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Author's Review

Croatian School on tRNA and Aminoacyl-tRNA Synthetases*

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Studies on transfer RNAs, as amino acid adapters in protein biosynthesis, and aminoacyl-tRNA synthetases, as enzymes that catalyze specific aminoacylation reactions, started in Croatia in the laboratory of Professor Željko Kućan in the early 1970s. This paper is an overview of the approaches and ideas that kept many of his collaborators on the same track for several decades.

INTRODUCTION

Starting with RNA

Nucleic acids structure, biochemistry and interactions with proteins have always been among the main research interests of Professor Željko Kućan. After his first important discovery that irradiated bacteria degrade their genetic material – DNA, which resulted in several excellent papers,[1–5] he visited the laboratory of the Nobel Prize laureate Fritz Lipmann, where he got interested in another nucleic acid – RNA. As Kućan mentioned in one of his reviews,[6] his arrival to Lipmann's laboratory at Rockefeller Institute, New York, in the late 1961 was just in time to witness the classical experiment of Francois Chapeville demonstrating the adaptor role of »soluble« RNA in the translation of genetic message.[7] That experiment was crucial for establishing the fundamental concept that the specificity in protein biosynthesis is primarily governed by the loading of every amino acid onto a cognate soluble RNA by an enzyme specific to that amino acid. The RNAs involved began to be known as transfer RNAs (tRNAs), and the activating enzymes as aminoacyl-tRNA synthetases (aaRSs).[8–12]

The Role of tRNA in Protein Biosynthesis

Their key function in protein biosynthesis and their many desirable properties (size, structure, availability, participation in a variety of interactions) have made tRNAs an attractive subject of research for almost five decades since their discovery. Still, the sixties and the seventies of the last century were probably the most exciting period of tRNA research ever. The first primary

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structure was determined by R. W. Holley *et al*..[13] Its striking feature was a high content of modified nucleotides. However, the tertiary structure discovery awaited another decade and went through the competition between crystallographers and other biophysicists using spectroscopic methods (NMR, neutron scattering). Finally, yeast $tRNA^{Phe}$, in two crystal forms was published in 1974 and remained for long the only source of knowledge of tRNA structures.[14,15] To this day, obtaining crystals of tRNA that diffract well is not a trivial undertaking! Many tRNA studies performed in the seventies aimed to answer the following questions: (i) Does the same, or highly similar, structure as in crystallized yeast $tRNA^{Phe}$, exist in other tRNAs involved in polypeptide chain elongation? (ii) Is the structure in solution similar to that in crystal? (iii) What is the mechanism of tRNA recognition by other macromolecules (proteins or nucleic acids)? (iv) Does tRNA undergo conformational changes while functioning in protein biosynthesis?

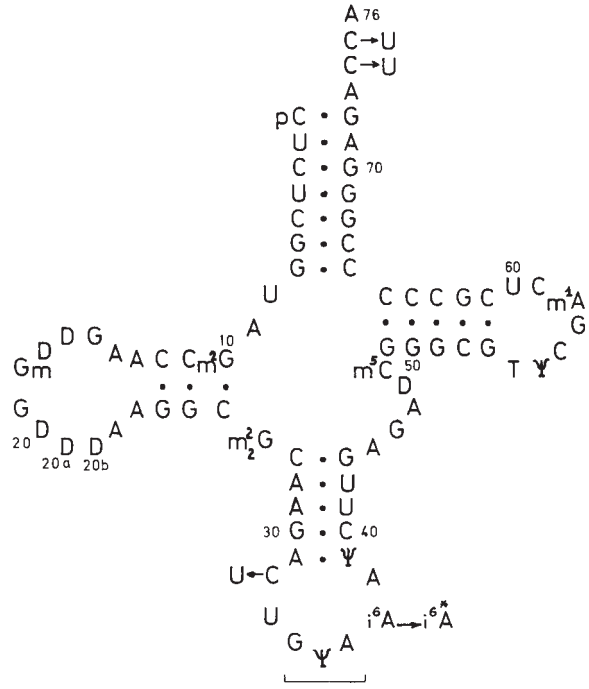

Figure 1. »Cloverleaf« structure of yeast $tRNA^{Tyr}$. The numbering system corresponds to the 76 nucleotides long yeast $tRNA^{Phe}$, so that additional two nucleotides appear as D20a and D20b. The anticodon, GΨA, is underlined at the bottom of the structure. Cytosine residues highly reactive in the bisulfite reaction are marked C→U. N^6-(Δ^2-isopentenyl)-adenosine (i^6A) at position 37 can be spin-labeled as described in the text.

STUDIES ON TRANSFER RNAs AND AMINOACYL-tRNA SYNTHETASES

tRNA Recognition by Aminoacyl-tRNA Synthetases and the Beginning of tRNA Investigation in Croatia

tRNA entered Kućan's primary research field during his collaboration with Robert Chambers, at New York University, School of Medicine, whose laboratory he joined in 1969. Knowing that the specific recognition of RNA sequences by proteins plays an important role in gene expression and protein biosynthesis, much as the specific recognition of DNA sequences, they were interested in elucidating the molecular bases by which the aminoacyl-tRNA synthetases select the cognate tRNAs with such a high degree of precision and discriminate between noncognate tRNA species. By producing a series of mutants of yeast $tRNA^{Tyr}$ (Figure 1), they tried to define which nucleotides were important for specific recognition by cognate tyrosyl-tRNA synthetase(TyrRS).[16,17] Those constituents are called yeast $tRNA^{Tyr}$ identity elements.

Tyrosine specific aminoacylation system was later brought to Zagreb, together with essential experimental methods required to study RNA : protein interactions. This marked the beginning of the »Croatian tRNA school« started at Ruđer Bošković Institute in 1973. The studies were later extended to the Faculty of Science, University of Zagreb, and have continued to these days.

Several general observations relevant to those studies are important. The experiments were done with purified, well characterized macromolecules (tRNAs and aaRS) of defined structure, and certainly deserved to be called molecular biology! Actually, for a long time, the tRNA system was the only one in which the RNA and the protein components were available in homogenous form for studies of RNA : protein recognitions.[18] The interest in correlating structure-function relationship prompted Kućan and collaborators to use structurally altered tRNAs generated by the bisulfite treatment. The bisulfite causes deamination of accessible cytosines and converts them into uracils, producing macromolecules with a certain number of natural bases exchanged into others (Figure 1). That was one of the first attempts of *in vitro* mutagenesis done in Croatian laboratories. Although the introduction of mutations was not completely site specific, but rather region specific, by conducting the reaction under different ionic conditions, tRNAs comprising higher or lower contents of alterations were produced. Those could be verified by nucleoside analysis after chromatographic separation of tRNA fragments, followed by hydrolysis. The second pioneering approach used to elucidate $tRNA^{Tyr}$: TyrRS recognition was footprinting of the RNA : enzyme complex,[19] which revealed the regions of tRNA making direct contact with the synthetase. In conclusion, those experiments showed that $tRNA^{Tyr}$ determinants important for the interaction with the synthetase and efficient aminoacylation were located in the aminoacceptor and anticodon regions.[16,17,19] Interestingly, essentially the same result was published by others a quarter of a century later.[20]

Studies of tRNA Conformational Changes by ESR-spectroscopy

Each tRNA molecule interacts with several small ions and macromolecules while functioning in protein biosynthesis.[21] As stated above, recognition by the aaRSs is a highly specific process, which together with specific codon : anticodon interactions ensures the accuracy of protein synthesis.[22] In some cases, like during the interaction with terminal nucleotidyl transferase (»CCA-enzyme«), the elongation factor Tu, the ribosome, and possibly while interacting with some of tRNA-processing (modifying) enzymes, the proteins must recognize general features common to all tRNA molecules. These interactions with small molecules or macromolecules are sometimes accompanied by conformational changes of tRNA, which can be monitored by biophysical methods. Electron spin resonance (ESR) seemed to be suitable for tracing region specific conformational changes, providing that tRNA was covalently labeled by a paramagnetic molecule (a spin-labeling probe, which is usually a nitroxide derivative comprising an unpaired electron) or paramagnetic ions (*e.g.,* Mn^{2+}) were bound.[23] Since the interest in collaborative research came from the neighboring laboratory at the Ruđer Bošković Institute, along with the offer to provide necessary knowledge and instrumentation, Kućan gladly accepted to participate in biophysical studies for several following years. However, spin-labeling of tRNA at specific sites was limited mostly to reactions of suitable nitroxide derivatives with reactive sulfur-containing nucleotides, modified uridine, or exposed uridine in the anticodon loop.[24,25] In order to study a number of macromolecular contacts that could result in local structural rearrangements, Kućan was interested in specific spin-labeling of yeast $tRNA^{Tyr}$ in the anticodon region.[26] The only conveniently positioned nucleotide with specifically reactive chemical groups was N^6-(A^2-isopentenyl)-adenosine (i^6A) (Figure 1), the hypermodified nucleotide in the partly stacked region, adjacent to the 3'-anticodon base. To ensure the specificity and the completeness of spin-labeling, first a synthesized nucleotide, and then the whole tRNA, was iodinated and then the iodine was replaced by the nitoxide spin-labeling probe. Many control experiments confirmed the specific attachment of the probe exclusively to i^6A. ESR-spectra of spin-labeled $tRNA^{Tyr}$ were recorded at various temperatures in the controlled ionic environment. The rotational correlation time, τ_c, plotted as a function of temperature, revealed that a change in the activation energy for spin-label motion occurred at the critical temperature, t_{cr} (Figure 2). That kink in the Arrhenius plot, reflecting a change in motional freedom of covalently attached spin label, was interpreted as the pre-melting conformational change of the anticodon region, since t_{cr} depended on the Mg^{2+} concentration and ionic strength, and was considerably below temperatures

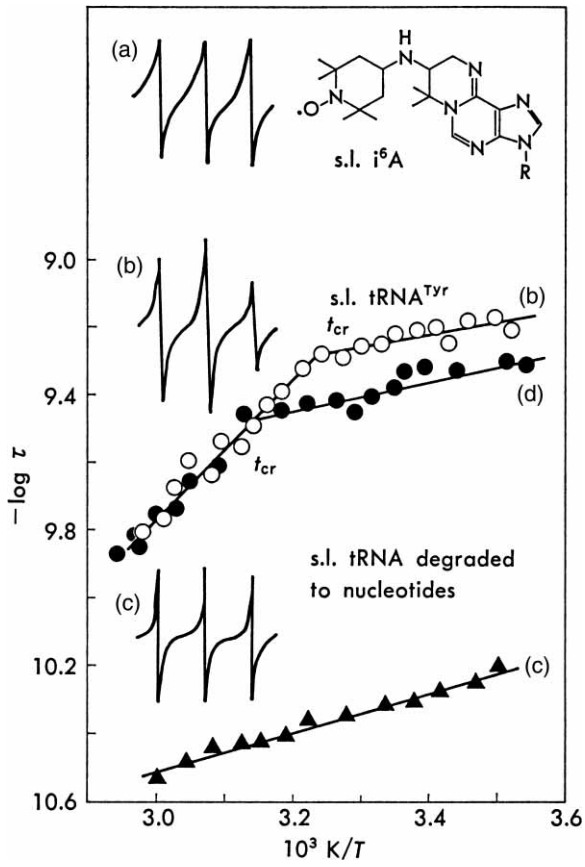

Figure 2. Arrhenius plot of -log τ_c vs. T^{-1} for spin-labeled $tRNA^{Tyr}$ with three (O) and five (●) bound spermine molecules, and for the same tRNA degraded to mononucleotides (▲). ESR spectra for spin-labeled i^6A (the structure is also shown; R, ribose), spin-labeled $tRNA^{Tyr}$, and spin-labeled $tRNA^{Tyr}$ degraded to nucleotides are inserted. The illustration was taken from Ref. 6.

needed to induce partial melting of the tRNA structure. The most likely molecular event to occur was the rearrangement of stacking interactions, or a change in the tilt of bases along the vertical axis. In a series of experiments, bound spin-label was successfully used to monitor temperature induced tRNA denaturation in the presence of various stabilizing agents,[19,26–32] including polycation spermine. The same approach was used later on for tracing the conformational changes during the interaction with other macromolecules.

The Role of Spermine in Stabilization of tRNA Structure and in Protein Biosynthesis

Polycations such as spermine and spermidine occur in all living cells. They are known to stabilize the tRNA structure and influence its activity.[33–36] Spermine was used to obtain $tRNA^{Phe}$ crystals for structural studies: two spermine molecules were indeed identified in specific positions of the $tRNA^{Phe}$ structure. Although it was known that a few spermine molecules bind to tRNA in solution, no specific data on this binding was available.

All attempts to locate the sites for spermine and spermidine binding to tRNA in solution have yielded only the information about the consequences, rather than the nature of this binding. With the spin-labeled $tRNA^{Tyr}$ available, which could provide information about the anticodon environment, Kućan and collaborators investigated the effect of gradually adding spermine to tRNA. They concluded that the binding of spermine was strong, specific and sequential, that is, after the binding of the first four spermine molecules, the next one was specifically directed into such a position in the $tRNA^{Tyr}$ molecule that it enhanced the stability of the anticodon region. Besides, bound spermine created new, strong and specific binding sites for divalent cations.[27,28]

In addition, spermine facilitates many biological processes. As noticed by Kućan and collaborators, it increased the rate of aminoacylation[30,31] and stimulated the codon-directed binding of tRNA to the ribosomal A and P sites. As a consequence, spermine prevented some erroneous tRNA-ribosome interactions.[31,32]

Conformational Changes of tRNA during Macromolecular Interactions with aaRS, EF-Tu, Oligonucleotides and Ribosome

Studies of various free tRNAs in solution by a number of chemical and physical methods suggest that all tRNAs have the same general three-dimensional structure, which is likely to be similar or identical to that found in the crystal form. The question of whether the conformation undergoes changes when tRNA interacts with other macromolecular components of protein-synthesizing machinery had been the subject of numerous studies, and suitable methods for detecting such changes had been searched for. General physical methods, high-resolution NMR and laser-excited Raman spectroscopy failed to detect small conformational changes in tRNA, such as those caused by aminoacylation.[37,38] Although such changes were reported to be detected by more specific probes, which monitored specific regions of tRNA, such as steroid or oligonucleotide binding,[39] a conformational difference caused by aminoacylation of yeast $tRNA^{Tyr}$ was not detected by ESR. But, if $tRNA^{Tyr}$ does not undergo a major conformational change in aminoacylation, this does not rule out such a change at a later stage in translation.[19] Kućan and collaborators investigated whether spin-label can sense the binding of synthetase. Although the binding of TyrRS to $tRNA^{Tyr}$ protects the anticodon G located at the 5'-side of the anticodon region against an attack by ribonuclease T_1, as described above,[19] the rotational correlation time of the spin-label attached adjacent to 3'-side of the anticodon remained unchanged.[40] This shows that the synthetase stretches over a considerable distance along the tRNA molecule, but interacts only with the 5'-side of the anticodon loop.

Aminoacylated spin-labeled tRNA, carrying a bulky probe at the i^6A position, formed a stable complex with the elongation factor Tu (EF-Tu × GTP), which resulted in spin label immobilization and consequently increased τ_c by at least 30 % over the control (Tyr-$tRNA^{Tyr}$ in the presence of EF-Tu × GDP).[40] Our finding that the sensitivity of the anticodon G-34 in Tyr-$tRNA^{Tyr}$ to RNase T_1 digestion was not changed by the ternary complex formation was in agreement with previous studies that EF-Tu does not interact with the anticodon region.[41] However, although the protein does not interact directly with the anticodon loop of Tyr-$tRNA^{Tyr}$, nevertheless it did appear to change the loop conformation. The immobilization was not due to increased molecular mass of the complex, because another type of complex, formed between yeast $tRNA^{Tyr}$ and *E. coli* $tRNA^{Val}$ having complementary anticodons, did not influence the spin-label motion.[40]

Involvement of the 3'-side of the anticodon loop of yeast $tRNA^{Tyr}$ in mRNA-free binding to ribosomes was also monitored by ESR.[42] The ESR spectrum of free $tRNA^{Tyr}$, characteristic of a rapidly tumbling nitroxide, changed to a spectrum with extensively broadened lines in the ribosome-tRNA complex. The original spectrum could be restored upon long incubations of the complex in the excess of unlabeled tRNA. ESR spectra suggested that the spin-label motion was drastically perturbed though not completely blocked in the ribosome-$tRNA^{Tyr}$ complex. Since ESR spectra of a spin-label attached to the opposite, *i.e.*, 5'-side of the anticodon loop, were only slightly perturbed as reported by Rodriguez *et al.*[43] indicated that the two sides of the anticodon loop face different environments when bound to the P site, which is in ageement with recent crystallographic studies.

The Stoichiometry of tRNA : Synthetase Complexes

The stoichiometry of TyrRS complexes with tRNA was of considerable interest for a long time, especially because TyrRS is a homodimeric enzyme, unlike other class I synthetases, which are predominantly monomers. However, it is rather unusual that dimeric TyrRS from yeast can form several types of complexes with its cognate $tRNA^{Tyr}$ molecules. Using the electrophoretic gel-shift assay, Rubelj *et al.*[44] detected a rather nonspecific complex, of assigned composition $TyrRS_2 \bullet (tRNA^{Tyr})_1$. At higher $tRNA^{Tyr}$/enzyme ratios, a faster moving complex was formed, approaching saturation at $tRNA^{Tyr}$ / enzyme = 1, *i.e.*, the assigned composition was TyrRS • $(tRNA^{Tyr})_1$. By changing the experimental conditions, I. Gruić Sovulj *et al.*[45] identified both TyrRS$(tRNA^{Tyr})_1$ and TyrRS • $(tRNA^{Tyr})_2$ complexes, which were stable enough to be detected by gel electrophoresis and mass spectrometry (MALDI MS). Based on the results of kinetic experiments and macromolecular composition studies, a simple model was made

in which yeast TyrRS exists as a symmetrical dimer, possessing two identical active sites, both capable of catalyzing the formation of tyrosyl adenylate, but which preferentially binds one molecule of $tRNA^{Tyr}$. [46]

A Personal View: What have we, several generations of Kućan's collaborators, learned working on the tyrosine specific aminoacylation system and what interesting characteristics of it have escaped our attention?

Many of us made a contribution to these well guided studies, and enjoyed the benefit of elucidating interesting phenomena in the field of biochemistry, biophysics and molecular biology of nucleic acids and protein synthesis. Looking back, I would have liked us to have been more successful in cloning the gene for *Saccharomyces cerevisiae* TyrRS, which was finally done by others.[47] Having had that gene in hands on time (*i.e.,* in the late 1980s) would have enabled us to generate the TyrRS overexpressing system, introduce desirable mutations and produce structurally altered variants. Recently, unique features of the tyrosine specific aminoacylation system have been widely exploited in biotechnology for construction of orthogonal tRNA : synthetase pairs suitable for site-specific incorporation of non-canonical amino acids into the proteins.[48,49] Although our results have certainly added to the expansion of that attractive concept, we were not ready to carry on comparably. However, following many Kućan's ideas as an important driving force, we have gradually switched to another intriguing aminoacylation system and continued the studies that led to further elucidation of the role of tRNA and aminoacyl-tRNA synthetases in protein synthesis.[50] Fast development of recombinant DNA techniques finally enabled us to combine *in vivo* and *in vitro* approaches. Our long-standing goal has been to examine and correlate the mechanisms of seryl-tRNA formation in different organisms and in different cellular compartments (cytosol, mitichondria and chloroplasts), with specific emphasis on the accuracy of serine incorporation into the proteins.

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SAŽETAK

Hrvatska škola tRNA i aminoacil-tRNA-sintetaza

Ivana Weygand-Đurašević

Istraživanje transfer RNA, kao adaptora u biosintezi proteina te aminoacil-tRNA-sintetaza, enzima koji kataliziraju aminoacilaciju tRNA, započelo je u Hrvatskoj početkom sedemdesetih, u laboratoriju profesora Kućana. Tijekom godina, ovom se problematikom bavilo niz njegovih suradnika. Ovo je pregled naših glavnih ciljeva, pristupa i rezultata.