

Identification of the major tRNA^{Phe} binding domain in the tetrameric structure of cytoplasmic phenylalanyl-tRNA synthetase from baker's yeast

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Received 29 October 1988

Native cytoplasmic phenylalanyl-tRNA synthetase from baker's yeast is a tetramer of the $\alpha_2\beta_2$ type. On mild tryptic cleavage it gives rise to a modified $\alpha_2\beta_2$ form that has lost the tRNA^{Phe} binding capacity but is still able to activate phenylalanine. In this paper are presented data concerning peptides released by this limited proteolytic conversion as well as those arising from exhaustive tryptic digestion of the truncated β' subunit. Each purified peptide was unambiguously assigned to a unique stretch of the β subunit amino acid sequence that was recently determined via gene cloning and DNA sequencing. Together with earlier results from affinity labelling studies the present data show that the Lys 172-Ile 173 bond is the unique target of trypsin under mild conditions and that the N-terminal domain of each β subunit (residues 1-172) contains the major tRNA^{Phe} binding sites.

Phenylalanyl-tRNA synthetase; tRNA^{Phe}-binding domain

1. INTRODUCTION

Cytoplasmic yeast phenylalanyl-tRNA synthetase was the first aminoacyl-tRNA synthetase for which an $\alpha_2\beta_2$ structure was proposed [1]. Functionally the enzyme behaves as a dimer, since it binds two moles per mole of tRNA^{Phe}, ATP and phenylalanine [2,3]. Similarly phenylalanyl- and glycyl-tRNA synthetases from *Escherichia coli* have this unusual $\alpha_2\beta_2$ quaternary structure [4,5]. On the other hand the majority of aminoacyl-tRNA synthetases are monomers or polymers made up of one type of subunit (for a recent review see [6]).

Protein sequence comparisons of aminoacyl-tRNA synthetases of well known crystallographic structures have led to the conclusion that functional domains are organised along the amino acid

sequence to give a modular structure to the protein [6]: the N-terminal domain is composed of alternating α helices and β strands, followed by a helical rich domain which binds the tRNA. Multimeric enzymes also have a third domain at the C-terminus which is responsible for polymerization as was shown for *E. coli* methionyl- and alanyl-tRNA synthetases [7-10]. It is difficult to extend this model to the yeast phenylalanyl-tRNA synthetase because its isolated α or β subunits are unable to catalyse the ATP-PP_i exchange and tRNA^{Phe} aminoacylation [11]. Its active site is more likely built by the subunits interface as suggested by the localization of phenylalanine binding sites [12]: indeed phenylalanine, either free or as phenylalanyl-adenylate, could be cross-linked with an equal efficiency to either type of subunit.

Previous studies by proteolytic modification of phenylalanyl-tRNA synthetase showed that the β subunit was responsible for most of the interactions with tRNA^{Phe}, since the binding of this ligand

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was lost after specific cleavage of β and conversely the binding of tRNA^{Phe} to phenylalanyl-tRNA synthetase strongly protected this β subunit against mild tryptic hydrolysis [13]. The data presented in this paper enable us to identify the site of proteolytic attack and indicate that most of the contacts with tRNA^{Phe} are located in the N-terminal domain of the β subunit outside the large region of homology found with the small subunit of the *E. coli* phenylalanyl-tRNA synthetase. Indeed, after the cloning and sequencing of the two genes encoding the α and β subunits of the yeast enzyme [14] we have analysed those peptides released on its mild tryptic digestion (as well as tryptic peptides of the truncated β' subunit) and localised them in the translated sequence of the β subunit.

2. MATERIALS AND METHODS

2.1. Enzymes and chemicals

Phenylalanyl-tRNA synthetase was purified from commercial baker's yeast [2]. Yeast tRNA^{Phe} was purified as reported [15]. Trypsin, carboxypeptidases A and B were from Worthington (Freehold, NJ, USA). Staphylococcal protease was from Miles Laboratory (Slough, England). Leucine aminopeptidase was from Sigma (St. Louis, MO, USA). Pepsin was from Merck (Darmstadt, FRG). Sephadex G50 (superfine) was from Pharmacia (Uppsala, Sweden), DEAE-cellulose was from Whatman (Maidstone, England). Cellulose plates (Polygram CEL 400, 20 × 20 cm) were from Macherey-Nagel (Dueren, FRG) and polyamide sheets (D.C. Fertigfolien F1700, micropolyamid 15 × 15 cm) from Schleicher and Schuell (Dassel, FRG). Dansyl chloride was from Serva (Heidelberg, FRG). Maleic acid was from Fluka (Buchs, Switzerland). Sequencing reagents were purchased from Pierce (Rockford, IL, USA). All other chemicals were obtained from Merck, analytical grade. Radioactive maleic acid was prepared as follows: 250 μ Ci [2,3-¹⁴C]maleic anhydride (Amersham, England, 50 mCi/mmol, CFA 490) was incubated overnight at room temperature with 50 μ l 1 N NaOH and then diluted with 250 μ l 1 M cold maleic acid adjusted to pH 7.0 with Tris base.

2.2. Limited proteolysis

Native phenylalanyl-tRNA synthetase (~100 mg) was dialysed in the cold against 1% ammonium bicarbonate (w/v) and incubated with trypsin for 30 min at 37°C with a ratio trypsin/enzyme of 1:5000 (w/w). Under these conditions complete conversion of the native $\alpha_2\beta_2$ enzyme to the modified $\alpha_2\beta'_2$ species was achieved as judged from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) [16]. The resulting solution was concentrated to a final volume of 4 ml using a Diaflo apparatus with a PM10 Amicon membrane. Then 50 ml of 1% ammonium bicarbonate solution (w/v) was added to the above 4 ml and the resulting mixture was concentrated again to about 4 ml. This operation was repeated four times and the combined ultrafiltrates (β - β' frac-

tion) were lyophilised. The trypsin-modified $\alpha_2\beta'_2$ enzyme was then S-succinylated under the conditions used for the native enzyme [11,17] in order to protect the thiol groups.

2.3. Separation of α and β' subunits

The S-succinylated α and β' subunits were separated from each other by means of gel-filtration on a column of Sepharose 6B. The column (2.5 × 150 cm) was equilibrated and eluted with a 0.1 M Tris-HCl buffer, pH 7.4, containing 6 M urea. Fractions of 4 ml were collected at a flow-rate of 10 ml/h. Protein was monitored by absorbance measurements at 280 nm and scintillation counting since radioactive maleic acid was used as the S-alkylating reagent [17].

2.4. Tryptic digestions and fractionation of tryptic digests

Fractions corresponding to purified α , β [17,18] and β' (this work) were extensively dialysed against 1% ammonium bicarbonate (w/v) to remove excess of reagents and urea, lyophilised, resuspended in a minimal volume of 1% ammonium bicarbonate (w/v) and hydrolysed by trypsin for 4 h at 37°C (with a ratio trypsin/protein of 1:50 (w/w)). The resulting tryptic digests were fractionated by a combination of gel-filtration on columns of Sephadex G50, ion-exchange chromatography on columns of sulphonated polystyrene using volatile buffers and thin-layer electrophoresis and chromatography on cellulose plates as described [17,18].

2.5. Peptide analysis and sequencing

Amino acid analysis of purified peptides was carried out on a Durrum D500 Analyser after total acid hydrolysis (incubation in 6 N HCl for 24 h at 110°C). Partial or complete sequences were deduced from the results of classical Edman's degradation, Edman-Chang's double coupling method or solid-phase sequencing together with the dansylation technique and digestions with carboxypeptidases A, B, Y and leucine aminopeptidase as reported [19–23].

3. RESULTS AND DISCUSSION

3.1. Identification of the limited cleavage site

From tryptic digests of β' 13 short tryptic peptides were purified to homogeneity including the unique cysteine containing tryptic peptide of β . Twelve of them could be unambiguously identified from amino acid analysis as they had already been obtained from β and sequenced [14,17,18]. The last one was easily assigned to the stretch 339–345 according to its composition. These 13 peptides are located between residues 207 and 483 as listed in table 1 and underlined in fig.1.

Peptides released by mild tryptic cleavage of native phenylalanyl-tRNA synthetase, namely the (β - β') fraction obtained from ultrafiltration (see section 2), were analysed by fingerprinting techniques [18]. Among all spots visualised on thin-layer cellulose plates, 13 gave a clear amino acid analysis

Table 1
Tryptic peptides of β , β' and β - β'

| Position | Sequence | β | β' | β - β' | Position | Sequence | β | β' | β - β' |
|----------|---------------|---------|----------|--------------------|----------|------------|---------|----------|--------------------|
| 5-11 | (QLEILKK) | | | + | 233-234 | VR | + | + | |
| 35-41 | (SALNSLK) | | | + | 235-238 | EEFR | + | + | |
| 42-45 | AHNSK | + | | + | 295-301 | TYMDNIK | + | | |
| 46-50 | LEFSK | + | | + | 302-308 | AVHEQGR | + | + | |
| 51-56 | (VDTVTY) | | | + | 309-315 | FGSIGYR | + | | |
| 57-60 | (DLTK) | | | + | 316-325 | YNWKPEECQK | + | + | |
| 75-77 | LVK | + | | + | 326-329 | LVLRL | + | | |
| 89-93 | DVMSK | + | | + | 339-345 | (MLHDLAK) | | | + |
| 94-100 | LGPQVGK | + | | + | 346-351 | DPKPTR | + | + | |
| 101-105 | VGQAR | + | | | 352-357 | LFSIDR | + | + | |
| 106-108 | AFK | + | | | 358-360 | VFR | + | | |
| 109-114 | NGWIAK | + | | | 392-399 | FMEEFFER | + | + | |
| 123-125 | (SAK) | | | + | 400-406 | MGVTGLR | + | + | |
| 147-157 | (NNSHLDSIDAK) | | | + | 453-455 | DLR | + | + | |
| 158-163 | (ILNDLK) | | | + | 471-472 | YK | + | | |
| 167-172 | LIAQGK | + | | + | 473-477 | VQNIR | + | + | |
| 207-209 | DLK | + | + | | 478-483 | ELLGHK | + | + | |

Parentheses mean that the corresponding residues were not sequenced. One letter symbols are used for amino acids

1 MSDFQLEILKKLDELDEIKSTLATFPQHGSQDVLSALNSLKAHNSKLEFSK
51 VDTVTYDLTKEGAQILNEGSYEIKLVKLIQELGQLQIKDVMSKLGPOVGK
101 VGQARAFKNGWIAKNASNELELSAKLQNTDLNELTDETQSILAIKNNSH
151 LDSIDAKILNDLKKRKLIAQGKITDFSVTKGPEFSTDLTKELETDLTSDMV
201 STNAYKDLKFKPYNFNSQGVQISSGALHPLNKVREEFRQIFFSMGFTEMP
251 SNQYVETGFWNFDALYVPQQHPARDLQDTFYIKDPLTAELPDDKTYMDNI
301 KAVHEQGRFGSIGYRYNWKPEECQKLVLRTHTAISARMLHDLAKDPKPT
351 RLFSIDRVFRNEAVDATHLAEFHQVEGLADYNITLGDLIKFMEEFFERM
401 GVTGLRFKPTYNPYTEPSMEIFSWHEGLQKWVEIGNSGMFRPEMLESML
451 PKDLRLVGLWGLSLERPTMIKYKVQNIRELLGHKVSLEDFIETNPAARLDED
501 LYE*

Fig.1. Translated amino acid sequence of the β subunit [14]. Sequences of short tryptic peptides of β' are underlined. Those purified from the (β - β') fraction are underlined twice. The likely point of limited proteolysis is indicated as T.

that could be correlated to a unique piece of the translated sequence. All these 13 peptides lie between residues 5 and 172 as indicated in table 1 and fig.1. Six of them were already identified in β digests [14]; of the seven others two are genuine tryptic peptides whilst the remaining five are obviously due to a combination of tryptic and chymotryptic-like cuts: 5-11, 35-41, 51-56, 57-60 and 123-125.

As seen before β has a blocked amino terminus whereas Ile was clearly identified as the unique amino terminal residue of β' [17]. This result strongly suggests that the mild tryptic cleavage must occur mainly if not exclusively in the amino terminal domain of each β subunit, thereby yielding a new free N-terminus from an initially N-blocked polypeptide chain. This is further supported by data reported in table 1: indeed all pep-

tides released on mild tryptic digestion (β - β') lie between residues 5 and 172 whilst those identified in β' digests are all located between residues 207 and 483. Moreover, Lys 172 is precisely followed by Ile 173. There is of course another Lys-Ile bond in this area (and no other Lys-Ile or Arg-Ile bonds in the whole sequence), but the fact that the tryptic peptide of β , Leu-Ile-Ala-Gln-Gly-Lys (167-172) was present in high yields in the (β - β') fraction favours the Lys-172-Ile-173 bond as the major site of limited proteolysis. Anyway, both Lys-Ile bonds (172-173 and 157-158) belong to a region rich in potential tryptic cleavage sites with a cluster of basic residues, Lys-Lys-Arg-Lys (163-166) and it is worth stressing that 21 out of the 41 lysines are located between residues 1 and 172. Finally table 2 also shows that the theoretical amino acid composition of the region 173-503 is in good agreement with that determined for β' [17].

Table 2
Amino acid composition of β'

| AA | DNA (173-503) | Protein |
|------------------|---------------|---------|
| Asp | 22 | |
| Asn | 14 | |
| Asx | | 40 |
| Thr | 22 | 22 |
| Ser | 19 | 23 |
| Glu | 27 | |
| Gln | 12 | |
| Glx | | 40 |
| Pro | 18 | 17 |
| Gly | 19 | 23 |
| Ala | 15 | 18 |
| Cys ^a | 1 | 2 |
| Val | 17 | 19 |
| Met ^a | 12 | 9 |
| Ile | 15 | 14 |
| Leu | 32 | 33 |
| Tyr | 13 | 13 |
| Phe | 22 | 20 |
| His | 9 | 10 |
| Lys | 20 | 25 |
| Trp ^b | 5 | 5 |
| Arg | 17 | 15 |
| Total | 331 | 348 |

^a Cys and Met were determined as cysteic acid and methionine sulphone respectively after performic oxidation of the protein

^b Trp was estimated after hydrolysis with methane sulphonic acid

For protein analyses numbers were calculated on the basis of an estimated M_r of 40000 and rounded to the nearest integer

3.2. Localization of the tRNA^{Phe} major binding sites

As described [13] the mild tryptic cleavage converts native phenylalanyl-tRNA synthetase to an $\alpha_2\beta'_2$ form still able to activate phenylalanine but unable to transfer it to tRNA^{Phe}. Indeed this modified tetramer does not bind tRNA^{Phe} and this loss of binding capacity must be correlated to the removal of about one third of each β subunit (apparent M_r 40000 for the truncated β' subunit). The α subunit was apparently unmodified by trypsin since no variation in its M_r could be detected by SDS-PAGE. Furthermore, after separation of α and β' subunits of this $\alpha_2\beta'_2$ modified species, tryptic digests of α yielded several peptides that could be aligned along the translated sequence from position 46 up to residue 556 [14]. This means that only a short segment of α , if any, might be removed by mild proteolysis.

The following data support the idea that this amino terminal domain of β , encompassing residues 1-172, contains the main tRNA binding sites. Firstly the removal of this domain by trypsin yields an $\alpha_2\beta'_2$ form that retains both the oligomeric structure and full catalytic activity with unaltered K_m values for ATP and phenylalanine [13]; this means that the loss of tRNA binding capacity on mild proteolysis cannot be explained by drastic changes that would alter the enzyme conformation. Secondly, earlier binding studies showed that UV irradiation of various (enzyme, tRNA^{Phe}) complexes always led to an exclusive covalent labelling of the β subunit: for instance s^4 U-tRNA^{Phe} could be joined to β and this suggests the existence of several contact areas between the protein and the ligand since s^4 U-tRNA^{Phe} contained 5 to 6 modified uridines statistically distributed along the nucleotide sequence; apparently the interaction between β and this modified tRNA did not involve the 3' end and the catalytic site because no loss of ATP-PP_i exchange activity was observed after photoirradiation of the complex [12].

On the other hand, the 3' CCA end of tRNA^{Phe} should interact with the catalytic site that is still present in the $\alpha_2\beta'_2$ form. Indeed, it was shown in this laboratory that a periodate oxidised tRNA^{Phe} could bind specifically to the β chain via Schiff base formation between the 2' and/or 3' aldehyde groups of this tRNA^{Phe} and the ϵ -amino group of a lysine residue. This lysine was identified as the C-

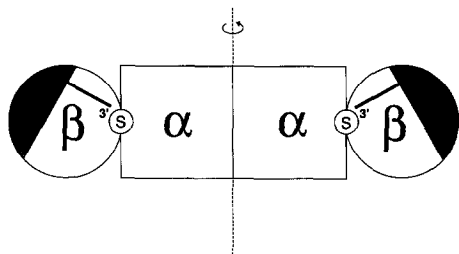


Fig.2. A hypothetical model for cytoplasmic phenylalanyl-tRNA synthetase from baker's yeast. The functional protomer is $(\alpha\beta)$ as the enzyme behaves as a functional dimer $(\alpha\beta)_2$. S is the active site at the interface between α and β subunits. The main tRNA^{Phe} binding domain is the amino terminal part of each β subunit (shaded region). The trypsin resistant $\alpha_2\beta_2$ core also contains the contact areas involved in subunit interactions.

terminal amino acid of the unique cysteine containing peptide of β [24]. This sequence is part of the β' domain of β and it must be located near or in the active site. Indeed labelling this lysine resulted in a complete loss of the aminoacylation activity whilst the ATP-PP_i exchange reaction remained unaffected [12]. It is worth stressing that this lysine 325 of β is located between two regions of high homology with the small subunit of phenylalanyl-tRNA synthetase from *E. coli* [14]. Therefore these regions could be important for either maintaining the active quaternary structure or building up the active site.

Examples of removal of functional domains in proteins with full retention of the other activities by the truncated molecule are well known. For instance, regions critical for tRNA recognition, located beyond the adenylate domain, can be deleted without impairing the amino acid activation function as shown in the case of tyrosyl- and alanyl-tRNA synthetases [25,26]. Similarly proteolytic digestion of the N-terminal domain of the subunit of aspartokinase-homoserine dehydrogenase from *E. coli* results in the loss of the kinase activity but does not affect the other function [27]. Therefore our data support the idea that the N-terminal domain of β encompassing residues 1–172 contains the main tRNA binding sites of yeast phenylalanyl-tRNA synthetase. Such a finding further illustrates the difference between this enzyme and its *E. coli* counterpart for which recent affinity labelling experiments showed that three lysine residues could interact with the CCA end of tRNA^{Phe}: these three lysines (residues 2, 61 and

106) are located in the amino terminal sequence of the large subunit [28]. To our knowledge there is no other reported case of an interaction between a given tRNA and the amino terminal region of its cognate aminoacyl-tRNA synthetase. In this respect yeast phenylalanyl-tRNA synthetase represents a rather unique species in that the bulk of the tRNA^{Phe} molecule interacts with the amino terminal third of each β subunit.

In conclusion, taking into account all the results recalled or reported in this paper a hypothetical structural model can be proposed for cytoplasmic phenylalanyl-tRNA synthetase from baker's yeast as described in fig.2: the amino terminal part of each β subunit represents the main tRNA binding domain and is not involved in either catalysis or subunits interactions. The trypsin resistant $\alpha_2\beta_2$ core contains the catalytic site as well as contact areas between subunits. The location of the catalytic site at the interface between α and β is supported by crosslinking experiments of ATP to β and of phenylalanine to both subunits [12]. The contact areas involve mostly if not exclusively the two α subunits as suggested by earlier results on purified α and β [29]: indeed it was shown that α and β could be separated from each other by chromatography on organomercurial columns in the presence of 6 M guanidine-HCl. A fully active enzyme could be renatured from a mixture of purified α and β . Under renaturing conditions α alone gave rise to polymeric structures whilst β remained in the monomeric form. Further experiments should be now carried out on phenylalanyl-tRNA synthetase from baker's yeast to test the validity of the proposed model.

Acknowledgements: This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS). The skillful technical assistance of Ms G. Nussbaum is gratefully acknowledged.

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