The early history of tRNA recognition by aminoacyl-tRNA synthetases

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1. Introduction

Discovery of aminoacyl-tRNA synthetases and importance of these enzymes for correct genetic code expression as well as early structural data and related enzymology will be reviewed. Despite structural diversity, all synthetases follow a two-step mechanism for tRNA aminoacylation. Specificity, however, is not absolute since synthetases were shown to catalyze mischarging reactions. These reactions are characterized by low catalytic rates and can lead to incomplete charging levels. Incomplete charging can also occur for cognate tRNA aminoacylation and reflects the equilibrium between the forward acylation and the reverse deacylation (enzymatic and chemical) reactions. Early strategies to characterize the structural features within a given tRNA that account for its preferential aminoacylation by the cognate synthetase will be reviewed. Among them were (i) sequence comparisons of tRNA (RNAs) recognized by a same synthetase, (ii) activity measurements of modified tRNAs (by chemical or enzymatic means) and (iii) reconstitutions of active tRNAs from fragments. As a result, the importance of anticodon and tRNA amino acid accepting arms were highlighted as well as that of the overall tRNA architecture that can be mimicked by tRNA-like structures. Altogether early data gave the robust background to the modern concept of identity.

2. Precursory findings

The pioneering work of Fritz Lipmann on the high-energy compounds ATP and acetyl-CoA is on the origin of the discovery of what we call today the aminoacyl-tRNA synthetases (aaRSs). When it was realized that the first step in protein formation might be amino acid activation by ATP (Hoagland 1955), biochemists rushed to find enzymes that would catalyze this reaction. These were rapidly found in several laboratories (e.g. those of Berg, Lipmann, Novelli, Zamecnik), the first enriched one being a tryptophan-activating enzyme from beef pancreas (Davie *et al* 1956). Further, when radioactive amino acids and ATP were incubated in cell-free extracts of *Escherichia coli* or rat liver, it was shown that the amino acids are converted to an acid-insoluble form (Berg and Offengand 1958; Hoagland *et al* 1958). In the suggested mechanism (Berg and Offengand 1958),

ATP + amino acid → adenyl~amino acid + PP (activation step)

adenyl~amino acid + RNA → RNA~amino acid + AMP (transfer step)

ATP + amino acid + RNA → RNA~amino acid + AMP + PP (overall reaction)

a soluble RNA is the acceptor of the activated amino acid. This RNA with an estimated average molecular weight of 20,000, in fact tRNA, was explicitly characterized in Paul Zamecnick's laboratory by Hoagland and coworkers (Hoagland *et al* 1958; Hoagland 1996; Kresge *et al* 2005).

Independently and pursuing different routes, the pioneers of molecular biology were thinking on gene expression and genetic code. In a famous letter send in 1955 to the "RNA Tie Club" Francis Crick predicted the existence of small *adaptor RNA molecules* that would carry their own amino acids and

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Abbreviations used: aaRS, aminoacyl-tRNA synthetase with the three-letter abbreviation for amino acids, e.g. AlaRS for alanyl-tRNA synthetase; tRNA, transfer RNA (called sRNA in the early literature), e.g. $tRNA^{Ala}$ for the molecule specific for alanine and alanyl-$tRNA^{Ala}$, when charged with alanine.

interact with the messenger *RNA template in a position for polymerization to take place* (Crick 1955). These adaptors, larger than predicted by Crick, are the tRNAs discovered in 1958.

The seminal experiment that established the biological importance of accurate tRNA aminoacylation for correct translation of the genetic code was run in 1962 (Chapeville *et al* 1962). It was designed to verify the adaptor hypothesis and showed that the coding properties of the adaptor is not determined by the amino acid it carries but by the interaction of the aminoacylated tRNA with the mRNA template. Demonstration came from the observation that both cysteinyl-$tRNA^{Cys}$ and alanyl-$tRNA^{Cys}$ (obtained by Raney Nickel reduction of the cysteinyl residue on $tRNA^{Cys}$) are amino acid donors in a ribosomal polypeptide synthesis system programmed with poly UG for cysteine incorporation. This implies that misaminoacylation of a tRNA would lead to incorporation of erroneous amino acid into proteins, and gave rise to the concept of a *second genetic code*, i.e. the signals/rules by which aaRSs recognize their cognate tRNAs, as first formulated by Lengyel (1966) and rejuvenated (de Duve 1988) after the characterization in $tRNA^{Ala}$ of base pair G3–U70 as the alanine identity determinant (Hou and Schimmel 1988; McClain and Foss 1988).

From these early findings it became obvious that tRNA recognition by aaRSs is the key problem and that its solution would rely on a precise molecular understanding of tRNA aminoacylation systems. Started then the race to decipher the peculiar structural and functional relationships tRNAs have with their cognate aaRSs. It is the purpose of this survey to outline the early stages (until the late eighties) of this race.

3. Structural background in the era of biochemistry

Before the advent of molecular biology technologies (1960–1985), purifying a tRNA was easier than purifying an aaRS. This comes from the peculiarity of tRNA to remain in the aqueous phase after phenol treatment of cellular extracts. Thus, the biochemical challenge is simplified, since isolation of an individual tRNA species can be done from a bulk of only 20 families of tRNA molecules in contrast to aaRSs that have to be purified from much more complex macromolecular mixtures.

Counter-current distribution (Dirheimer and Ebel 1967; Doctor 1967) and column chromatographies on BD-cellulose (Gillam *et al* 1968) or reverse phase supports (RPC) (Pearson *et al* 1971) were much used to obtain the large amounts of pure tRNA needed for the early structural investigations. Yeast $tRNA^{Ala}$ and $tRNA^{Phe}$ could be readily isolated by counter-current distribution, because of peculiar solubility properties. In the case of $tRNA^{Phe}$ its hydrophobic Y-base conferred strong binding to BD-cellulose allowing its easy separation from the bulk of the other tRNAs. Yeast $tRNA^{Ala}$ was the first to be sequenced (Holley *et al* 1965) but the yeast $tRNA^{Phe}$ was the first whose 3D structure was solved by X-ray crystallography in three laboratories (Kim *et al* 1973; Robertus *et al* 1974; Stout *et al* 1978). These studies revealed that tRNA is folded in a cloverleaf that organizes in a L-shaped 3D-architecture (for historical details see Clark 2006; RajBhandary and Köhrer 2006). Notable are the small-angle X-ray scattering investigations done in the early sixties on bulk *Escherichia coli* tRNA that predicted the L-shape (Witz 2003) and the chemical *tour de force* with the total synthesis of the gene of yeast $tRNA^{Tyr}$ (Agarwal *et al* 1970).

Today, the early purification procedures of tRNA are no more used. The modern methods are faster and easier to perform. However, all of them, be it *in vitro* transcription of synthetic tRNA genes, *in vivo* overproduction of specific tRNAs, or purification by hybridization, suffer from drawbacks like production of unmodified or partially modified molecules and low purification yields in the case of hybridization methods. As an unfortunate consequence, experiments requiring pure and fully modified tRNA molecules are presently hampered.

Structural information on aaRSs came later, partly due to biochemical difficulties and the lack of dedicated methods for purifying the gram amounts of pure material needed for sequencing and crystallization. Researchers refrained to work on animal or plant aaRSs particularly prone to proteolysis. But on the other hand, several groups worked out large-scale procedures using up to 50–100 kg of bacterial or yeast cells (Bruton *et al* 1975; Kern *et al* 1977). The first surprise came when the structural diversity of aaRSs became apparent despite the same function of these enzymes. Molecular weights were found in the range ~40–400 kDa with monomeric, dimeric or tetrameric organizations (Schimmel 1987). Typical examples are yeast monomeric ValRS and dimeric LysRS (Rymo *et al* 1972) and tetrameric $\alpha_2\beta_2$ PheRS (Fasiolo *et al* 1970). A proteolyzed but active fragment of *E. coli* MetRS gave the first aaRS crystals (Waller *et al* 1971), soon followed by crystals of full-length yeast LysRS (Lagerkvist *et al* 1972) and *Bacillus stearothermophilus* TyrRS (Reid *et al* 1973). Sequence data came later due to difficulties in obtaining proper peptidic fragments, the first known sequence being that of dimeric *B. stearothermophilus* TrpRS, the smallest aaRS with a subunit of 37 kDa (Winter and Hartley 1977).

Knowledge of the 3D structure of a synthetase and especially that of a complex with tRNA was a long awaited dream. Early aaRS crystals were of poor quality and the initial attempts to crystallize a complex appeared hopeless. Shifting from mesophilic to thermophilic organisms gave a first answer to improve crystal quality and led to solve

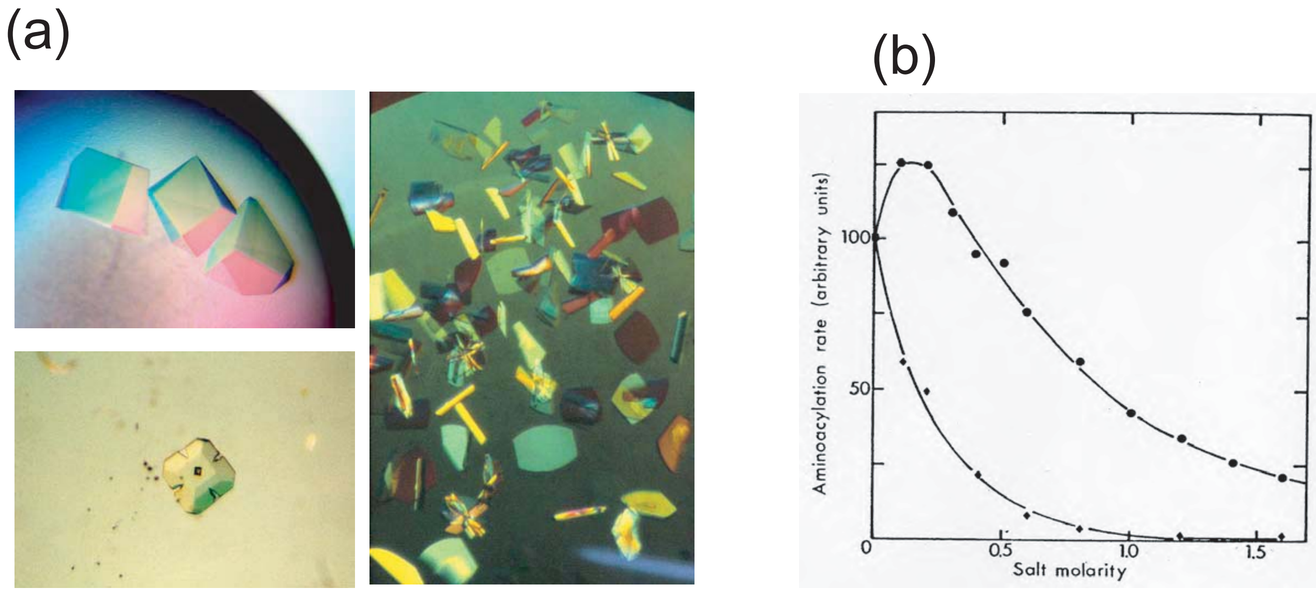

Figure 1. Towards the structure of a tRNA/aaRS complex. **(a)** Crystals of the complex between yeast AspRS and its cognate tRNA^{Asp} obtained in the presence of ammonium sulphate (Giegé *et al* 1980). The figure displays at the left two cubic crystal forms of poor diffraction quality and at right good quality orthorhombic crystals for structure analysis. **(b)** Demonstration that crystallization conditions permit efficient aspartylation of tRNA^{Asp} (Giegé *et al* 1982). When NaCl is added, aminoacylation rates drop rapidly (◆); in contrast they remain high under crystallization conditions at high concentration of $(NH_4)_2SO_4$ being only reduced by a factor of ~5 (●).

the structure of *B. stearothermophilus* TyrRS (Irwin *et al* 1976). The surprise was the presence in this protein of a nucleotide-binding fold, i.e. a Rossmann-fold as found in dehydrogenases (Rossmann *et al* 1974). Attempts to crystallize a complex were first conducted with non-ionic crystallizing agents which turned out to be negative because they were based on a wrong rationale. Even though salts dissociate tRNA/aaRS complexes, because ionic contacts become disrupted (Loftfield and Eigner 1967), we found that association as well as aminoacylation activity are maintained in the presence of ammonium sulphate at high concentration (>1.0 M). This led to the crystallization of the first tRNA/aaRS complex (Giegé *et al* 1980, 1982; Lorber *et al* 1983) (figure 1). The effect is restricted to ammonium sulphate, the only known salt to sustain strong hydrophobic associations at high concentration although at low concentration it disrupts ionic contacts, as do the other salts. This remarkable property permitted to grow many other crystals of RNA/protein complexes (Dock-Bregeon *et al* 1999) and consequently to solve their structures, the first ones being those of the glutamine (Rould *et al* 1989) and aspartate (Ruff *et al* 1991) systems.

4. A phenomenological view on tRNA aminoacylation

Until the late seventies~~th~~, enzymology of tRNA aminoacylation was subject of large controversy. As first suggested by Paul Berg and later advocated by Alan Mehler, the reaction would occur in two steps consisting of rapid formation of adenylate followed by the transfer of the aminoacyl-moiety to tRNA (Berg and Offengand 1958; Mehler and Mitra 1967). In the alternate mechanism proposed by Robert Loftfield, activation and transfer would occur in a concerted manner (Loftfield and Eigner 1969). The existence of three aaRSs (ArgRS, GlnRS and GluRS) requiring tRNA for $[^{32}P]PP_i$-ATP isotope exchange (Ravel *et al* 1965; Mitra and Mehler 1966) was the main argument in support of the concerted mechanism. The dilemma was solved in 1980 when it was demonstrated that tRNA^{Glu} charging by *E. coli* GluRS occurs via two steps, even though this enzyme requires tRNA for amino acid activation (Kern and Lapointe 1980). The explicit proof was the cleavage of ATP in a stoichiometric amount to GluRS without concomitant synthesis of Glu-tRNA^{Glu} at the very early time of tRNA^{Glu} charging.

Mischarging and incomplete charging of tRNA were two unexpected phenomena that attracted attention. Indeed, amino acid and tRNA recognition by aaRSs turned out to be not very specific. Linus Pauling raised the question of amino acid recognition in 1957, before the overall scheme of protein synthesis was known. He argued that stereochemical discrimination between related amino acids like valine and isoleucine by simple molecular recognition would not be sufficient to prevent errors in protein synthesis (Pauling 1957). His prediction was verified and editing mechanisms preventing misactivated amino acids to be utilized in protein

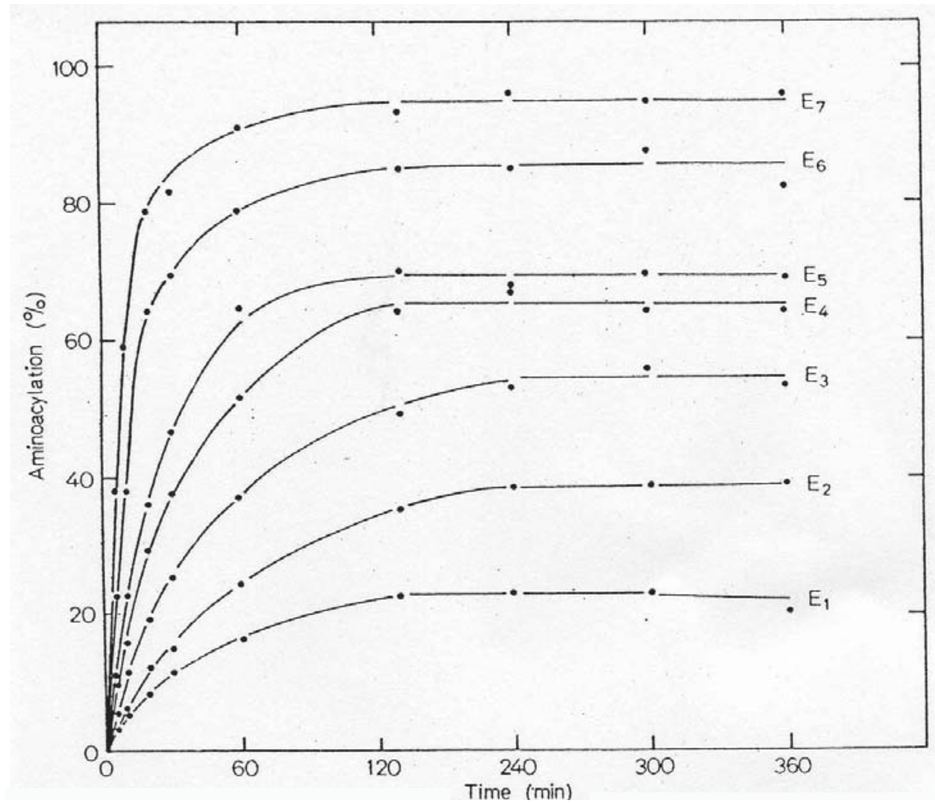

Figure 2. tRNA aminoacylation plateaus as a function of aaRS concentration. The data concern mischarging of yeast initiator $tRNA^{Met}$ by yeast ValRS. Notice the increase of plateau levels when ValRS concentration increases from 0.06 μM (E_1) to 2.5 μM (E_7) (Dietrich *et al* 1976).

synthesis were discovered (reviewed by Hendrickson and Schimmel 2003).

Mischarging of tRNA was discovered *in vitro*, with a variety of aaRSs, like AspRS, IleRS, PheRS and ValRS, able to catalyze such reactions (reviewed by Giegé *et al* 1993). These reactions are characterized by low rates, are particularly sensitive to experimental conditions (pH, salts, spermine, $[Mg^{2+}]$/[ATP] ratio) and are facilitated in heterologous systems as well as by the presence of organic solvents (ethanol or DMSO). It was thus proposed that loosening tRNA and/or aaRS structures facilitates false recognitions and consequently tRNA mischarging (Yarus 1972). Today we know that tRNA mischarging occurs also *in vivo* under certain circumstances (Swanson *et al* 1988; Rogers and Söll 1990) and can even be a biological necessity in organisms lacking AsnRS or GlnRS (Ibba *et al* 1997). While studying mischarging reactions and aaRS activities, researchers often noticed aminoacylation plateaus reflecting incomplete tRNA charging (figure 2). The phenomenon, often misunderstood, is dependent on aaRS concentration and explained by the chemical fragility of the ester bond between the charged amino acid and the 3'-terminal ribose of tRNA, i.e. its pH sensitivity. In fact plateaus reflect equilibria between the forward acylation and the reverse deacylation (enzymatic and chemical) reactions (Bonnet and Ebel 1972; Dietrich *et al* 1976).

Binding constants of tRNA for aaRSs were found to be in the range of 10^6–10^8 M^{-1} with small discrimination between cognate and non-cognate complexes, as also reflected by small $\Delta G°$ differences. Complex formation is entropy driven with large positive $\Delta S°$ ($\geq$ 20 cal mol^{-1} K^{-1}), with highest values for non-cognate complexes. Specific binding involves likely release of water from the contact interface while non-specific binding is stabilized by electrostatic interactions as suggested by $\Delta H°$ values close to 0 in the case of cognate and >> 0 kcal mol^{-1} in that of non-cognate complexes (Lam and Schimmel 1975; Krauss *et al* 1976). Fast kinetics and other physico-chemical experiments showed that cognate complex formation proceeds in two steps accompanied by conformational changes, in contrast to a one step mechanism for non-cognate complexes (Krauss *et al* 1976; Riesner *et al* 1976). Further information came from steady-state kinetics of tRNA aminoacylation reactions. Cognate and non-cognate systems turned out to differ predominantly by k_{cat} effects. Taken together this led to the concept of kinetic specificity according to which specificity of tRNA aminoacylation relies more to subtle adjustments of the aaRS catalytic site (reflected by k_{cat}) than to the recognition between the two macromolecules (reflected by K_m) (Ebel *et al* 1973; Giegé *et al* 1993). In agreement with this view, it could be shown later, in the case of yeast PheRS, that the body of $tRNA^{Phe}$ deprived of its 3'-terminus can activate the catalytic site of PheRS and thereby triggers aminoacylation of free adenosine or CpCpA (Renaud *et al* 1981).

In a more biological perspective, studies on the regulation of aaRSs showed the importance of controlling their biosynthesis for optimal cellular growth and accuracy of *in vivo* tRNA aminoacylation. Regulation of aaRSs was exemplified in 1985 for *E. coli* ThrRS and PheRS in the laboratory of Marianne Grunberg-Manago: for ThrRS it is autogenous (Springer *et al* 1985) and for PheRS it occurs by attenuation in a phenylalanyl-$tRNA^{Phe}$-dependent mechanism (Springer *et al* 1983). Note that deregulation of aaRS expression, as found with overexpressed GlnRS, can lead to tRNA mischarging (Swanson *et al* 1988).

5. Early findings from structure-function approaches

The search of unifying features in the tRNA/aaRS world occupied many researchers. Evidence came from Sylvain Blanquet's laboratory with the observation that bacterial aaRSs (i.e. IleRS, MetRS, TrpRS or TyrRS) can be inactivated by affinity labelling with periodate oxidized ATP (Fayat *et al* 1978) or tRNA (Fayat *et al* 1979). Inactivation is the consequence of the formation of a Schiff's base between the oxidized ATP or tRNA and a lysine residue from the enzyme active site. Since all the investigated proteins contained a KMSKS signature sequence (Hountondji *et al* 1986), it was concluded that all aaRSs can be affinity labelled with periodate oxidized substrates. The conclusion turned out to be wrong since oxidized yeast $tRNA^{Asp}$ could not be specifically cross-linked to cognate AspRS via the Schiff's base formation (Théobald *et al* 1988). Explanation came after the discovery of the partition of aaRSs into two classes (Cusack *et al* 1990; Eriani *et al* 1990), the first one containing the KMSKS signature, the second one lacking it.

Many approaches were employed to find how tRNA contacts aaRSs. Photo-cross-linking (Schoemaker and Schimmel 1974) and tritium-labelling (Schoemaker and Schimmel 1976) identified tRNA regions in vicinity of aaRSs. The general picture that emerged was that aaRSs *interact with the general area in which the two helical branches of the L-shaped tertiary structure fuse together, with additional interactions on other parts of the tRNA as well* (Schoemaker *et al* 1975). Conceptualizing structural and functional data, Sung-Hou Kim proposed in 1975 the symmetry recognition hypothesis according to which *the internal symmetry of tRNA coincides with that between unit domains of the enzyme* (Kim 1975). Footprinting approaches were of particular interest. We systematically used ethylnitrosourea, a phosphate-alkylating probe, to determine the phosphate groups in tRNA protected by aaRSs. The method was worked out with yeast $tRNA^{Val}$ and $tRNA^{Phe}$ in complex with ValRS and PheRS (Vlassov *et al* 1983) and generalized to study the interaction of several *E. coli*, yeast, plant and animal tRNAs in complex with their cognate aaRSs (reviewed by Giegé *et al* 1993). Data revealed idiosyncratic recognition pattern, but in all cognate complexes investigated, phosphates in anticodon stems were found protected against alkylation. *A posteriori*, the protection patterns roughly agree with the known crystal structures of the complexes.

Identification within tRNA sequences of residues important for aminoacylation was a major goal. In the seventies, with the increasing number of known tRNA sequences and the large panel of available aminoacylation data (cognate charging in homologous or heterologous systems and mischarging), researchers started to compare the sequences of tRNAs aminoacylated by the same aaRS. This approach identified putative important residues in tRNAs such as U35 and G73 conserved in tRNAs aspartylable by yeast AspRS (Asp, His, Gln and Glu tRNAs) and A35 and A73 in tRNAs valylable by *B. stearothermophilus* ValRS (Val, Phe, Ile and Met tRNAs) (Ebel *et al* 1973; Giegé *et al* 1974). These residues were later characterized as aspartate and valine identity determinants (reviewed by Giegé *et al* 1998b). Following a similar rationale and comparing all known sequences showed a remarkable conservation of residue 73 in tRNAs specific for the same amino acid. This observation led to the discriminator hypothesis and the proposal that base 73, presently called the discriminator base, *serves as the primary site to subdivide the tRNAs into groups for recognition purposes* (Crothers *et al* 1972). Interestingly, non-cognate tRNAs which are easily mischarged, fall into these groups, having the same discriminator base as the cognate tRNAs (Ebel *et al* 1973; Giegé *et al* 1993).

Following different routes, geneticists discovered mutant tRNAs that are mischarged and translate stop codons (Shimura and Ozeki 1973). For instance amber suppressor $tRNA^{Tyr}$ mutants from *E. coli* can translate the UAG stop codon as glutamine as the result of their mischarging by GlnRS (Celis *et al* 1973). The mutations are all located in the last base pairs of the amino acid accepting stem and at discriminator position 73. Other investigations gave significance to anticodon, as for instance the discovery of an *E. coli* $tRNA^{Gly}$ species with an anticodon mutation that produces a 10^4-fold decrease of its glycylation rate (Squires and Carbon 1971).

6. Aminoacylation capacity of tRNA after chemical modification

Chemical approaches were widely used to study the recognition of tRNA by aaRSs (reviewed by Ebel 1968; Schulman 1979). Hydroxylamine modifying U, dimethyl sulphate alkylating C, A and G and especially bisulphite converting C to U were frequently used probes. With *E. coli* $tRNA^{Glu}$ it was shown under optimized conditions that exposed cytidines react to completion (Singhal 1971); so the bisulfphite treatment of a tRNA can be equivalent to a mutagenesis experiment. In refined protocols to get unequivocal interpretations, molecules completely inactivated by a chemical treatment were removed from the samples before activity measurements and sequence analysis of the modified tRNA. This was of prime importance with bisulphite which can convert the 3' terminal CCA to a toxic UUA sequence (Schulman 1979).

In yeast $tRNA^{Val}$, for instance, modification of exposed residues led to decreased aminoacylation activity when C36 in the anticodon was targeted (Chambers *et al* 1973). Likewise, initiator $tRNA^{Met}$ from *E. coli* lost activity after C34 and/or A35 modification in the anticodon (Schulman and Goddard 1973). Note that this approach allowed also identification of residues not involved in aminoacylation. For instance, C to U transitions in the D-loop and unexpectedly even in the terminal CCA of *E. coli* $tRNA^{Arg(ICG)}$ have no effect on arginylation in contrast to the deamination of C35 that inactivates the tRNA (Chakraburtty 1975).

Earlier experiments used exclusively native tRNAs with modified nucleotides in contrast to what is often done today with the unmodified transcripts. Specific probes such as borohydride, kethoxal, or irradiation by UV light were used to target D, mG or thiolated residues, respectively (reviewed by Chambers 1971). All U residues in yeast tRNA, including D and *Ψ*, could be replaced by *in vivo* incorporation of 5-FU (Giegé *et al* 1969). In most cases, aminoacylation capacity was not affected (Chambers 1971), a disappointing output for early investigators but that turned out to be essential later in allowing studies on identity with unmodified tRNA transcripts. However, effects on aminoacylation mediated by modified residues were observed. One example is photo crosslinking between s^4U8 and C13 in *E. coli* $tRNA^{Val}$ that decreases the affinity of the modified tRNA for ValRS (Yaniv *et al* 1971). Further, treatment of bulk *E. coli* tRNA with BrCN produced decreased glutamate and glutamine acceptance, likely as the result of the cyanation by BrCN of the 2-thio-U derivatives in the first anticodon position of $tRNA^{Glu}$ and $tRNA^{Gln2}$ (Seno *et al* 1974). Mechanistic studies showed that modification of 5-[methyl-amino-methyl]-2-thio-U34 (mam^5s^2U) in $tRNA^{Glu}$ reduces ~20-fold the steady-state charging rate and displaces the rate-determining step of the glutamylation process (Kern and Lapointe 1979).

Synthetic DNA oligomers corresponding to *E. coli* $tRNA^{Phe}$ or $tRNA^{Lys}$ have been synthesized in two versions, with either dT or dU at positions occupied by U residues in the native tRNAs. These tDNAs had a terminal rA76 in the case of $tDNA^{Phe}$ and dA76 in $tDNA^{Lys}$ according to the requirements for aminoacylation at ribose 76 (for details see Sprinzl 2006). All molecules could be charged by either phenylalanine or lysine by the cognate aaRSs (Khan and Roe 1988). Remarkably, aminoacylation efficiencies as expressed by V_{max}/K_m were efficient, with losses of at most 13-fold.

7. Aminoacylation capacity of tRNA with reshaped structure

Molecular microsurgery of tRNA with enzymatic procedures using among others T4 polynucleotide kinase and T4 RNA ligase was the first powerful technology that allowed replacing residues in anticodon loops with any desired nucleotide or oligonucleotide. This method was used to prepare a large variety of variants of yeast $tRNA^{Phe}$ and $tRNA^{Tyr}$ with single or multiple substitutions in their anticodon loops (Bruce and Uhlenbeck 1982; Bare and Uhlenbeck 1985, 1986). With $tRNA^{Phe}$, strongest effects on aminoacylation by pure PheRS were found for Gm34 replaced by A with 10-fold decrease of K_m and only a small effect on V_{max} (Bruce and Uhlenbeck 1982). With $tRNA^{Tyr}$, single substitution of G34 or *Ψ*35 by A had the strongest effects which were increased for double substitutions, suggesting that each position acts independently for TyrRS recognition. But, substitution of modified *Ψ*35 by U or modified U derivatives had weak effects on tyrosylation (Bare and Uhlenbeck 1986). Interestingly, the mischarging rate by PheRS of $tRNA^{Tyr}$ with *Ψ*35 replaced by A increased 10-fold (Bare and Uhlenbeck 1985), in agreement with the importance of A35 in phenylalanine identity.

Study of the aminoacylation capacity of tRNA fragments or of molecules reconstituted with fragments was applied to investigate alanine, methionine, phenylalanine, and valine specificities. This approach was widely used by Bayev, Chambers, Ebel, Nishimura, Reid, Zachau and their colleagues (figure 3) and led to conclusions supported by the present understanding of tRNA identity rules (Giegé *et al* 1998b; Giegé and Frugier 2003). In the pioneering work done in Moscow, yeast $tRNA^{Val}$ was fragmented in halves and quarters by enzymatic dissection. Association of the fragments reconstituted active tRNA molecules, showing that D-, anticodon- and T-loops need not to be intact for ValRS functional interaction. But valylation capacity was lost after removal of A35 and C36 from the 3'-CCA half-fragment, indicating that anticodon residues are important for valylation (Mirzabekov *et al* 1969a,b, 1970, 1971).

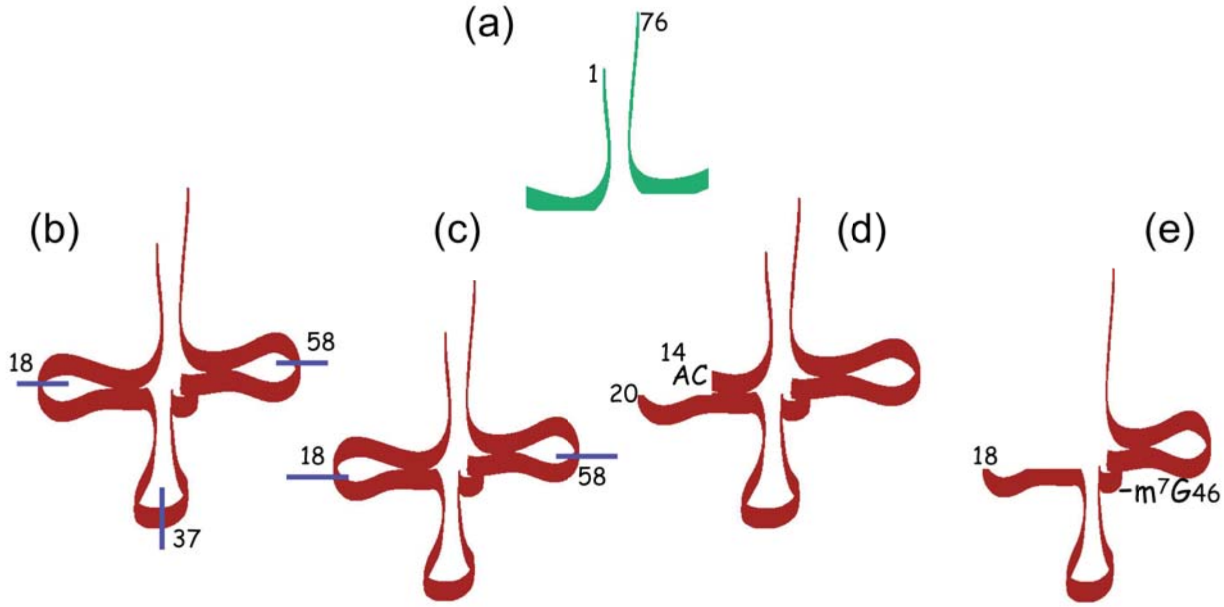

Figure 3. Five examples of fragmented tRNA molecules that keep aminoacylation capacity. **(a–d)** Reconstituted molecules: **(a)** yeast $tRNA^{Ala}$, **(b)** $tRNA^{Val}$, **(c)** yeast $tRNA^{Phe}$ and **(d)** *E. coli* initiator $tRNA^{Met}$. **(e)** Three-quarter of yeast $tRNA^{Phe}$ with single stranded acceptor end and excised m^7G46.

Similar reconstitutions were done with *E. coli* $tRNA^{Val}$ (Oda *et al* 1969).

Unexpected results came from Chamber's group when alanine activity was found in reconstituted acceptor stem of yeast $tRNA^{Ala}$ (Imura *et al* 1969a, b). From this Chambers concluded *that the specific recognition site for yeast tRNA*^{Ala} *is located in the acceptor stem* (Chambers 1969). The result, however, was criticized because of low alanylation plateaus which in fact are explained by the plateau theory (see above). About 20 years later, Chamber's belief was confirmed when it was found that the G3-U70 base pair, present in the reconstituted acceptor stem, is the alanine identity determinant (Hou and Schimmel 1988; McClain and Foss 1988).

With *E. coli* initiator $tRNA^{Met}$, reconstitution was achieved by annealing a 3'-three-quarter fragment with different 5'-quarter fragments (wild-type or lacking one or several 3'-residues from the D-arm). Activity assays showed that aminoacylation progressively drops when the 5'-quarter is stepwise deprived of its terminal 3'-residues, indicating that D-loop is important for methionylation (Seno *et al* 1970).

Active yeast $tRNA^{Phe}$ could be reconstituted by association of half-molecules produced by chemical chain scission after excision of the Y-base in the anticodon loop at position 37 (Philippsen *et al* 1968) or half and quarter fragments obtained by enzymatic cleavage of the tRNA in the D- and T-loop (Schmidt *et al* 1970). Interestingly, an inactive 3'-three-quarter fragment of yeast $tRNA^{Phe}$ acquired phenylalanylation capacity after removal of m^7G46 in the variable region (Renaud *et al* 1979). Restoration of activity was explained by a structural relaxation of the modified fragment facilitating its adaptation on PheRS. *A posteriori* one understands activity of the fragment that encompasses all the phenylalanine identity determinants (G20, G34, A35, A36 and A73) (Sampson *et al* 1989).

8. Aminoacylation of plant viral genomic RNAs

A serendipitous observation in the early seventies was the demonstration of the valylation capacity of the genomic RNA from turnip yellow mosaic virus (TYMV) (Pinck *et al* 1970), soon followed by that of the tyrosylation capacity of brome mosaic virus (BMV) RNA (Hall *et al* 1972) and histidylation capacity of tobacco mosaic virus (TMV) RNA (Oberg and Philipson 1972). It was hypothesized that the 3'-terminal structure of the plant viral RNAs recognized by aaRSs is folded in a tRNA-like structure (TLS) mimicking tRNA. However, sequencing did not reveal canonical tRNA features, despite aminoacylation of the viral RNAs is very efficient as explicitly shown for the valylation of TYMV RNA by yeast ValRS (Giegé *et al* 1978). The mystery was solved by Cornelius Pleij and his coworkers in Leiden who proposed a plausible model of the TYMV TLS. In this model, a novel RNA folding principle, the pseudoknot, allows building of a tRNA acceptor stem with a single strand of RNA (Rietveld *et al* 1982; Pleij *et al* 1985). Using chemical probing data and the crystallographic

RNA	K_m (μM)	k_{cat} (min^{-1})	Loss (x-fold)
yeast $tRNA^{Val}$	0.12	143	1
TYMV TLS	0.32	23	16
yeast $tRNA^{Metf}$	7.00	0.78	$>10^4$

Figure 4. Functional and structural mimicry of the TYMV TLS with canonical tRNA. **(a)** Efficient valylation of the TLS by yeast ValRS. Loss of catalytic activity correspond to ratios $(k_{cat}/K_m)_{cognate}/(k_{cat}/K_m)_{non\ cognate}$ (adapted from Giegé *et al* 1978). **(b)** Folding of the TLS in a L-shaped conformation with emphasis on the pseudo-knotted structure of the valine accepting end (adapted from Dumas *et al* 1987).

knowledge on tRNA allowed proposing a computer model of the TYMV TLS (figure 4) that perfectly mimics the L-shape of a canonical tRNA (Dumas *et al* 1987). Early and recent information of TLSs are reviewed in Fechter *et al* (2001). They have to be taken into account for understanding the molecular strategy used by aaRSs to recognize and aminoacylate tRNAs.

9. From biochemistry to gene technology

The period covered by this survey was the flourishing era of biochemistry enriched by enzymology, genetics, physical chemistry and early crystallography. The advent of gene technologies was a turning point for the field at the end of the seventies. Paul Schimmel at MIT applied these nascent methods to study the large *E. coli* AlaRS. The remarkable finding was the linear arrangement of the AlaRS gene in specific domains that *may account for the diverse sizes of the aaRSs* (Jasin *et al* 1983). This modularity correlates with the functional steps of the tRNA aminoacylation process, with adenylate formation sustained by a ~400 amino acid N-terminal fragment, and tRNA aminoacylation requiring an additional domain of 76 amino acids. The remaining part of the 875 amino acid long sequence is required for tetramer assembly with a oligomerization site located near the C-terminus of the monomer. Likewise in England, systematic site directed mutagenesis was employed to dissect the structure and activity of the TyrRS from *B. stearothermophilus* (Fersht 1987) and to propose an experiment-based model of the interaction of $tRNA^{Tyr}$ with TyrRS where the tRNA spans over the two subunits of the protein (Bedouelle and Winter 1986).

10. Early general conclusions

Initial investigations on tRNA recognition by aaRSs were essentially conducted on isolated systems often considered as idiosyncratic entities. A wealth of results accumulated, often seemingly contradictory (e.g. charging versus mischarging, poor interaction specificity versus kinetic specificity) or of unexpected nature (e.g. activity of reshaped tRNAs, chargeability of tDNAs or TLSs), and attempts to establish a robust paradigm taking into account the ensemble of available information were scarce. The diversity of aaRS properties and the relative lack of information on aaRS and tRNA sequences and 3D structures could explain this fact. Nevertheless testable hypothesis were proposed and several general conclusions could be drawn on the basis of the results outlined in this survey:

- Despite structural diversity, aaRSs catalyze tRNA aminoacylation in a two-step process. This does not preclude system specific idiosyncratic mechanistic differences.
- Interaction between aaRS and tRNA takes place through discrete steps involving scattered recognition sites. The recognition process is accompanied by conformational changes in both macromolecules with best mutual adaptation for the cognate complexes. Kinetic effects and relative concentrations of tRNA and aaRS are important for tuning aminoacylation specificity.
- Primary recognition sites exist in the amino acid acceptor stem as illustrated with the alanylation of a reconstituted $tRNA^{Ala}$ acceptor stem (Chambers 1971). They exist also in anticodons as first proposed by Lev Kisselev (reviewed by Kisselev 1985). Altogether major interaction with aaRSs would be *along and around the diagonal side of the tRNA structure* (Rich and Schimmel 1977).
- tRNA modifications are seldom essential for aminoacylation and crucial recognition elements can be single tRNA bases. Identity determinants as characterized later and defined presently (Giegé *et al* 1998b), include residues suggested by the early investigations or are located within tRNA areas that were found important for aminoacylation.
- The ribose-phosphate backbone and the canonical cloverleaf folding of tRNA are not absolutely required for aminoacylation. The tRNA structure can be reshaped without loosing aminoacylation capacity and this occurred even in the course of evolution, since TLS structures based on alternate folding principles exist in nature. In other words mimicry of tRNA, as conceptualized later (Giegé *et al* 1998a), was discovered early.

Figure 5. Jean-Pierre Ebel (1922–1990).

These overall conclusions from the eighties are globally valid today. With the explosion of functional and structural genomic data and exploration of tRNA identity by modern technologies, they became refined and more robust. As a final remark, I would like to point out that many of the early results that appeared bizarre or just not understood gain meaning when examined with the present knowledge (for recent readings see e.g. Giegé *et al* 1998b; Beuning and Musier-Forsyth 1999; Giegé and Frugier 2003; Ibba *et al* 2005; Ryckelynck *et al* 2005; Xie and Schultz 2005).

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I dedicate this survey to the memory of Jean-Pierre Ebel (figure 5) who initiated the field in Strasbourg. I thank also my past and present coworkers at IBMC and many colleagues around the world, who contributed to the understanding of tRNA recognition by aminoacyl-tRNA synthetases.

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