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Relaxing the substrate specificity of an aminoacyl-tRNA synthetase allows in vitro and in vivo synthesis of proteins containing unnatural amino acids

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Abstract It has previously been demonstrated that the unnatural amino acid *p*-Cl-phenylalanine can be attached to $tRNA^{Phe}$ by a modified phenylalanyl-tRNA synthetase with relaxed amino acid substrate specificity. We show that this modification to the translational machinery of *Escherichia coli* is the only requirement for the incorporation of either *p*-Cl- or *p*-Br-phenylalanine into full-length luciferase in vitro. The incorporation of *p*-Cl-phenylalanine was also demonstrated in vivo using a suitably modified host strain. These results represent the first description of the incorporation into a protein in vivo of an unnatural amino acid which is normally rejected by the cellular translational machinery.

Key words: *p*-Cl-Phenylalanine; Phenylalanine; Phenylalanyl-tRNA synthetase; Chaperone; *Escherichia coli*

1. Introduction

A number of methods have recently been developed which expand the use of site-directed mutagenesis to allow the site-specific incorporation of unnatural amino acids in vitro [1,2,3]. The success of such techniques has demonstrated the ability of the translation machinery to recognise a wide range of non-cognate aminoacylated tRNAs in vitro. Over the same period it has also become clear that the non-cognate pairing $tRNA^{Gln}$-Glu, which is found in mitochondria, chloroplasts and some eubacteria, is excluded from the process of translation [4,5]. In this work we set out to investigate whether other non-cognate pairings would also be excluded in vivo and if they were not, how they would affect the synthesis and activity of a heterologous target protein. As it has previously been demonstrated that amino acid analogues sufficiently similar to their natural counterparts are both aminoacylated and incorporated into proteins (reviewed in [6]) we chose unnatural amino acids which are not normally substrates for any of the aminoacyl-tRNA synthetases.

The function of the aminoacyl-tRNA synthetases is to catalyse the attachment of amino acids to their cognate tRNAs (for reviews see [7,8]), a process whose fidelity is maintained both at the level of substrate discrimination and proofreading of non-cognate intermediates and products [9]. It has previously been shown that replacement of Ala-294 by Gly in *Escherichia coli* phenylalanyl-tRNA synthetase ($PheRS^1$) α subunits results in the normally non-cognate amino acid, *p*-Cl-phenylalanine (*p*-Cl-Phe), becoming a substrate for attachment to $tRNA^{Phe}$ [10]. It was also shown that *p*-Cl-Phe is taken up by *E. coli* and that it can be toxic to cells producing A294G PheRS [10,11], although it was not clear whether this resulted from incorporation of the unnatural amino acid into proteins or depletion of the cellular $tRNA^{Phe}$ pool. In the work reported here we have investigated whether *p*-Cl-Phe and a second unnatural amino acid, *p*-Br-Phe, are incorporated into a target protein and if this incorporation affects the overall efficiency of translation. The choice of American firefly (*Photinus pyralis*) luciferase as the target protein, which contains 29 phenylalanine codons [12], also enabled us to readily assess any effects of unnatural amino acid incorporation on enzymatic activity.

2. Materials and methods

2.1. Bacterial strains and plasmids

All in vivo labelling experiments were performed using the *E. coli* strains KA3 (*pheS*A294G) and KA4 which are isogenic except for the *pheS* allele [10]. KA3 produces PheRS containing wild-type β subunits and mutant α subunits with the A294G replacement whereas KA4 produces wild-type PheRS. The plasmid pBEST*luc* (Promega, Madison, WI) was used as the source of the American firefly luciferase gene. DNA templates for in vitro coupled transcription/translation were prepared from *E. coli* strain HB101 previously transformed with pBEST*luc*. In order to allow the regulation of in vivo synthesis of luciferase in strains KA3 and KA4, they were co-transformed with pBEST*luc* and pET24a (Invitrogen, San Diego, CA).

2.2. Reagents

p-Bromo-L-phenylalanine was from Fluka Chemie AG (Buchs, Switzerland), *p*-chloro-D,L-phenylalanine from Sigma (St. Louis, MO), *p*-chloro-D,L-[1-^{14}C]phenylalanine and translation grade [^{35}S]methionine from NEN-DuPont (Boston, MA) and L-[U-^{14}C]phenylalanine from Amersham International (Amersham, UK). Firefly luciferase from *Photinus pyralis* was obtained from Boehringer (Mannheim, Germany), while GroEL, GroES, DnaK, DnaJ, and GrpE were all from StressGen (Victoria, B.C., Canada). Wild-type and A294G PheRS were purified as previously described [10]. Anti-luciferase antibodies were from Promega (Madison, WI), anti-GroEL antibodies from Epicentre Technologies (Madison, WI) and anti-DnaK antibodies were kindly provided by Dr. B. Bukau (Zentrum für Molekulare Biologie, Universität Heidelberg, Germany).

2.3. In vitro transcription/translation

Cellular extracts (S30) for coupled transcription/translation were prepared either from *Salmonella typhimurium* strain SK419 as described previously [13] or from *E. coli* SL119 (obtained from Promega, Madison, WI). While both extracts showed comparable levels of luciferase synthesis, the *E. coli* extract gave significantly higher background and hence the *S. typhimurium* extract was used in all experiments described. Reactions were performed essentially as described [14] except that the amino acid composition was altered and additional enzymes included as indicated for particular experiments. Protein was prepared for polyacrylamide gel electrophoresis (PAGE) as described [14]. DNA templates were prepared from overnight cultures of *E. coli* HB101/pBEST*luc* grown on LB medium containing 25 μg/ml ampicillin. Plasmids were isolated using Qiagen plasmid kits (Qiagen Inc., Chatsworth, CA) followed by linearization with *Xho*I restriction enzyme. Linearized

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templates were then extracted with phenol and methylene chloride and finally resuspended in TE buffer at a final concentration of approximately 0.5 μg/μl. 1.5 μg template was then used in a final reaction volume of 50 μl.

2.4. Visualisation of radiolabelled proteins

^{35}S-Labelled proteins were visualised using a Molecular Dynamics SF Phosphorimager. ^{14}C-Labelled proteins were visualised as follows. Polyacrylamide gels were treated for 30 min in each of the following: 45% ethanol/5% acetic acid/2% glycerol, then water, then 1 M sodium salicylate. Gels were dried at 60°C and exposed to X-ray film at minus 80°C for 2–6 weeks.

2.5. Immunoblots

Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose filters as described [15]. Proteins cross-reacting with the appropriate antibodies were detected by chemiluminescence using a Western Blotting Kit from Boehringer (Mannheim, Germany).

2.6. Immunoprecipitation of luciferase

100 μl Pansorbin cells (Calbiochem, La Jolla, CA) were washed twice with 900 μl of 20 mM HEPES, pH 7.5, and finally resuspended in 1 ml of the same buffer. 5 μl of anti-luciferase was added and the cells incubated with shaking at room temperature for 20 min followed by three washes in 900 μl of 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100. Cells were resuspended in 950 μl of 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF) to which was added either 30 μl of in vitro translation product or 250 μl of cell lysate. Cells were incubated overnight with shaking at 4°C followed by washing in 900 μl of each of buffer 1 (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 5 mM EDTA), buffer 2 (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 0.1% SDS) and buffer 3 (10 mM Tris-HCl, pH 8, 0.1% Triton X-100). Cells were finally resuspended in 20 μl of standard SDS-PAGE loading buffer and incubated at 90°C for 5 min. Supernatants were analysed on a 10% polyacrylamide gel.

2.7. In vivo incorporation of phenylalanine analogues

A 10-ml culture was grown overnight at 30°C in M9 medium containing ampicillin (100 μg/ml) and kanamycin (30 mg/ml) to an absorbance at 600 nm (A_{600}) in the range of 1–1.5. The culture was centrifuged and the cells were re-suspended and diluted in M9 medium containing ampicillin (50 μg/ml), kanamycin (30 μg/mg), tryptophan (1 mM) and tyrosine (0.1 mM) to give a final A_{600} of 0.5. The culture was then grown to an A_{600} in the range 0.7–0.9 prior to the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After a further 10 min incubation either *p*-chloro-D,L-[^{14}C]phenylalanine or [^{14}C]phenylalanine was added to a final concentration of 600 μM (8.7 mCi/mmol) of the L-enantiomer. Cultures were then incubated until the A_{600} reached approximately 1.1. The cells were harvested and lysates prepared as described before [16] except that PMSF was added to the lysis buffer at a final concentration of 1 mM.

2.8. Luciferase activity determinations

Luciferase activity was assayed in appropriately diluted samples using the Luciferase Assay System supplied by Promega (Madison, WI) in a scintillation counter with all channels open.

3. Results

3.1. In vitro incorporation of phenylalanine analogues into luciferase

The incorporation of *p*-Cl-Phe into luciferase during in vitro translation was investigated as follows. S30 extracts were incubated at 35°C for 2 h with template DNA, premix lacking phenylalanine, *p*-Cl-[^{14}C]Phe (1 mM) and PheRS (0.2 mM). 30 ml aliquots were then removed and immunoprecipitated with anti-luciferase prior to SDS-PAGE and visualisation (Fig. 1). Two bands were visible, one corresponding to full-length luciferase (61 kDa) and the second to a smaller 48 kDa internal start-site luciferase (Promega, Madison, WI). The addition of exogenous PheRS slightly reduces the overall level of luciferase synthesis and may result from a less favourable interaction between *S. typhimurium* tRNA^{Phe} with *E. coli* PheRS than with *S. typhimurium* PheRS. As *p*-Br-[^{14}C]Phe was not available, the incorporation of *p-Br-Phe* was investigated indirectly by [^{35}S]methionine labelling. S30 extracts were incubated at 35°C for 30 min with template DNA and a premix lacking phenylalanine but containing low concentrations of unlabelled methionine (20 mM) in order to deplete the endogenous phenylalanine pool prior to the initiation of protein labelling. A postmix containing [^{35}S]methionine (10 μCi/ml), phenylalanine, *p*-Br-Phe (1 mM), wild-type or A294G PheRS (0.2 mM), and IPTG (1 mM) was then added and the reaction incubated for a further 90 min. Samples were analysed by SDS-PAGE followed by phosphorimaging and gave comparable results to those described above for *p*-Cl-Phe incorporation (data not shown). This data shows that *p*-Cl- and *p*-Br-Phe are both incorporated into luciferase exclusively in the presence of A294G PheRS and this incorporation does not affect the level of translation of full-length protein.

3.2. Enzymatic activity of luciferase containing p-Cl- or p-Br-Phe

In order to compare the enzymatic activity of luciferase containing Phe and that containing *p*-Cl- or *p*-Br-Phe, identical reactions to those described before were performed except that unlabelled amino acids were used. Samples were removed after 2 h and the activity assayed as described above. Although comparable levels of luciferase were observed after the incorporation of *p*-Cl-Phe, *p*-Br-Phe or Phe in the presence of A294G PheRS (controlled by immunoblotting, results not shown), the incorporation of the unnatural amino acids reduced activity by over 99%.

As it has previously been demonstrated that a complex of the

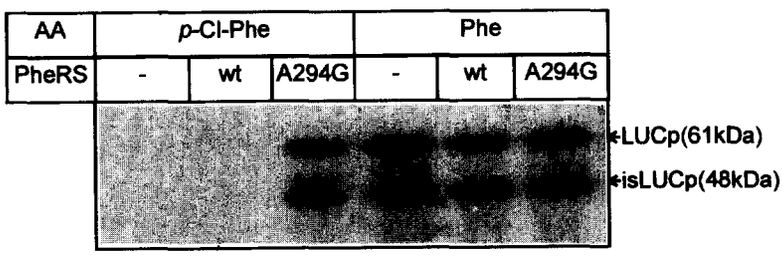

Fig. 1. Immunoprecipitation with anti-luciferase immunoglobulins of ^{14}C-labelled in vitro translated luciferase. Reactions were performed as indicated in the text. 'AA' refers to the ^{14}C-labelled amino acid and 'PheRS' refers to the purified PheRS protein added to the premix (wt = wild type; A294G = A294G PheRS). LUCp refers to the full-length luciferase protein and isLUCp to a truncated internal start-site form (see text for details).

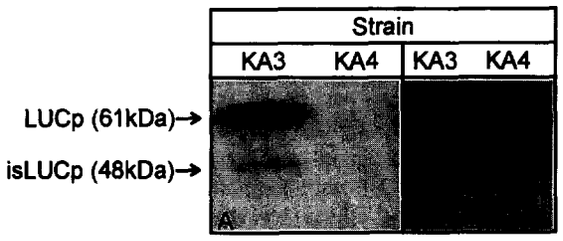

Fig. 2. In vivo labelling of luciferase with *p*-Cl-[^{14}C]Phe. *E. coli* strains KA3 (which produces A294G PheRS) and KA4 (which produces wild-type PheRS) were used for *p*-Cl-[^{14}C]Phe labelling of luciferase as indicated in the text. Samples of cell lysates were then used for both immunoprecipitation and immunoblotting with anti-luciferase. Panel A is an autoradiograph of immunoprecipitated luciferase, while panel B is an immunoblot. LUCp refers to the full-length luciferase protein and isLUCp to a truncated internal start site form (see text for details). It is worth noting that the ratio of full-length to truncated luciferase is considerably higher in vivo than in vitro.

chaperones DnaK, DnaJ and GrpE is able to restore the enzymatic activity of thermally deactivated luciferase [17], the incorporation reactions for *p*-Cl-Phe and Phe were repeated with the addition of various chaperones. In all cases the addition of chaperones did not alter activity (Table 1). As a control of chaperone activity, samples were also removed at the end of the reactions, diluted into luciferase renaturation buffer [17] containing chaperones and ATP, heat treated for 10 min at 42°C (inactivation period) and then incubated for 90 min at room temperature (recovery period). While all samples were inactive following heat treatment, partial restoration of enzymatic activity was only observed in proteins which did not contain *p*-Cl-Phe (Table 1).

3.3. *In vivo incorporation of* p-*Cl-Phe into luciferase*

The incorporation of *p*-Cl-Phe into luciferase in vivo was investigated using the *E. coli* strains KA3, which contains the chromosomal *pheS*A294 mutation, and KA4, which contains the wild-type *pheS* gene. Both strains were transformed with the plasmids pBEST*luc* and pET24a in order to allow IPTG-inducible luciferase expression. Luciferase expression, *p*-Cl-Phe labelling and cell lysate preparation were performed with both strains as described above. Aliquots from the cell lysates were used for both immunoprecipitation and immunoblotting (Fig. 2). While comparable amounts of luciferase were produced in both strains, *p*-Cl-Phe was only incorporated into luciferase by strain KA3. This was confirmed by assaying the enzymatic activity in the lysates which was 75% lower in KA3 than in KA4 in agreement with the in vitro results which also showed a reduction in activity upon *p*-Cl-Phe incorporation. The residual 25% activity can be explained by the production of luciferase prior to the addition of *p*-Cl-Phe and the presence of endogenous Phe in the cells.

The effect of luciferase expression and the incorporation of *p*-Cl-Phe into cellular proteins on chaperone levels was also investigated by immunoblotting. Comparison of the changes with and without IPTG and *p*-Cl-Phe addition indicates that while luciferase production results in increased levels of DnaK, but not GroEL, the incorporation of *p*-Cl-Phe does not lead to any additional changes in chaperone levels (data not shown).

4. Discussion

4.1. *Incorporation of* p-*Cl-Phe and* p-*Br-Phe into luciferase*

The incorporation of *p*-Cl- and *p*-Br-Phe into luciferase during in vitro translation of the luciferase gene is clearly shown in Fig. 1. The only modification required to a standard S30 extract to achieve this was the addition of A294G PheRS to catalyze the attachment of the unnatural amino acid to $tRNA^{Phe}$. This was confirmed in vivo by expressing luciferase in the presence of *p*-Cl-Phe using an *E. coli* strain containing the *pheS*A294G mutation (Fig. 2). These results indicate that the only barrier to the incorporation of *p*-Cl-Phe into cellular proteins is at the level of substrate discrimination by PheRS. There is apparently no discrimination by elongation factor Tu, whose function is to correctly present aminoacyl-tRNAs to the ribosome for mRNA decoding [18], or by the ribosomal A site, a phenomenon previously demonstrated for a number of unnatural amino acids [19,20]. The production of comparable levels of predominantly full-length luciferase with or without *p*-Cl-Phe incorporation indicates that no exclusion occurs and that, additionally, the unnatural amino acid does not cause early termination of translation. Similar observations have previously been made for the incorporation of amino acid analogues such as azaleucine [21] and furanomycin [22], both of which are substrates of wild-type aminoacyl-tRNA synthetases. By contrast, the results described here represent the first description of the incorporation into a protein in vivo of an unnatural amino acid which is normally excluded from the translation machinery of *E. coli* by virtue of its chemical structure.

Table 1
The effect of chaperones on luciferase activity during in vitro translation and on the reactivation of thermally inactivated luciferase

Amino acid added	Chaperones[a]	Relative luciferase activity (%)	
		Translation product[b]	After inactivation and renaturation[c]
p-Cl-Phe	GroEL/GroES	0.12	0
p-Cl-Phe	DnaK/DnaJ/GrpE	0.17	0
p-Cl-Phe	DnaK/DnaJ/GrpE/GrEL/GroES	0.09	0
p-Cl-Phe	–	0.08	0
Phe	GroEL/GroES	93	5.8
Phe	DnaK/DnaJ/GrpE	100	10
Phe	DnaK/DnaJ/GrpE/GrEL/GroES	86	9.5
Phe	–	94	0

[a] For both the translation and reactivation reactions chaperones were added in the ratio luciferase:DnaK:DnaJ:GrpE:$(GroEL)_{14}$:$(GroES)_7$ 1:5:2:5:1:1 as previously described [21]. ATP was added at a final concentration of 1 mM.
[b] All in vitro translation reactions were performed as for the reactions in Fig. 1 except that A294G PheRS (0.2 mM) was present in all cases.
[c] All samples had zero activity after heat inactivation.

4.2. Characterization of luciferase containing p-Cl-Phe

While *p*-Cl-Phe essentially acts as a homologue of Phe during translation in the presence of A294G PheRS, the incorporation of the unnatural amino acid dramatically reduces enzymatic activity both in vitro and in vivo. The likely explanation is that the replacement of Phe by *p*-Cl-Phe disrupts a hydrophobic core structure in luciferase, which would in turn be expected to destabilize the protein [23]. This is supported by the work of Kajiyama and Nakano [16] on the thermostability of *Luciola cruciata* luciferase, which shares 67% amino acid sequence homology with American firefly luciferase [24]. They showed that the thermostability of a series of single amino acid substitutions correlated with the hydrophobicity of the substituted residue. Given that *p*-Cl-Phe could be expected to substitute for phenylalanine at a number of positions, it was not surprising that this caused significant structural disruption and consequently abolished activity.

Luciferase has previously been shown to form productive folding interactions with the chaperone complex DnaK/DnaJ/GrpE [17], as was confirmed here by the reactivation of wild-type luciferase following heat inactivation (Table 1). Some reactivation of wild-type protein was also observed in the presence of GroEL/GroES, in agreement with the observation that the eukaryotic GroEL homologue TRiC can interact with luciferase during folding [25]. Neither of the chaperone complexes had any effect on the enzymatic activity of *p*-Cl-Phe-containing luciferase, regardless of whether they were added before polypeptide chain synthesis or prior to deactivation of full-length protein. In vivo, the expression of luciferase led to an increase in the levels of both DnaK and to a lesser extent GroEL, but no additional effect was observed when *p*-Cl-Phe was incorporated. Similarly, the addition of *p*-Cl-Phe to strain KA3 without the induction of luciferase expression had no detectable effect on chaperone levels. Taken together the in vivo and in vitro results suggest that *p*-Cl-Phe-containing luciferase either is not recognised as a substrate by these chaperones or is recognised but cannot be folded to an active form.

4.3. Future prospects

The results described demonstrate that an unnatural amino acid can be incorporated into proteins in vivo as a direct result of a modification in the substrate specificity of an aminoacyl-tRNA synthetase. The data also highlights the need to further refine such a system to allow site-specific replacements which would be expected to be less disruptive to the structure and activity of target proteins. It can be envisaged that this could be achieved through two additional modifications to the substrate specificity of PheRS, a wild-type copy of which would also have to be present to ensure continued Phe incorporation. First, Phe itself would have to be excluded as a substrate and second, the mutant PheRS would have to show an increased affinity for a nonsense suppressor $tRNA^{Phe}$ compared with the wild-type. Such a mutant PheRS would then allow the site-specific incorporation of *p*-Cl-Phe at codons mutated to the appropriate termination codon [6].

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References

[1] Cornish, V.W. and Schultz, P.G. (1994) Curr. Opin. Struct. Biol. 4, 601–607.
[2] Sonar, S., Lee, C.P., Coleman, M., Marti, T., Patel, N., Liu, X., Khorana, H.G., RajBhandary, U.L. and Rothschild, K.J. (1994) Nature Struct. Biol. 1, 512–517.
[3] Benner, S. (1994) Trends Biotechnol. 12, 158–163.
[4] Schön, A., Kannangara, C.G., Gough, S. and Söll, D. (1988) Nature 331, 187–190.
[5] Stanzel, M., Schön, A. and Sprinzl, M. (1994) Eur. J. Biochem. 219, 435–439.
[6] Ibba, M. and Hennecke, H. (1994) Bio/Technology 12, 678–682.
[7] Carter Jr., C.W. (1993) Annu. Rev. Biochem. 62, 715–748.
[8] Delarue, M. and Moras, D. (1993) Bioessays 15, 675–687.
[9] Jakubowski, H. and Goldman, E. (1992) Microbiol. Rev. 56, 412–429.
[10] Ibba, M., Kast, P. and Hennecke, H. (1994) Biochemistry 33, 7107–7112.
[11] Kast, P. (1994) Gene 138, 109–114.
[12] De Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R. and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737.
[13] Santero, E., Hoover, T., Kenner, J. and Kustu, S. (1989) Proc. Natl. Acad. Sci. USA 86, 7346–7350.
[14] Lesley, S.A., Brow, M.A.D. and Burgess, R.R. (1991) J. Biol. Chem. 266, 2632–2638.
[15] Loferer, H., Bott, M. and Hennecke, H. (1993) EMBO J. 12, 3373–3383.
[16] Kajiyama, N. and Nakano, E. (1993) Biochemistry 32, 13795–13799.
[17] Schroeder, H., Langer, T., Hartl, F.-U. and Bukau, B. (1993) EMBO J. 12, 4137–4144.
[18] Barciszewski, J., Sprinzl, M. and Clark, B.F.C. (1994) FEBS Lett. 351, 137–139.
[19] Hohsaka, T., Sato, K., Sisido, M., Takai, K. and Yokoyama, S. (1993) FEBS Lett. 335, 47–50.
[20] Hohsaka, T., Sato, K., Sisido, M., Takai, K. and Yokoyama, S. (1994) FEBS Lett. 344, 171–174.
[21] Lemeignan, B., Sonigo, P. and Marlière, P. (1993) J. Mol. Biol. 231, 161–166.
[22] Kohno, T., Kohda, D., Haruki, M., Yokoyama, S. and Miyazawa, T. (1990) J. Biol. Chem. 265, 6931–6935.
[23] Lim, W.A. and Sauer, R.T. (1989) Nature 339, 31–36.
[24] Masuda, T., Tatsumi, H. and Nakano, E. (1989) Gene 77, 265–270.
[25] Frydman, J., Nimmesgern, E., Ohtsuka, K. and Hartl, F.U. (1994) Nature 370, 111–117.