{p.f.r.}\beta\beta$). Inactive PRS material eluted later and was only detected by complementation of the fractions with purified α subunit.

(ii) The hybrid enzyme formed in vitro upon complementation of NP37 crude extract with p.f.r. α subunit was centrifuged on sucrose gradients. It was found that active PRS material sedimented more slowly than the intact $\alpha_2\beta_2$ enzyme. Detailed analysis revealed that PRS material in this position is either a trimer ($\alpha_2\beta$) or a disaggregated tetramer [6].

Thus, we failed to isolate a stable tetramer containing both the t.s. and the p.f.r. α subunits. Apparently this enzyme species is only formed in situ in the test tube upon addition of purified p.f.r. α to the NP37 crude extract or in vivo in the heterogenote strain. The presence of the t.s. α subunit in the hybrid PRS, however, does not allow this enzyme species to wtihstand temperatures above 35°C or centrifugal forces or other separation techniques.

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