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The proofreading
mechanism of
isoleucyl-tRNA
synthetase

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Abstract <p>The molecular mechanism with which isoleucyl-tRNA synthetase edits misactivated valine was investigated. In an attempt to distinguish between two proposed models, rapid enzyme kinetics was studied <i>in vitro</i> by the use of quench-flow technique.</p>		
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The proofreading mechanism of isoleucyl-tRNA synthetase

Magnus Johansson

Sammanfattning

Vid kopiering och avläsning av genetiskt material är noggrannheten extremt viktig. Den totala felfrekvensen då en gen översätts till en kedja av aminosyror som bildar ett protein är endast ett fel på mellan 1000 till 10000 ihopsatta aminosyror. Översättningen av en gen sker med hjälp av stora makromolekylkomplex som kallas ribosomer. Aminosyror kommer till ribosomerna bundna till speciella transportmolekyler, tRNA, och laddningen av aminosyror på tRNA-molekyler, som i flera påvisade fall har lägre felfrekvens än 1 på 10000, sker med hjälp av ett för varje aminosyra unikt enzym, aminoacyl-tRNA-syntetas. Trots substratlikheter, där för vissa aminosyrapar endast en metylgrupp skiljer, sker laddningen av aminosyror på tRNA-molekyler med mycket hög noggrannhet, vilket inte kan förklaras enbart med olika gynnsam bindning. Denna höga noggrannhet är möjlig tack vare en energikrävande korrekturläsning-mekanism. Isoleucyl-tRNA-syntetasets selektion mot valin har visats använda en sådan korrekturläsning-mekanism, men trots trettio års studier är fortfarande inte de molekylära mekanismerna bakom selektionen kända. I denna studie undersöktes denna mekanism i ett försök att skilja de två rådande modellerna åt; endera laddas valin först felaktigt på isoleucins tRNA för att sedan snabbt korrigeras bort, eller så sker korrekturläsningen redan då valinet aktiverats på enzymet i form av valyl-adenylat.

Examensarbete 20 p, Molekylär bioteknikprogrammet

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1 INTRODUCTION

1.1 Background

1.1.1 *Specificity in protein biosynthesis*

Thousands of chemical reactions must take place in a cell for one correct protein molecule to be made. First, in the process called transcription, the template DNA gene is transcribed by an RNA-polymerase to create a messenger RNA (mRNA) encoding a protein. For an average gene of 1000 basepairs this means that the polymerase must pick out and incorporate the correct ribonucleoside-triphosphate 1000 times. In the following protein biosynthesis, the translation, the ribosome catalyses the polymerisation of amino acids into a protein. The twenty different amino acids used for protein synthesis come bound to separate transfer RNA molecules (tRNAs) of which there are one, or several, for each amino acid. The ribosome must be able to discriminate between these 20 different tRNA families in each of the catalysing steps depending on the blueprint - the mRNA. The fidelity of the translation step is hence both depending on the discrimination of tRNA by the ribosome, as well as on the accurate binding of an amino acid to its corresponding tRNA - the aminoacyl-tRNA synthesis.

So, what is so special about these reactions? High specificity is important in all reactions occurring in a cell, isn't it? Well, the problem with some of the above mentioned reactions are the similarity of the substrates. In transcription and replication of DNA, the polymerases discriminate between four rather similar nucleoside-triphosphate substrates ATP, UTP, CTP, and GTP, or dATP, dTTP, dCTP, and dGTP, respectively. The selection mechanism here is dependent on the hydrogen bond base-pairing of the nucleosides to the template DNA strand. In translation, the selection is also making use of the base-pairing in the codon-anticodon recognition, occurring when the anticodon site on the tRNA is probed against the mRNA codon. A codon is a triplet on the mRNA coding for one specific amino acid, and it can encode 64 (four nucleotides in three positions, 4^3) different keywords. As there are only 20 amino acids, in most cases several codons will be translated into the same amino acid. For the aminoacylation of tRNAs there is a family of enzymes, aminoacyl-tRNA synthetases, which recognise the substrates, amino acids and tRNAs, and catalyse the binding. To be able to pick out the correct pair to be bound there is one unique enzyme for each amino acid to be bound to a tRNA.

The selection of the correct tRNA family by the aminoacyl-tRNA synthetase is most probably not a big issue as the tRNAs are quite big molecules (Ibba and Soll, 1999), and specific RNA-to-protein interactions most certainly have evolved in the approximately 2500-5500 Å² large areas of contact (Nadassy *et al.*,

1999). However, the other reactions mentioned above all include the selection of one substrate (or a family of substrates as in the codon- anticodon recognition) out of several rather similar substrates, and these reactions happen all the time, very fast, in all living cells. The evolutionary pressure has of course made these reactions very accurate, even though the accuracy is still a compromise of need for the cell to survive and compete with others on one hand, and the ability to adapt to new environments via mutations on the other. However, the structural differences of the substrates are not always enough to thermodynamically account for the high accuracy apparent *in vivo*.

1.1.2 Proofreading

Thermodynamically the error frequency of a reaction in which a site can bind both cognate and non-cognate substrate is greater than or equal to $e^{-\frac{\Delta G_{CN}}{RT}}$, where ΔG_{CN} is the largest difference in free energy decrease between the non-cognate substrate binding and the cognate substrate binding. Linus Pauling was in the fifties one of the first to observe the paradox in the accuracy observations in biosynthetic processes (Pauling, 1957). For an aminoacyl-tRNA synthetase's selection of amino acids differing in only one methyl group (*e.g.* glycine/valine or valine/isoleucine) he calculated the error rate to be as high as 1 in 5 using 4.2 kJ/mol as the hydrophobic binding energy for a methyl group. Even though later results have shown the binding energy of a methyl group to proteins to be 14.2 kJ/mol (Fersht *et al.*, 1980), this would still only correspond to a discrimination factor of ~200. *In vivo*, however, isoleucyl-tRNA synthetase (IRS) correctly charges the tRNA^{Ile} with isoleucine 10000 times as efficient as with valine (Hopfield *et al.*, 1976).

The theoretical solution to this problem came after studies of the mechanism of aminoacylation of tRNA^{Ile}. Both isoleucine and valine form relatively stable complexes with AMP and IRS according to scheme (1). However, when tRNA^{Ile} is added, valine rapidly disappears from the complex (Fig. 1b), while isoleucine is correctly loaded onto the tRNA (Baldwin and Berg, 1966) (Fig. 1a).



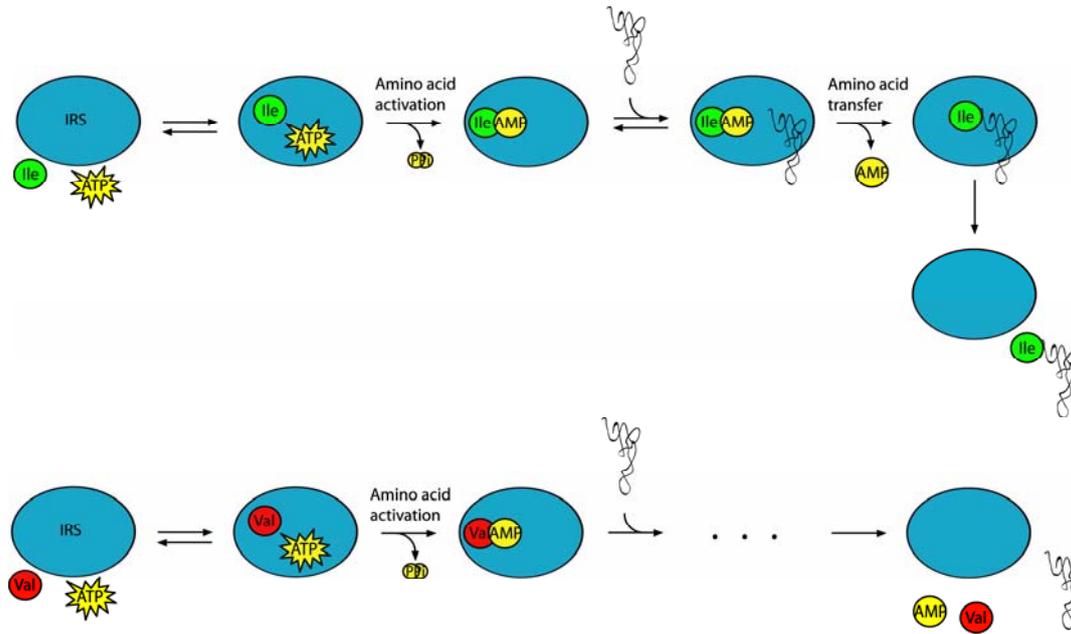
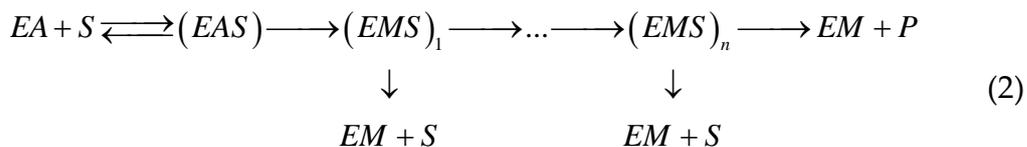


Figure 1. Isoleucyl-tRNA synthetase activates both isoleucine and valine in the form of aa-AMP. But, whereas isoleucine is transferred to added tRNA^{Ile} (a, upper scheme), the valyl-adenylate is rapidly hydrolysed when tRNA^{Ile} is added (b, lower scheme).

These results led to Hopfield's proposal in 1974 of a kinetic proofreading mechanism occurring in the biosynthetic processes. According to Hopfield's scheme (scheme (2)) a non-cognate substrate, S , passing the initial binding discrimination of the enzyme, E , can in one or several steps exit the main path. To contribute to the overall selection these editing steps must be preceded by practically irreversible steps, e.g. ATP- or GTP hydrolysis (in scheme (2) A hydrolyses to give M (Berg *et al.*, 2001)) (Hopfield, 1974).



According to this model the cell pays a little extra energy to get the specificity needed, and by measure the ratio between ATP/GTP hydrolysis and aminoacylation of tRNA in the presence of non-cognate substrates it would also be possible to find proofreading mechanisms elsewhere (as shown for several aminoacyl-tRNA synthetases by Yamane and Hopfield (1977)).

A similar model of how accuracy is achieved in biosynthesis was also proposed by Ninio almost the same time as Hopfield published his theory (Ninio, 1975). Ninio preferred to look upon the mechanism as a time delay in the step

subsequent to substrate binding, resulting in faster dissociations and improved accuracy. However, both models yield the same results, and the crucial demands, such as an irreversible step (e.g. by coupling to ATP/GTP hydrolysis) and one or several substrate exit gates, are the same.

The molecular mechanisms of proofreading was however not explained by these models and it took until 1976 before Fersht and Kaethner could present some experimental data showing the proofreading at work - in the aminoacylation of tRNA^{Val} (Fersht and Kaethner, 1976a). Mixing preformed threonyl-adenylate, complex-bound to valyl-tRNA synthetase, with tRNA^{Val} in the quench-flow apparatus, they could see a transient formation of mischarged Thr-tRNA^{Val}, indicating that indeed threonine is incorrectly charged onto the tRNA^{Val}. However, immediately after being charged, the tRNA^{Val} is deacylated again in an editing step also catalysed by the enzyme. These findings are fully compatible with a one-proofreading-step version of Hopfield's model.

In the years after, experimental evidence of proofreading mechanisms in several other aminoacyl-tRNA synthesis reactions came (Fersht and Dingwall, 1979a; Jakubowski, 1980; Lin *et al.*, 1984), and recently also the proposal of a 3'→5' exonuclease activity of RNA polymerase (Thomas *et al.*, 1998), similar to that of the 3'→5' exonuclease activity of DNA polymerase (Brutlag and Kornberg, 1972), all in analogy with Hopfield's and Ninio's theories (Hopfield, 1974, Ninio, 1975).

1.1.3 *The problem of IRS*

When the mechanism of proofreading by valyl-tRNA synthetase was presented by Fersht and Kaethner in 1976 (Fersht and Kaethner, 1976a), research continued on other synthetases where the same accuracy problem was thought to occur. Misactivations and subsequent editing of amino acids were discovered for alanyl-, leucyl-, methionyl-, and phenylalanyl-tRNA synthetases (Tsui and Fersht, 1981; Englisch *et al.*, 1986; Fersht and Dingwall, 1979b; Lin *et al.*, 1984). However, there were also examples of synthetases, such as cysteinyl- (Fersht and Dingwall, 1979c) and tyrosyl-tRNA synthetases (Fersht *et al.*, 1980) that are so selective in the initial activation reaction that no editing mechanism is needed, and actually has not yet been found. Following his studies on VRS, Fersht published some new results on proofreading in 1977, and this time he had examined IRS (Fersht, 1977). However, even though his procedure was the same as for VRS he was not able to trap any, or at least very little of (within the error range), mischarged tRNA^{Ile} in the form of valyl-tRNA^{Ile} and he could therefore not conclude that the mechanism is the same as in the case of threonine and VRS. Instead he proposed two models based on his results: either the hydrolysis step, taking place after the transfer of valine to tRNA^{Ile}, is at least 125 times faster than the aminoacyl transfer; or the main part of the

misactivated valyl adenylate is hydrolysed, tRNA^{Ile}-dependent, before the transfer occurs, and only a minor part is incorrectly charged onto tRNA^{Ile} and thereafter hydrolysed.

Based on his results on isoleucyl-tRNA synthetase's rejection of valine Fersht proposed in 1978 a double-sieve proofreading model for aminoacyl-tRNA synthetases (Fersht and Dingwall, 1979d). According to this, substrates that are similar to the cognate one, and smaller, bind to a first recognition site. Then there is a second site on the enzyme, a hydrolysis site, in which only the smaller non-cognate substrate fits. This would explain why *e.g.* valine is rejected by IRS. In 1998 the publication of crystal structures of IRS in complex with isoleucine or valine also confirmed this model (Nureki *et al.*, 1998). The structure of IRS and isoleucine showed a binding only in the catalytic transfer site (the Rossmann-fold), while the structure of the enzyme together with valine showed two binding sites for the amino acid; one in the transfer site, and one in the so called CP1 fragment. The latter has in mutational studies been shown to be crucial for the proofreading reaction (Lin *et al.*, 1996) (Fig. 2). The two sites are, however, about 34 Å apart (Silvian *et al.*, 1999) and the question arose how the incorrect amino acid could move from one site to the other. The structure of IRS complexed with tRNA^{Ile} and mupirocin (an antibiotic substance inhibiting IRS by mimicing the isoleucyl adenylate) gave a suggestion: by the change of conformation in tRNA from a hairpin style to a helical conformation the CCA-end of the tRNA shuttles between the active site and the editing site, enabling post-transfer editing of a misacylated tRNA (Silvian *et al.*, 1999). There is, however, also some results from kinetic studies with a fluorescent probe indicating that valyl-adenylate too is shuttling between the two sites, implying pre-transfer proofreading (Nomanbhoy *et al.*, 1999).

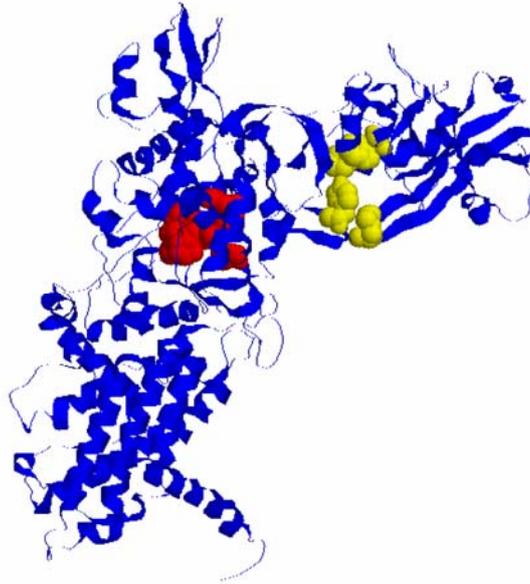


Figure 2. Structure of isoleucyl-tRNA synthetase from *Thermus thermophilus*. Spacefill residues in red have been shown to bind both isoleucine and valine, whereas spacefill residues in yellow only bind valine (Nureki *et al.*, 1998).

Today, almost 30 years after Fersht's proposals, still no one has been able to completely distinguish between the two models of IRS's selectivity mechanism for valine, even though the subject has been extensively investigated. Indications of the post-transfer editing capability of IRS date back to Fersht's studies in 1977 (Fersht, 1977) and Eldred's and Schimmel's in 1972 (Eldred and Schimmel, 1972) who both added separately mischarged Val-tRNA^{Ile} to IRS and noted hydrolysis of the substrate, even though Fersht reasoned that in his study the observed hydrolysis rate of 10 s⁻¹ would not be enough (Fersht, 1977) (Eldred and Schimmel have no quantitative analysis of the hydrolysis rate). However, in Fersht's model he also states that perhaps this slow rate is due to the need of a conformational change of the tRNA when it is added separately. Yet another interesting result from Fersht's study is that the ATP pyrophosphatase activity of IRS in the presence of ATP and valine, is exactly the same as the rate of charging of isoleucine on tRNA^{Ile}, for a wide span of pH and temperature (1.2 s⁻¹ in 25 °C at pH 7.78). As he has also shown aminoacylation to be the rate limiting step (Fersht and Kaethner, 1976b) this strongly suggests that valine is transferred to the tRNA^{Ile} before being edited. Fersht's and Kaethner's studies of threonylation of tRNA^{Val} by VRS (Fersht and Kaethner, 1976a), as mentioned above, and the rapid hydrolysis of mischarged tyrosyl-tRNA^{Phe} by phenylalanyl-tRNA synthetase (Lin *et al.*, 1984) also supports the theory of post-transfer editing as all three aminoacyl-tRNA synthetases face similar problems. Apart from the fluorescent probe studies mentioned above (Nomanbhoy *et al.*, 1999), pre-transfer-editing supporters mostly refer to a study from 1996 by Hale and Schimmel (Hale and Schimmel,

1996). In these experiments a DNA-aptamer was selected that promoted the hydrolysis of Val-AMP on pre-formed IRS·Val-AMP complexes. As the aptamer itself was not a substrate for aminoacyl transfer the authors reasoned that the hydrolysis must occur in a pre-transfer mode. Finally, there is also a study showing the tRNA-independent hydrolysis of mischarged cysteine and homocysteine by IRS indicating the presence of such a pathway in IRS-catalysed aminoacylation (Jakubowski and Fersht, 1981).

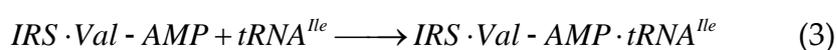
1.2 Objective of the study

There is no convincing evidence of either of the two proofreading models proposed. The hydrolysis of preformed Val-tRNA^{Ile} by IRS, noted by both Fersht and Eldred & Schimmel as mentioned above, does not tell whether this reaction is the main editing step or just a minor “cleaning-up” step. The aptamer study too, lacks confidence as the mechanism of hydrolysis, promoted by a DNA-aptamer selected for this purpose, not necessarily is the same as the naturally occurring mechanism, promoted by tRNA (The aptamer might even have hydrolysing properties itself). Therefore, in this study the proofreading properties of the isoleucyl-tRNA synthetase was investigated in an attempt to solve the almost 30 year old issue: is the misactivated valine completely transferred to tRNA before getting edited, or is the major part of it already hydrolysed when still bound to AMP. To be able to distinguish these two models from one another, the editing mechanism of IRS was studied in a cell-free system using highly purified protein biosynthesis components from *Escherichia coli*. To follow the rapid enzyme-catalysed reactions quench-flow technique was employed.

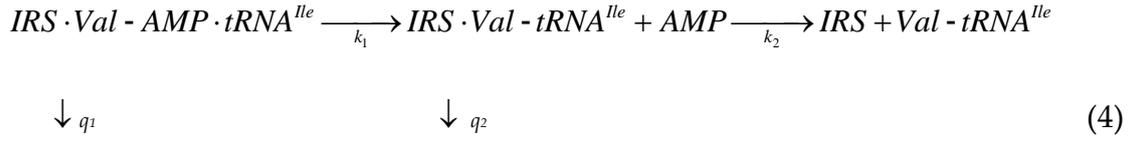
1.3 Approach

Using quench-flow technique it is possible to achieve quick mixing and quenching of reagents, enabling the study of fast reactions. By mixing preincubated isoleucyl-tRNA synthetase, ATP and valine with tRNA and isoleucine, it is possible to measure the time evolution of the hypothetical formation of incorrectly made Val-tRNA^{Ile}. After the first round of hydrolysis, the valine will be chased by the quicker binding of isoleucine and the cognate reaction will be the only one present.

Assuming rapid formation of valyl-AMP-synthetase-tRNA complex according to scheme (3), the incorrectly charged valine will disappear from the complex in one or several ways.



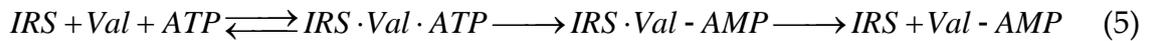
According to a model with two editing steps, scheme (4), the amount of misacylated tRNA^{Ile} respective to time will follow equation A.



$$\begin{array}{c}
IRS + Val + AMP + tRNA^{lle} \qquad \qquad IRS + Val + tRNA^{lle} \\
[IRS \cdot Val - tRNA^{lle}](t) = [IRS \cdot Val - AMP \cdot tRNA^{lle}]_0 \cdot \frac{k_1}{q_2 - k_1 - q_1} \left(e^{-(k_1+q_1)t} - e^{-q_2 t} \right) \quad (A)
\end{array}$$

The dissociation rate of mischarged tRNA from the enzyme, k_2 , is assumed to be zero. If there is no pre-transfer editing step, this would correspond to setting the q_1 rate in equation A equal to zero. Similarly, if there is no post-transfer editing, q_2 in equation A is equal to zero, even though this is not very likely as IRS-catalysed hydrolysis of externally produced Val-tRNA^{lle} has been shown to occur (Fersht, 1977; Eldred and Schimmel, 1972).

However, to be able to draw any kind of quantitative conclusions out of such an experiment the concentration of pre-activated valine in complex with the synthetase when the reaction starts, $[IRS \cdot Val - AMP \cdot tRNA^{lle}]_0$, needs to be known. Earlier studies have shown the spontaneous dissociation rate of Val-AMP from IRS to be 0.04 s⁻¹ (Fersht, 1977) or 0.05 s⁻¹ (Jakubowski and Fersht, 1981), but these experiments were performed under rather different conditions and there are anyway no results on the K_M for the amino-adenylate part of the reaction. Therefore, the properties of a reaction following scheme (5) first need to be understood, *i.e.* the Michaelis-Menten constant, K_M^{Val} , is needed to be able to calculate the fraction of total enzyme concentration that is occupied by Val-AMP at a given valine concentration, and the dissociation rate by which this valyl-adenylate dissociates from the enzyme to know how long the mixture can be incubated before it runs out of substrate.



1.4 Experimental techniques

1.4.1 Radioactive labelling

To be able to follow the reactions ³H- or ¹⁴C-labelled substrates were used. As these isotopes only emit β -radiation of low energy all the analysis of radioactivity was done in the presence of scintillation liquid in an LC6500 scintillation counter (Beckman-Coulter). The scintillation liquids, Quicksafe Flow 2 (Zinsser Analytic) for liquid samples, Filtersafe (Zinsser Analytic) for samples on wet nitrocellulose filters, and Ready Protein+ (Beckman-Coulter) for dry GF-C filters, all contain fluorophores which absorb the β -radiation of the

sample and emit γ -radiation detected by the photo multipliers in the scintillation counter.

1.4.2 Quench-flow technique

A quench-flow apparatus allows rapid mixing of two reagents, followed after some preset time, by the rapid addition of a quencher liquid (acid in the experiments described below). Small fractions of the two reagents, held in syringes, are being pushed into mixing, and a reaction delay loop, by a computer controlled step-motor. After passing the loop, where the reaction takes place, the sample gets mixed with the quencher agent and is collected for analysis.

1.4.3 Thin Layer Chromatography, TLC

In thin layer chromatography, small volumes of samples are spotted near the end of a plate coated with a thin layer of the stationary phase, *e.g.* silica particles or fibrous cellulose (as used in this study). This end of the plate is then lowered into the mobile phase which will start moving upwards on the plate by capillary forces dragging the samples with it. Depending on the solubility of the samples in the mobile phase, and their binding properties to the stationary phase, the compounds in the samples will migrate by different rates on the plate.

1.4.4 HPLC using MonoQ ion-exchange column

High Performance Liquid Chromatography, HPLC, is a widely used form of liquid chromatography used for separations of compounds in solution. A small amount of sample is injected into the mobile phase and pumped through a separation column including the stationary phase. The MonoQ column (Amersham Biosciences) used in this study, is a strong anion exchanger, thus, it contains positively charged groups, immobilised onto a matrix, binding negatively charged sample compounds (in this study adenosine mono-, di-, or tri-phosphates). The elution of the sample is done by adding a weakly binding eluent (such as Cl⁻) at a high concentration gradient, chasing out the compounds to be separated.

2 MATERIALS AND METHODS

DNAse I, ATP, [³H]ATP, [¹⁴C]ATP, [³H]Ile, [³H]Val, and [¹⁴C]Val were from Amersham Biosciences. Putrescine, spermidine, myokinase, phosphoenolpyruvate, and nonradioactive amino acids were from Sigma. Pyruvate kinase was from Roche Applied Science.

All experiments were performed at 37 °C in polymix buffer, containing 5 mM magnesium acetate, 5 mM ammonium chloride, 95 mM potassium chloride,

0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate (pH 7.3), and 1 mM dithioerythritol.

tRNA^{le} was purified in-house from crude *E. coli* tRNA according to Lee and Marshall (1986) with minor modifications.

2.1 Purification of IRS from overexpressing *E. coli* cells

Buffer TMK0 contained: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTE, and 10% (v/v) glycerol. Buffer TMK80 and buffer TMK1000 contained the same thing as TMK0 with the addition of 80 mM or 1000 mM KCl, respectively.

6.6 g of IRS overproducing cells (grown in-house) were re-suspended in 40 ml buffer TMK0 (+ 0.2 mM PMSF) and broken by French press. The French press was run once again with 30 ml of TMK0 (+ 0.2 mM PMSF) buffer to lyse all the cells, and then 80 µg DNase I was added to digest the DNA. To remove cell membranes etc. the French-pressed cells were centrifuged in an SS34 (Sorvall) rotor for 20 min at 19000 rpm (4 °C) and the pellet was discarded.

2.1.1 DEAE- Sepharose ion-exchange column

A 130 ml DEAE Sepharose CL-6B (Amersham Biosciences, Uppsala) column was washed with one column volume of TMK1000, and pre-equilibrated with approximately 3 volumes of TMK0. The 70 ml supernatant was then applied at 2 ml/min speed and the column was washed with TMK0 buffer until the UV absorbance (at 280 nm) had dropped to the base line. The sample was then eluted using a 1 l gradient from 0 M to 0.5 M KCl in TMK buffer at 2 ml/min speed and fractions of 11 ml were collected.

Fractions containing IRS were screened for by adding 10 µl of fractions to 90 µl of a mixture containing [³H]Ile, tRNA^{le}, 1 mM ATP, and 10 mM phosphoenolpyruvate in polymix. The samples were incubated for 30 s at room temperature and the reaction was then quenched with 5 ml of trichloroacetic acid, TCA, (5% + cas-aa (casein amino acids, added to prevent unspecific binding of the labelled amino acid)). The samples were filtrated through GF-C filters (Whatman), washed three times with 5 ml TCA, and the filters were then analysed for their ³H-content in the scintillation counter.

The purity of the fractions containing IRS was determined by SDS-PAGE. 7.5 µl of aliquots were mixed with 7.5 µl SDS (2% final, plus dye: bromophenol blue) and applied to a 10% polyacrylamide gel. Gel electrophoresis was run for approximately one hour at 200 V. The gel was taken out and stained using Commassie Brilliant Blue G250 (Sigma), and then destained using 12% isopropanol, and 5% acetic acid in water.

2.1.2 Ammonium sulphate precipitation

Fractions containing IRS were pooled and 18.4 g of ammonium sulphate (+1 mM EDTA) was added. The 66 ml protein mixture was stirred for 10 min and then centrifuged 30 min at 18000 rpm in an SS34 rotor. The pellet was discarded and another 10.2 g of ammonium sulphate (+1 mM EDTA) was added to the supernatant which was again stirred for 10 min and centrifuged 30 min at 18000 rpm in an SS34 rotor. The precipitate was this time collected and dissolved in TMK0 buffer to approximately 6 ml.

2.1.3 Gel filtration using Superdex 200

A 320 ml Superdex 200 column (Pharmacia, Uppsala) was equilibrated with 2 volumes of TMK80 buffer at 1.5 ml/min. The 6 ml protein solution was applied and eluted with TMK80 buffer 1.5 ml/min. Fractions of 10 ml (up to 100 ml elution volume) and 4 ml (after 100 ml elution volume) were collected.

Fractions containing IRS were screened for, and analysed for purity, by SDS-PAGE as above.

2.1.4 QFF ion-exchange column

A 100 ml QFF column (Q Sepharose Fast Flow column, Amersham Biosciences) was washed with one column volume of TMK1000 buffer, and equilibrated with three column volumes of TMK0 buffer. Fractions from Superdex 200, containing IRS, were pooled and applied to the column at 2 ml/min. The column was washed until the UV absorbance reached the base line and a 600 ml gradient from 0 M to 0.5 M KCl in TMK0 buffer was developed at 2 ml/min.

Fractions containing IRS were screened for, and analysed for purity, by SDS-PAGE as above. The fractions containing IRS were pooled and concentrated by ultra filtration through Millipore filters (63.5 mm, 30 kDa, and 25 mm, 10 kDa). The protein was stored in poly mix containing 50% Glycerol at -20°C .

2.2 Analysis of biosynthesis components

2.2.1 Determination of active isoleucyl-tRNA synthetase concentration

To obtain the active concentration of the IRS stock solution, two separate experiments were performed; a substrate titration and a chasing experiment.

In the substrate titration different concentrations of [^3H]Ile (ranging from 1 μM to 128 μM) were incubated with IRS (stock solution diluted 16 times), ATP (2 mM), and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$) for 30 seconds. The samples were filtered through nitrocellulose filters, which were then washed with

3 times 1 ml of polymix. The ^3H -activity of the filters was then analysed in the scintillation counter.

In the second experiment a mixture of IRS, [^3H]Ile (50 μM), ATP (2 mM), and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$) was rapidly mixed with tRNA^{Ile} (8 μM) and Ile (10 mM) using a quench-flow instrument (KinTek Corp. Austin, USA). The samples, quenched with TCA (15%), were filtered through GF-C filters and washed with three times 5 ml TCA (5% with the addition of cas-aa). The ^3H -activity of the filters was analysed in the scintillation counter.

2.2.2 Determination of the active tRNA^{Ile} concentration

To obtain the concentration of tRNA^{Ile} in the stock solution the absorbance at 260 nm was measured.

To obtain the concentration of active tRNA^{Ile} a charging experiment was performed. Different dilutions of tRNA^{Ile} (ranging from 100% to 0.0195%) were added to a 19 times bigger volume of a mixture of IRS (0.1 μM), [^3H]Ile (200 μM), ATP (1 mM), phosphoenolpyruvate (10 mM), pyruvate kinase (0.1 mg/ml) and myosin kinase (4 $\mu\text{g}/\text{ml}$). The mixture was incubated for 10 minutes and quenched by the addition of approximately 5 ml TCA (5% + cas-aa). The samples were filtered through GF-C filters and washed with three times 5 ml TCA (5% + cas-aa). The ^3H -activity of the filters was analysed in the scintillation counter.

The tRNA^{Ile}-batch was concentrated by ultra filtration through vertical Millipore filters (10 kDa), and again measured for its active concentration by the charging experiment as mentioned above.

2.3 Kinetic experiments

2.3.1 Kinetics of IRS-catalyzed isoleucylation of tRNA^{Ile}

Five different [^3H]Ile concentrations (1, 2, 5, 10, 25 μM) were incubated in the presence of tRNA^{Ile} (1 μM), IRS (9.3 nM), ATP (2 mM) and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$). Aliquots were removed at different timepoints (ranging from 5 s to 30 s) and immediately quenched with cold TCA (5 ml of 5% TCA with the addition of cas-aa to each 20 μl sample). The samples were filtered through GF-C filters, washed with three times 5 ml TCA (5% + cas-aa), and the ^3H -activity of the filters was analysed in the scintillation counter.

2.3.2 Kinetics of the valyladenylate reaction

The valyl adenylate reaction was investigated by incubating valine at four different concentrations (1000, 500, 250, 125 μM) in the presence of [^{14}C]ATP (500 μM), IRS (1 μM) and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$). Aliquots were

removed at different timepoints, added to KOH (0.3 M final) and incubated for 10 min at room temperature to quench the reaction and hydrolyse any formed valine adenylate. The hydrolysis was interrupted by the addition of formic acid (12.5% final), the samples were centrifuged for 15 min at 14000 rpm, and the supernatant was collected. The amount of AMP formed was analysed by adding the supernatants to a TLC plate (Polygram CEL 300, 0.1 mm) and running the chromatography in a 0.5 M KH_2PO_4 buffer (pH 3.5) for approximately 2.5 h. Standards containing 10 nmol of each of ATP, ADP and AMP were also added to each spot before the TLC runs. The migrations of the standards were analysed under UV light (giving dark spots where there are adenosines, as these absorb the fluorescence of the plate) and pieces of the plate containing the adenosines were cut out, added to scintillation liquid, and the ^{14}C -content of the TLC-pieces was analysed in the scintillation counter.

The same reaction was also studied by ion exchange chromatography using a 1 ml MonoQ column (Amersham Biosciences, Uppsala). The reagents (valine concentration now ranging from 625 μM to 10000 μM) were incubated the same way as mentioned above, but this time the reaction was interrupted by diluting 10 μl samples in 490 μl ice cold buffer A (20 mM Tris-HCl, pH 7.5) and then immediately filtering them through nitrocellulose filters. The filtrates were collected and more buffer A was added so that the volume of all samples was 1.5 ml. Samples of 400 μl , with the addition of 2, 4, 6 nmol AMP, ADP, and ATP, respectively, were then applied to the MonoQ column (pre-equilibrated with 5 ml buffer A at 1 ml/min) and eluted by a 5 ml 0.15-0.4 M NaCl gradient (1 ml/min). Between each run the column was washed with 2 ml buffer A containing 1 M NaCl, and equilibrated with 5 ml buffer A at a flow rate of 1 ml/min. Fractions containing the adenylates were collected, added to scintillation liquid, and analysed for their ^{14}C -content using the scintillation counter. The experiment was repeated once for valine concentrations of 5 mM and 10 mM.

2.3.3 *The IRS catalysed valylation of tRNA^{Ile}*

The formation of non-cognate Val-tRNA^{Ile} was studied using quench-flow techniques. One syringe contained IRS (19.8 μM), [^3H]Val (1 mM), ATP (2 mM), and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$), and the other syringe contained tRNA^{Ile} (20 μM), Ile (1 mM), and inorganic pyrophosphatase (5 $\mu\text{g}/\text{ml}$). Equal volumes of the two reagents were rapidly mixed, the reactions were quenched after different times by the addition of TCA (15%), and the samples were collected in tubes containing approximately 5 ml of TCA (5% with the addition of cas-aa). The samples were then filtrated through GF-C filters and afterwards washed four times with approximately 5 ml of TCA (5% + cas-aa) each time. The filters were analyzed for their ^3H -content in the scintillation counter.

2.4 Data analysis

Experimental data was fitted to equations using the Marquardt algorithm implemented in Origin 7 (OriginLab Corp.)

3 RESULTS

3.1 Components

3.1.1 Purification of IRS

Purification of IRS from overexpressing *E. coli* cells was done in several steps; two different ion-exchange columns, one gel filtration, and one ammonium sulphate precipitation. The UV-absorbance chromatograms for the column runs (measured at 280 nm), and the results of the SDS-PAGE analyses made after each column run are shown in figures 3-5. Fractions kept and pooled after each separation were as follows: fractions number 34-39 from DEAE Sepharose column, fractions number 34-41 from Superdex 200 column, and fractions number 37-46 from QFF column.

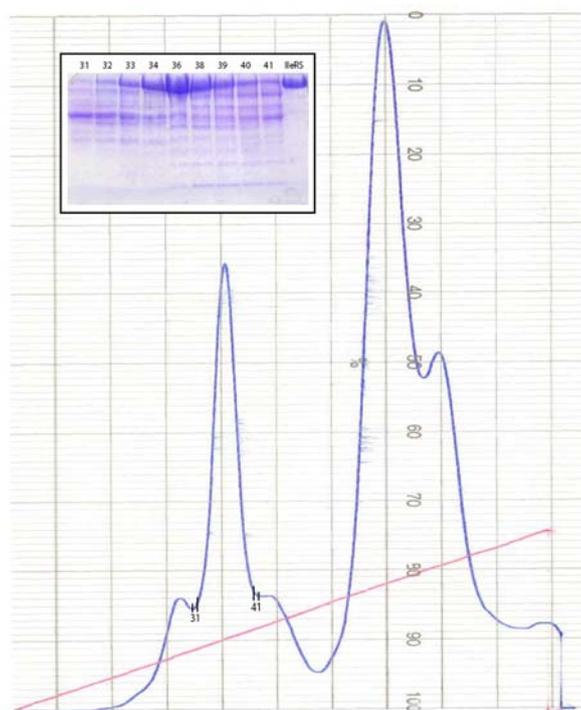


Figure 3. Purification of IRS by DEAE Sepharose ion-exchange column. Cell extract containing IRS added to the column and eluted with a 0 to 0.5 M KCl gradient (red line). The SDS-PAGE picture shows the content of fractions in the first peak (31-41 as indicated in the chromatogram. The band to the extreme right shows purified IRS).

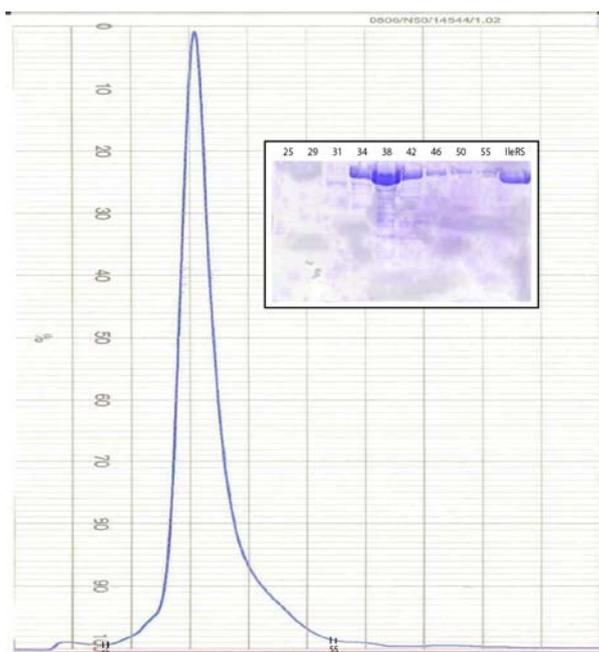


Figure 4. Fractions 34-39 from DEAE Sepharose column (Fig. 3) containing IRS pooled, further purified by ammonium sulphate precipitation, and added to a Superdex 200 gel filtration column. The SDS-PAGE picture shows the content of fractions in the peak (25-55 as indicated in the chromatogram. The band to the extreme right shows purified IRS).

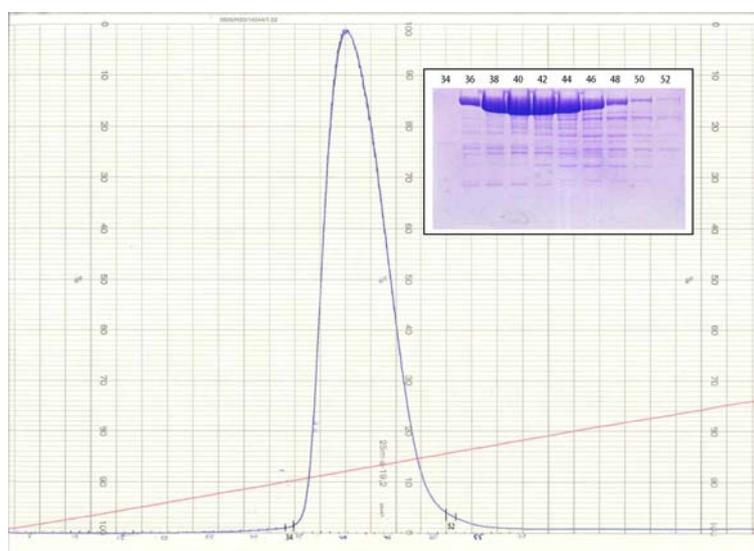


Figure 5. Fractions 34-41 from Superdex 200 column (Fig. 4) containing IRS pooled, and added to a QFF ion-exchange column. The SDS-PAGE picture shows the IRS content of fractions in the peak (34-52 as indicated in the chromatogram).

3.1.2 Determination of active isoleucyl-tRNA synthetase concentration

By incubating a dilution of IRS with different concentrations of [³H]Ile in the presence of excess ATP the amino acid will form an aminoadenylate, Ile-AMP, on the enzyme according to scheme 1, following equation B.

$$[Ile - AMP \cdot IRS] = \frac{[Ile] \cdot [IRS]_0}{[Ile] + K_D} \quad (B)$$

From the estimated plateau in figure 6 the total amount of enzyme in the reaction, $[IRS]_0$, was determined and, hence, the stock concentration of active isoleucyl-tRNA synthetase was determined to be approximately 7 μ M.

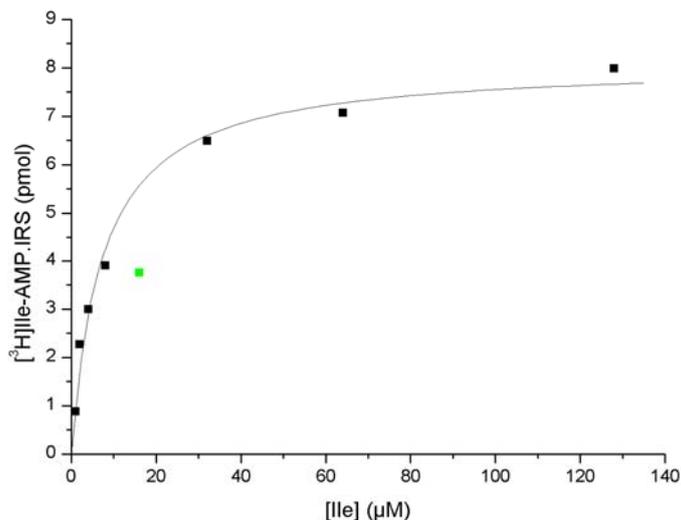


Figure 6. Formation of $[^3\text{H}]\text{Ile-AMP-IRS}$ complex in 30 s at different $[^3\text{H}]\text{Ile}$ concentrations in the presence of 2 mM ATP, 5 $\mu\text{g/ml}$ inorganic pyrophosphatase, and an unknown concentration of IRS (stock solution diluted 16 times in reaction volumes of 18 μl). The line was obtained by fitting the data to equation B (The green data point was not taken into account in the fitting).

The active enzyme concentration was also measured in another separate chase experiment. In this, the transfer of isoleucine from Ile-AMP to Ile-tRNA^{Ile} was followed (Fig. 1a). By allowing the formation of $[^3\text{H}]\text{Ile-AMP-IRS}$ in one syringe of the quench-flow apparatus (Representing the two first steps in figure 1a) and then mix this with a solution containing tRNA^{Ile} and a large amount of Ile (non-labelled Ile in a final concentration much higher than the final concentration of $[^3\text{H}]\text{Ile}$) one round of enzyme catalysis of $[^3\text{H}]\text{Ile-tRNA}^{\text{Ile}}$ is made. When the enzyme recycles, the $[^3\text{H}]\text{Ile}$ is competed by the non-labelled Ile and the overall amount of $[^3\text{H}]\text{Ile-charged tRNA}^{\text{Ile}}$ will follow equation C.

$$[[^3\text{H}]\text{Ile-tRNA}^{\text{Ile}}](t) = [IRS]_0 \cdot (1 - e^{-k \cdot t}) \quad (C)$$

From figure 7 the total concentration of synthetase in the reaction, $[\text{IRS}]_0$, was determined, and the stock concentration of active synthetase was calculated to be $8.4 \mu\text{M}$.

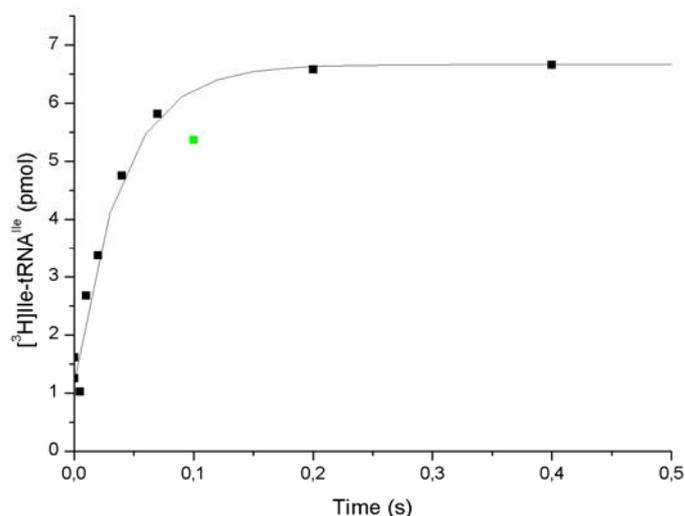


Figure 7. IRS-catalysed isoleucylation of tRNA^{Ile} by preincubated $[\text{H}^3]\text{Ile}\text{-AMP}\cdot\text{IRS}$ complex ($50 \mu\text{M}$ $[\text{H}^3]\text{Ile}$, 2 mM ATP, $5 \mu\text{g/ml}$ inorganic pyrophosphatase, and stock solution of IRS (diluted 18 times) incubated in $37 \text{ }^\circ\text{C}$ for approximately 1 min) in the presence of $8 \mu\text{M}$ tRNA^{Ile} and 10 mM Ile (non-radioactive). The line was obtained by fitting the data to equation C (The green data point was not taken into account in the fitting).

3.1.3 Determination of active tRNA^{Ile} concentration

The absorbance of the tRNA^{Ile} stock solution at 260 nm was measured to get its total RNA content ($1 A_{260} = 0.12 \text{ mM}$ nucleotides (Sambrook *et al.*, 1989)). However, due to possible degradation of the tRNA , the concentration of active tRNA^{Ile} in the stock was examined. By incubating different dilutions of the tRNA stock solution for 10 minutes in the presence of IRS, $[\text{H}^3]\text{Ile}$, and large excess of ATP, all the available tRNA^{Ile} was assumed to become aminoacylated with $[\text{H}^3]\text{Ile}$ by the enzyme (The reaction in figure 1a is driven to the extreme right), and the amount of $[\text{H}^3]\text{Ile}\text{-tRNA}^{\text{Ile}}$ formed is the same as the amount of the limiting tRNA^{Ile} added. From figure 8a the stock concentration of tRNA^{Ile} was estimated to be $66 \mu\text{M}$ which corresponds to approximately 4% of the total amount of nucleotides in the stock.

The tRNA^{Ile} stock was later concentrated using ultra filtration and therefore the charging experiment was performed again using the same conditions. The active concentration of the stock was then increased to approximately $130 \mu\text{M}$ (Fig. 8b).

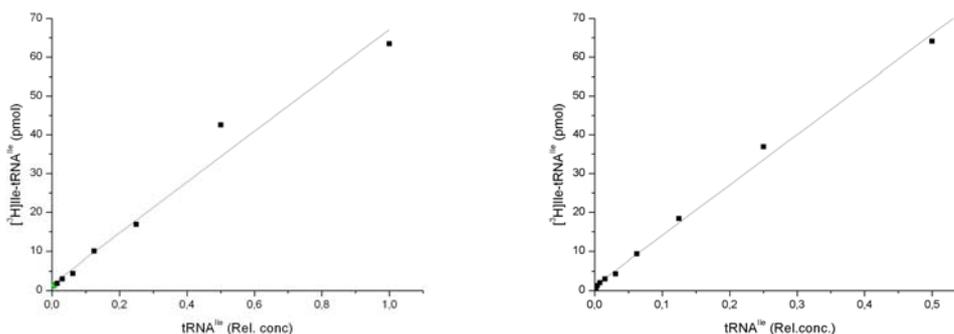


Figure 8. IRS-catalysed isoleucylation of tRNA^{Ile} from different dilutions of tRNA^{Ile} stock before (a, left panel), or after (b, right panel) concentration using ultra filtration. The 30 min reactions took place in a solution containing 0.1 μM IRS, 200 μM [³H]Ile, 1 mM ATP, 10 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, and 4 μg/ml myeline kinase.

3.2 Kinetic experiments

3.2.1 Kinetics of IRS-catalysed isoleucylation of tRNA^{Ile}

In order to examine the cognate reaction of IRS in terms of kinetics, the rate of isoleucylation of tRNA^{Ile} was determined for five different amino acid concentrations (Fig. 9). According to Michaelis-Menten kinetics the rate of product formation, v , as a function of the limiting substrate, S , follows equation D.

$$v = \frac{j}{[E]_0} = \frac{[S] \cdot k_{cat}}{[S] + K_M} \quad (\text{D})$$

In equation D $[E]_0$ is the total concentration of enzyme, k_{cat} is the turnover number of the enzyme (the maximum number of substrate molecules turned into product molecules per unit time), and K_M is the concentration of limiting substrate at which the enzyme is half saturated (Fersht, 1999). Fitting the rates of figure 9 into equation D (Fig. 10) gives a k_{cat}^{Ile} value of 1 s⁻¹ for the enzyme and a K_M^{Ile} of 7.3 μM for the isoleucine limited reaction on the enzyme.

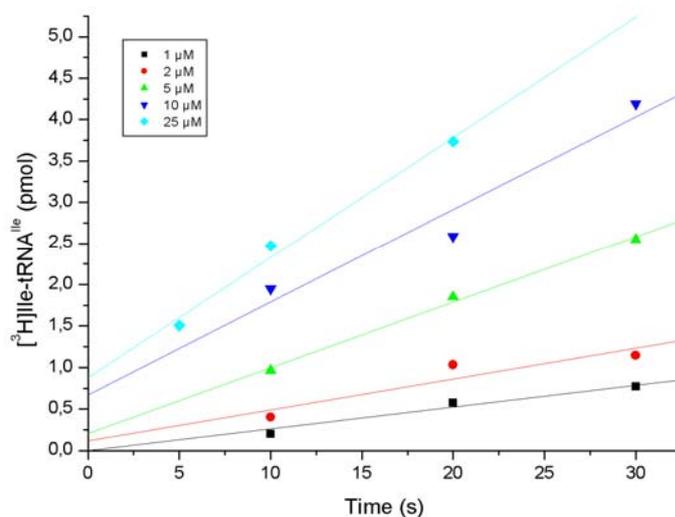


Figure 9. Rate of IRS-catalysed isoleucylation of tRNA^{Ile}. Different [³H]Ile concentrations ranging from 1 μM (■) to 25 μM (◆) were incubated in the presence of 1 μM tRNA^{Ile}, 9.3 nM IRS, 2 mM ATP, and 5 μg/ml inorganic pyrophosphatase for different times.

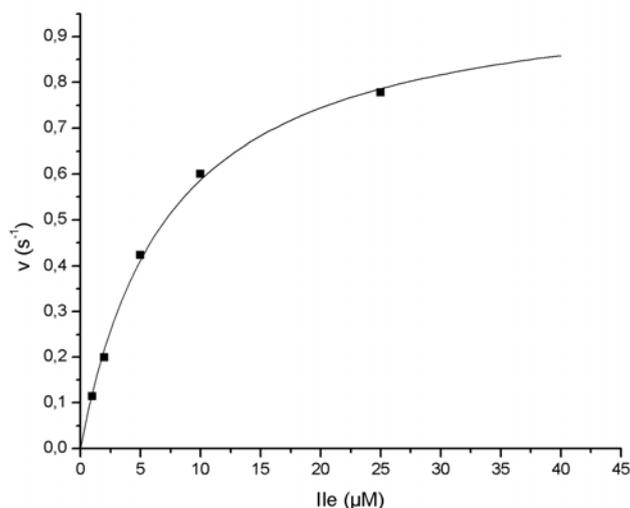


Figure 10. Michaelis-Menten plot of the rates of IRS-catalysed isoleucylation of tRNA^{Ile} (from Fig. 9). The line was obtained by fitting the data to equation C.

3.2.2 Kinetics of the valyl-adenylate reaction

In the study of the valyl-adenylate formation and its dissociation from the isoleucyl-tRNA synthetase the amount of AMP and Val-AMP produced from ATP was examined, either by separation of the nucleotides on TLC, or on a MonoQ-HPLC system. As Val-AMP hydrolyses rather quickly (Fersht, 1977) at room temperature, and there was no obvious separation of AMP and Val-AMP on either TLC or the MonoQ-column (data not shown), the total amount of

AMP together with any possible Val-AMP was treated as the product of the reaction.

From the experiment where TLC-separation was used it was not possible to get any accurate quantitative analysis as the concentration range of valine obviously was too low (Fig. 11 and 12). However, from the series of experiments employing the MonoQ-column for separation (Fig. 13, 14, and 15) the dissociation rate of Val-AMP was estimated to be 0.025 s^{-1} and K_M^{Val} was approximately 3 mM .

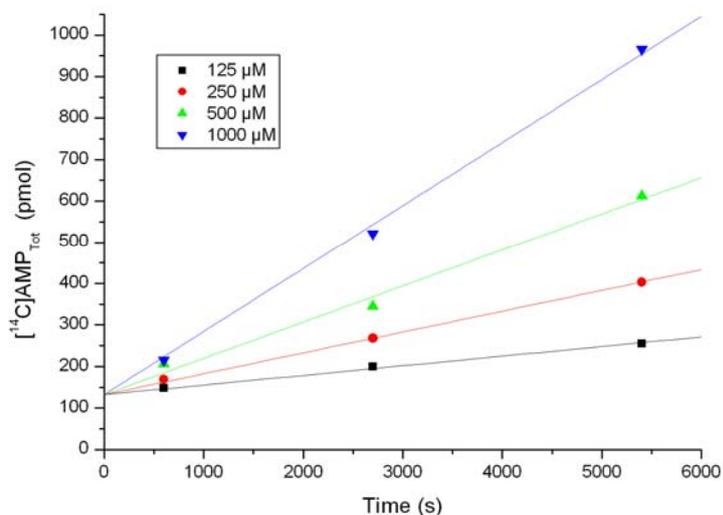


Figure 11. Rate of $[^{14}\text{C}]\text{AMP}$ formation in a solution containing $1 \mu\text{M}$ IRS, $500 \mu\text{M}$ $[^{14}\text{C}]\text{ATP}$, $5 \mu\text{g/ml}$ inorganic pyrophosphatase, and valine at different amino acid concentrations ranging from $125 \mu\text{M}$ to $1000 \mu\text{M}$.

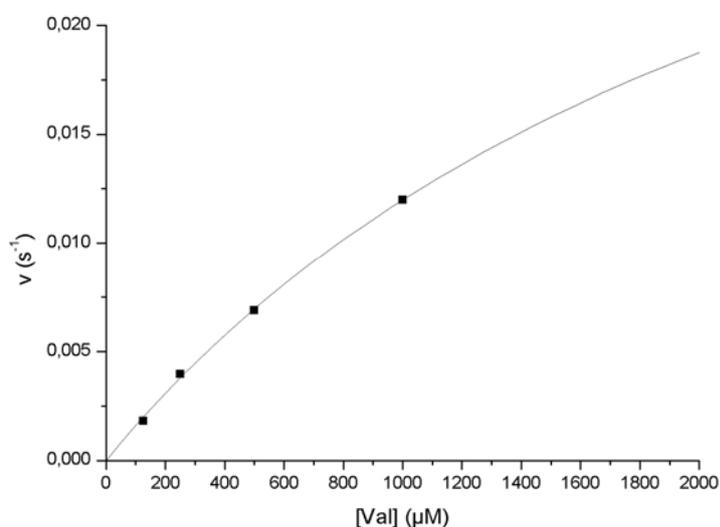


Figure 12. Michaelis-Menten plot of the rates of the IRS catalysed formation of $[^{14}\text{C}]\text{AMP}$ from $[^{14}\text{C}]\text{ATP}$ (from Fig. 11). The line represents the data fitted to equation D.

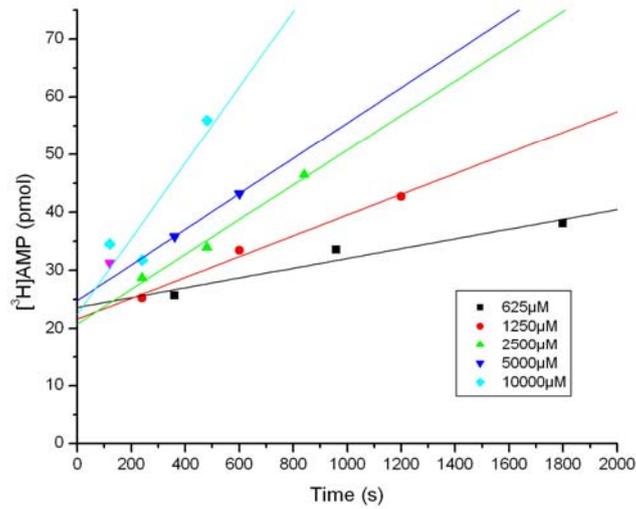


Figure 13. Rate of $[^3\text{H}]\text{AMP}$ formation in a solution containing $1\ \mu\text{M}$ IRS, $500\ \mu\text{M}$ $[^3\text{H}]\text{ATP}$, $5\ \mu\text{g/ml}$ inorganic pyrophosphatase, and valine at different amino acid concentrations ranging from $625\ \mu\text{M}$ to $10000\ \mu\text{M}$. The triangle in pink was not included in the fitting.

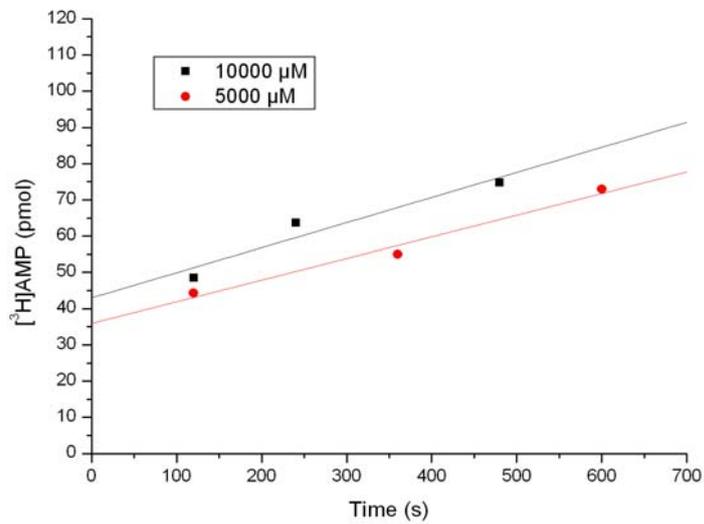


Figure 14. Rate of $[^3\text{H}]\text{AMP}$ formation in a solution containing $1\ \mu\text{M}$ IRS, $500\ \mu\text{M}$ $[^3\text{H}]\text{ATP}$, $5\ \mu\text{g/ml}$ inorganic pyrophosphatase, and $5\ \text{mM}$ or $10\ \text{mM}$ valine.

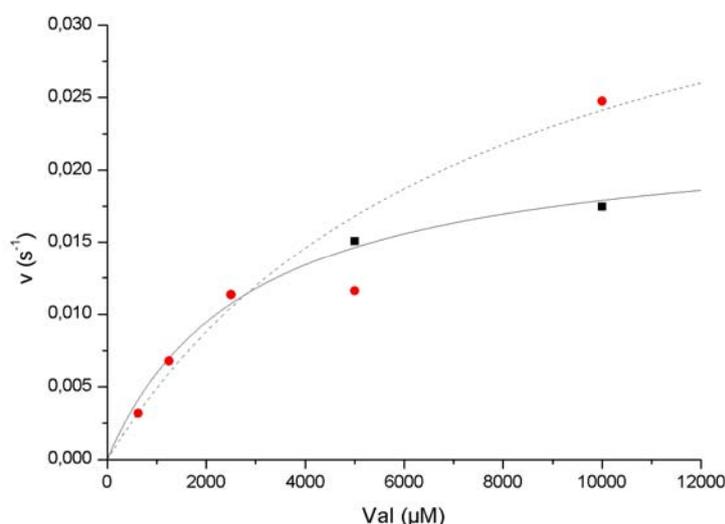


Figure 15. Michaelis-Menten plot of the rates of the IRS catalysed formation of $[^3\text{H}]\text{AMP}$ from $[^3\text{H}]\text{ATP}$. ● : rates from Fig. 13, ■ : rates from Fig. 14. The two lines represent the data fitted to equation D leaving out the 5 mM and 10 mM points from Fig. 13 (lower line), or the 10 mM point from Fig. 14 and both the 5 mM points (upper line) respectively.

3.2.3 The IRS catalysed valylation of tRNA^{Ile}

According to the literature, if any tRNA^{Ile} is mischarged by valine, the hydrolysis of this compound is very quick (at least 150 s^{-1} (Fersht, 1977)). Therefore in an attempt to catch any of the hypothetically mischarged tRNA the time evolution of the reaction was studied in the sub second range. By using the K_M result from the study of the valyladenylate reaction the amount of pre-activated valine on the enzyme could be estimated according to equation E.

$$[\text{IRS} \cdot \text{Val} - \text{AMP}]_0 = \frac{[\text{Val}] \cdot [\text{IRS}]}{[\text{Val}] + K_M^{\text{Val}}} \quad (\text{E})$$

Then, as stated under “Approach” above, assuming a rapid binding of tRNA^{Ile} to the complex the result of the experiment should fit into equation A, where $[\text{IRS} \cdot \text{Val} - \text{AMP} \cdot \text{tRNA}^{\text{Ile}}]_0 \approx [\text{IRS} \cdot \text{Val} - \text{AMP}]_0$ from equation E

In the experimental setup (Materials and Methods), the valine concentration was set to 1 mM. Using the approximate K_M^{Val} value of 3 mM (Fig. 13) this would correspond to a 25% activation of the amino acid on the synthetase (58.5 pmol activated out of 234 pmol total in one data point). In figure 16 the approximate amount of mischarged tRNA^{Ile} reaches 6 pmol, thus corresponding to an incorrect transfer of at least 10% of the pre-activated valine.

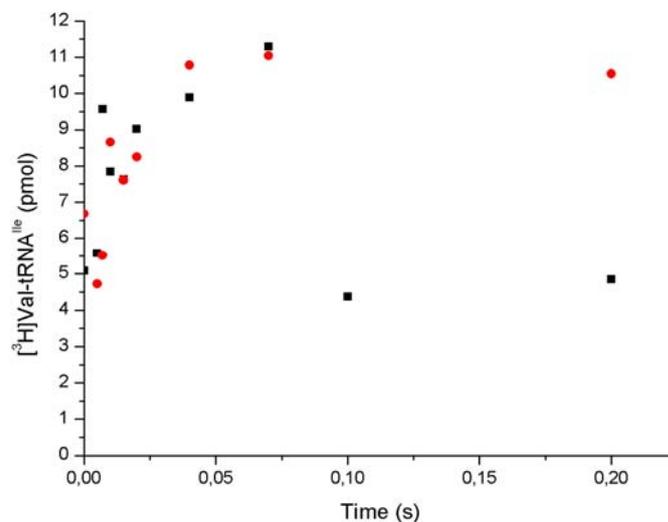


Figure 16. Amount of [³H]Val incorrectly charged on tRNA^{Ile}. The plot shows two separate experiments (■ and ●) in which small volumes of two reagent mixtures, containing 19.8 μM IRS, 1 mM [³H]Val, 2 mM ATP, and 5 μg/ml inorganic pyrophosphatase, and 20 μM tRNA^{Ile}, 1 mM Ile, 2 mM ATP, and 5 μg/ml inorganic pyrophosphatase respectively, were rapidly mixed and allowed to react for different times.

4 DISCUSSION

One of the key reactions preceding translation is the aminoacylation of tRNAs. Despite substrate similarities, such as for valine and isoleucine, aminoacyl-tRNA synthetases charge an amino acid onto a tRNA with an error frequency of only 1 in 10000 (Hopfield *et al.*, 1976). This high level of accuracy is achieved by the use of an energy consuming editing mechanism, proofreading, as proposed by Hopfield (Hopfield, 1974), and Ninio (Ninio, 1975). In the rejection of valine the isoleucyl-tRNA synthetase has in several studies been shown to use such an editing mechanism (Baldwin and Berg, 1966; Eldred and Schimmel, 1972; Fersht, 1977; Jakubowski and Fersht, 1981). However, despite almost thirty years of studies, the molecular mechanism of this editing step is still not understood; either, the incorrectly activated valine is transferred from AMP to a tRNA^{Ile} molecule, and then this binding is rapidly hydrolysed, or, the transfer to tRNA^{Ile} only occurs to a minor extent and the valine-AMP binding is hydrolysed in a tRNA^{Ile} promoted reaction.

In this study the properties of highly purified *E. coli* isoleucyl-tRNA synthetase were investigated *in vitro*. The final purity of the enzyme is not completely apparent in figure 5 as the polyacrylamide gel is heavily overloaded. However, after the preceding purification step the protein solution looked purer (figure 4),

and most probably some of the impurities shown in figure 5 were also removed in the ultra filtration using filters with a cut-off at 30 kDa.

As no obvious plateau was reached in the isoleucine titration of IRS (Fig. 6), the activity of the purified isoleucyl-tRNA synthetase was investigated in yet another experiment, the chase experiment. From the result of this experiment (Fig. 7) the concentration of active enzymes was estimated to 8.4 μM , which lies in the proximity of the one achieved in the titration experiment, 7 μM . From the experimental procedure however, the value obtained from the chase experiment seemed more confident why in the following experiments an enzyme stock concentration of 8.4 μM was assumed.

For comparisons with earlier studies the cognate reaction was first studied; For isoleucylation of tRNA^{Ile} the turnover number, k_{cat}^{Ile} , of approximately 1 s⁻¹, and the K_M^{Ile} value of 7.3 μM (Fig. 10) corresponds well with those presented by Fersht in 1977 (1.2 s⁻¹, and 7.3 μM (Fersht, 1977)). However, when preformed Ile-AMP in complex with IRS is mixed with tRNA^{Ile}, the transfer of isoleucine from the adenosine to the tRNA molecule is significantly higher (25 s⁻¹, Fig. 7). This is not consistent with Fersht's observations (Fersht, 1977; Fersht and Kaethner, 1976b) in which both the rate of the total reaction (from Ile, IRS, tRNA, and ATP), and the rate of tRNA charging from pre-formed isoleucyl-adenylate on the enzyme, are the same. To some extent this difference in rate might be explained by the difference in temperature; Fersht's studies were made at 25 °C, while the experiments presented here were all performed at 37 °C as this is the temperature for which *E. coli* enzymes are optimised. However, the difference might also be due to the different buffer systems and, as shown by Fersht and Kaethner, the reaction rate is highly dependent on pH (Fersht and Kaethner, 1976b). By decreasing from pH 7.78 to pH 5.87 Fersht and Kaethner observed a decrease in transfer rate from 1.47 s⁻¹ to 0.38 s⁻¹. All the experiments in this degree work were performed in pH 7.3.

In order to examine the IRS catalysed valylation of tRNA^{Ile} the kinetic properties of the initial valyl-adenylate formation were investigated. Surprisingly, the binding reaction of valine to the enzyme was very difficult to saturate. Earlier results indicate the K_M for valine to be in the size of 400 μM (Jakubowski and Fersht, 1981) or 500 μM (Fersht, 1977), but in the results from both applied separation methods here (TLC and MonoQ column, see Materials and Methods above) the K_M value was far higher (Fig. 12, and Fig. 15). If this is true it could to some extent explain why Fersht only found a small amount of mischarged Val-tRNA^{Ile}, as he used only 2 mM of valine and if he thought this was a saturating concentration. The large uncertainty in the data, and the fact that the two separation methods were not optimised to separate AMP and

unspecifically produced ADP, suggest that perhaps this is an artificially high K_M . The dissociation rate of valyl-adenylate from the enzyme, 0.025 s^{-1} , on the other hand, was lower than that of 0.04 s^{-1} as reported by Fersht (1977), or 0.05 s^{-1} as reported by Jakubowski and Fersht (Jakubowski and Fersht, 1981).

Using the somewhat uncertain K_M^{Val} value of 3 mM for the activation of valine on the synthetase, the amount of pre-activated complex in figure 16 was estimated to be around 60 pmol. This indicates that at least 10% of the misactivated valine is incorrectly transferred to the tRNA^{le}. However, if there is a rapid hydrolysis of Val-tRNA^{le}, not obvious in figure 16 though, all of the activated amino acid might as well have been transferred to the tRNA^{le}. As it is not obvious whether the valylated tRNAs are hydrolysed (as implied by the squares, ■, at times 0.1 s and 0.2 s, Fig. 16), or remain stable (implied by the circle, ●, at 0.2 s, Fig. 16) one cannot exclude the possibility that the ³H-counts trapped on the GF-C filters are due to non-specific binding of [³H]Val to precipitated tRNAs, synthetases, or even the filter itself. As the total [³H]Val in one sample is 11800 pmol, the 5 pmol on the filters at time point zero only corresponds to 0.04% of the total amount. However, there is a significant increase of [³H]Val being caught on the filters as the reaction proceeds, indicating that something else is happening.

To achieve a better resolution of the process depicted in figure 16, the signal-to-background ratio needs to be increased. In the experiments presented here the ³H-counts per fraction of activated complex present, $[IRS \cdot Val - AMP]_0$, are maximised. As the fraction of activated complex increases linearly with respect to enzyme concentration, but only hyperbolically with respect to valine concentration (equation D), an increased stock concentration of enzyme is the preferred way to increase the signal.

However, even if the experiment would be optimised and a nice looking peak would appear, this might still not be enough. Given a peak-like curve, as in the transient formation of Thr-tRNA^{Val} (Fersht and Kaethner, 1976a), it is possible to estimate the transfer rate, k_1 , and the one-step editing parameter q_2^I of equation A by non-linear regression (Index I indicates the one-step editing model. The q_1^I rate is thus equal to zero). The problem is that exactly the same curve is achieved by setting the two-step model parameters q_1^{II} and q_2^{II} according to: $q_1^{II} = q_2^I - k_1$ and $q_2^{II} = k_1$. If, however, the rate, q , with which the activated valine, Val-AMP, disappears is known (*i.e.* $q = k_1 + q_1$ in equation A) equation A turns into equation F.

$$[IRS \cdot Val - tRNA^{le}](t) = [IRS \cdot Val - AMP \cdot tRNA^{le}]_0 \cdot \frac{q - q_1}{q - q_2} (e^{-q_2 t} - e^{-q t}) \quad (\text{F})$$

Fitting the hypothetical peak into equation F would then solve the question as q_1 in the case of a one-step editing mechanism is equal to zero.

From the study presented here it is not possible to draw any conclusions as to whether the editing step of valine on IRS occurs in a pre- or post-transfer mechanism. Nevertheless, in the attempts to trap incorrectly valylated tRNA^{le} (Fig. 16), the results show a distinct formation of something that is believed to be Val-tRNA^{le}. Up to this point no one has been able to achieve a significant amount of this non-cognate product, and if the data in figure 16 is not due to an artefact, *e.g.* due to unspecific binding, this study has led at least one step further in the understanding of the proofreading mechanism of isoleucyl-tRNA synthetase.

5 ACKNOWLEDGEMENTS

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