The crystal structure of tRNA

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1. Introduction

Because of its importance in decoding, its small size (~25 000 Da or 73-93 nucleotides) and its availability, tRNA became a favoured example of a nucleic acid molecule in the 1960s for both sequence determination and 3D-structural elucidation. The first primary structural determination of a tRNA, that of yeast alanine tRNA by Robert Holley's group at Cornell University (lthaca, NY, USA) in 1965, earned Holley a share in the 1968 Nobel Prize for Physiology or Medicine. By contrast, despite its great importance for establishing the foundation of structural nucleic acid chemistry at atomic resolution and for giving insight into the mechanism of protein synthesis and transfer of genetic information, the determination of the 3D structure of the tRNA (in 1974) has not been recognized with such distinction. The topic certainly was confused by the intense competition among many research groups that ended in strong rivalry between the two groups that first solved successfully the crystal structure: these being led by Aaron Klug at the MRC Laboratory of Molecular Biology (LMB), Cambridge, UK, and Alex Rich at MIT, Department of Biology, Cambridge, MA, USA. Indeed, in December 1968 the *New Scientist* wrote an article called "The race for transfer RNA" reporting that any one of several research groups could bring off one of the biggest coups in 'classical' molecular biology by obtaining crystals of tRNA large enough and ordered enough to allow a structural determination by X-ray crystallography (Chedd 1968).

How did I become involved (Clark 2001, 2005)?

2. Decoding and sequencing

After a PhD working on the chemistry of phosphinositides with Dan Brown in the Department of Organic Chemistry at Cambridge University, UK (1961), I learned biochemistry during two postdoctoral periods: one with Jack Buchanan at MIT's Division of Biochemistry (1961-1962) and the other with Marshall Nirenberg at the NIH's National Heart Institute, Bethesda, MD, USA (1962–1964). It was in the laboratory of Nirenberg that he and Heinrich Matthaei synthesized polyPhe, directed by poly U, using a cell-free system, thus deciphering the first codon for an amino acid and starting the race for the elucidation of the genetic code. The experience I gained in Nirenberg's laboratory led, in 1964, to a staff position at the MRC LMB's Division of Molecular Genetics, then co-headed by Francis Crick and Sydney Brenner. I returned to Cambridge with alacrity as the Americans thought that the coding problem was essentially solved. However, researchers in Cambridge realized that there should be signals for both initiation and termination of protein synthesis. Therefore, I started working on nonsense codon (a codon specifying chain termination) elucidation and separation of suppressor tRNAs (tRNAs which could decode nonsense codons) with John Smith and Brenner. However, my attention was soon captured by the 'strange' tRNA, shown to be formylmethionyl-tRNA (fMet-tRNA), recently discovered by Kjeld Marcker and Fred Sanger. I was able to put my experience of decoding and cell-free protein synthesis to good use in a close collaboration with Marcker over the next six years.

My task was to purify triplet oligonucleotides and nonradioactive initiator tRNA with the aim of elucidating the codons for initiation. Our work showed that fMet-tRNA was a prokaryotic initiator because, on analysis, fMet was found at the N-terminal end of polypeptides synthesized under the direction of synthetic and natural mRNAs.

Because Marcker was in Sanger's division at the LMB, he was conversant with the new rapid tRNA sequencing methods developed by Sanger, George Brownlee and Bart Barrell. A severe sequencing problem with tRNAs was the high occurrence of modified bases that could only sometimes be detected by the radioactive method. Thus Marcker's group and my group emphasized different aspects of tRNA biochemistry. His group was concerned with the rapid sequencing of methionine tRNAs, whereas my group was developing large-scale purification methods to allow identification of any modified bases in specific tRNAs, which

required milligrams of purified tRNA species. Fortunately, the sequencing of the $tRNA^{Met}$ species proceeded quickly as this initiator tRNA did not contain many modified bases. After a few months, the primary structure was determined. However, we were still in the process of developing large-scale purification methods for tRNAs, especially for the initiator tRNA. We were becoming less interested in identifying new modified bases and more interested in understanding the molecular mechanism of protein biosynthesis. With the splendid structurally inclined atmosphere of the LMB in the mid-1960s we knew that it would be necessary to determine 3D structures of protein synthesis components. This area has spurred me on for the past 30 years.

3. Purification and crystallization

Actually, it was in the Autumn of 1966 that we began to work on the project of separating different tRNA species on a large enough scale to obtain a single species of methionine tRNA with the aim of crystallizing this tRNA as an intact molecule, or even as a fragment, for crystallographic analysis and tertiary structure determination. The crystallization project had, in particular, the enthusiastic support of Crick.

The large-scale purification of pure species of tRNA was no mean feat in the mid-1960s, particularly considering that >50 tRNA species with similar properties needed to be separated. We noted that the method of countercurrent distribution (CCD) used by Holley's group in the purification of $tRNA^{Ala}$ separated two methionine tRNAs. With my chemist's background I soon set up this method in the laboratory. Marcker and I then showed (Clark and Marcker 1966) that the two types of $tRNA^{Met}$ were the initiator $tRNA^{Met}$ and the elongator $tRNA_m^{Metf}$ (figure 1). The large-scale purification of the methionine tRNAs was published in 1969 in collaboration with Bhupendra P (Doc) Doctor, Brian Wayman, Suzanne Cory and Philip Rudland (Doctor *et al* 1969). We produced >100 mg of pure initiator tRNA from 10 g of unfractionated tRNA.

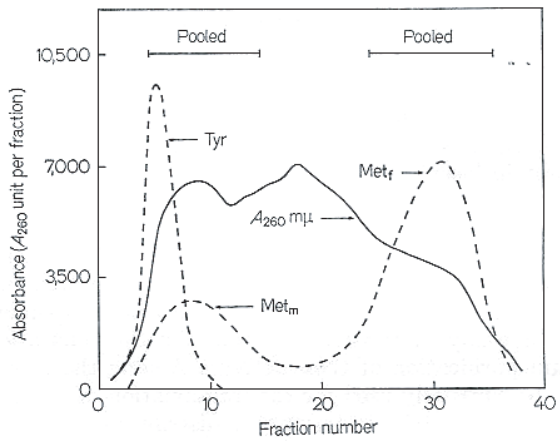

Figure 1. Counter current distribution of *E. coli* tRNA.

My initial crystallization attempts did not produce identifiable crystals of tRNA but rather crystals of various salts. My simple techniques involved the precipitation of tRNA with standard oligonucleotide- or nucleotide precipitating agents such as ethanol, acetone or dioxan. My interest was faltering when, in the early Spring of 1968, a procedure by a German postdoctoral worker, Hasko Paradies, purporting to have crystallized yeast $tRNA^{Ser}$, was given to me by Ken Holmes at LMB. These claims were never verified by X-ray data, and neither ourselves nor Paradies successfully used this method to crystallize the initiator tRNA. It was discovered later that his published tRNA crystals were, in fact, protein (Hendrikson *et al* 1983).

Nevertheless, his crystallization claim stimulated my group to put more systematic efforts into crystallization of the initiator tRNA. At the same time, our group and potential collaborators got together to discuss more systematic approaches. Indeed, I have notes from these days that our discussions with crystallographers such as John Finch, Tom Steitz and David Blow in the laboratory were continued with Holmes, his colleague Shirley Morris and my PhD student Philip Rudland, in the Prince Regent pub.

Meanwhile, my systematic crystallization attempts using specific salt forms of tRNA succeeded. At first, I obtained small spherical crystal aggregates called spherulites using initiator tRNA. Morris also obtained spherulites using a sample of impure $tRNA^{Lys}$. A solution of the purified uncharged $tRNA^{Metf}$ as a mixed magnesium potassium salt was dialysed against water to remove the excess of inorganic cations. This was then lyophilized and redissolved in water to make a 2-5% solution that was then equilibrated with an atmosphere of dioxan (35% v/v with water) in a desiccator. This procedure produced spherulites in ~16 h, and these spherulites were indicative of disordered crystalline forrns, thereby strongly suggesting that single crystals were possible.

A microscopic study of the spherulites revealed that they became more ordered in time, although the overall shape of the spherulite was maintained. Microcrystals could also be directly produced if the tRNA solution was equilibrated for several days with a lower concentration of dioxan. The microcrystals disappeared in 30 min when a trace of ribonuclease A was added to the mother liquor, indicating the ribonucleic acid nature of the microcrystals. These microcrystals, although far too small for single-crystal analysis, did allow powder X-ray photographs to be taken and analysed by Holmes and Klug. The analysis yielded probable unit cell dimensions and space group identifying crystal symmetry characteristics, thus giving evidence that the microcrystals were true 3D crystals and not liquid

crystals. Thus, the first documented crystals of tRNA were authenticated and published in *Nature* in September 1968 (Clark *et al* 1968). Further progress now needed large single crystals of a pure tRNA species.

Following the demonstration that a tRNA molecule could be crystallized, the tRNA field became very competitive. The Oak Ridge National Laboratory (Oak Ridge, Tenn, USA), under the aegis of the US Atomic Energy Commission, developed new chromatography methods based on reversed phase chromatography, purified separate species of tRNA on a large scale and offered them to anyone interested in crystallization.

4. Competition

In the Autumn of 1968, the race for tRNA structure determination involved Hans Zachau's group in Munich working on yeast $tRNA^{Ser}$, Fritz Cramer's group in Göttingen collaborating with Holmes's group (by that time moved to Heidelberg) on yeast $tRNA^{Phe}$, a collaboration between the late Bob Bock's group in Madison and the late Paul Sigler's in Chicago, a collaboration between Alex Rich at MIT and Don Caspar at the Boston Children's Cancer Research Foundation, and Jacques Fresco's group in Princeton. In the UK we also had competition from the Department of Biophysics at King's College, London.

After publication of the Cambridge crystallization conditions I was soon able to obtain single crystals of both $tRNA^{Val}$ and the initiator tRNA. With the advent of single crystals of tRNA 50 x 20 x 20 μm in dimensions, Crick encouraged me to join forces with Aaron Klug's crystallography group, thus starting a friendly and productive collaboration. The crystals were extremely fragile and sensitive to environmental conditions so I grew them in quartz capillary tubes, which could be sealed and used directly for X-ray analysis. That we collected any data at all was a result of the persistence of John Finch, who worked heroically in the cold room trying to obtain good resolution pictures from the crystals.

By the end of 1968, there were already publications in *Science* and *Nature* of single tRNA crystals from the groups of Cramer, Bock, Rich and Fresco. Another article in the *New Scientist* in January 1969 described the competitive nature of the field and made the point that the 3D structure of tRNA would not be solved in the near future because of the various problems several experienced groups had in producing and handling a variety of tRNA crystals. Indeed, Arnold Hampel and Bock grew yeast $tRNA^{Phe}$ crystals as large as 0.5 x 2.0 mm while, in Cambridge, I was obtaining only small crystals 0.15-0.2 mm in diameter. Furthermore, Rich and Sung-Hou Kim at MIT grew crystals of the initiator *Escherichia coli* tRNA obtained from Oak Ridge from a chloroform-water mixture. These crystals were as large as 1.0 x 1.7 x 0.6 mm. However, none of these publications showed that the crystals were 'crystallographer's' crystals capable of giving high resolution X-ray data. We were soon in the same situation as the other laboratories with respect to crystal size by obtaining a variety of large single crystals, as my group was purifying its own tRNAs and producing an increasing number of tRNA species. Unfortunately, things then became more and more depressing. First, our lead in tRNA production was wiped out by the US National Laboratory at Oak Ridge, and then our crystals proved to be somewhat disordered, giving resolution no better than 7 Å. I called these crystals mere bags of water.

Thus, there was a lull in our crystallographic data production for three years. However, the unstinting support of the British Medical Research Council provided space, people, equipment and materials to help us try to solve the tRNA structure and win the race. My group grew to include five research assistants (Bill Whybrow, Bob Coulson, Ray Brown, Daniela Rhodes and Margaret Prentice) and several PhD students, who purified tRNAs and helped with the crystallization, and postdoctoral worker Jane Ladner from Caltech, who soon converted from an NMR spectroscopist to a crystallographer.

At the later stages of tRNA production and crystallographic analyses, there was more and more structural collaboration with Klug's group, in particular with John Finch and a new postdoctoral worker Jon Robertus, for structural analysis.1t was to my chagrin that we never produced ordered crystals of initiator tRNA to better than ~10 Å resolution.

5. Structural determination

Because of the lack of production of good crystals from *E. coli* tRNAs for analysis, we started obtaining samples from outside the laboratory (e.g. from either Oak Ridge, Strasbourg or commercial sources). By 1972, we were able to obtain beautiful crystals of several *E. coli* tRNA species but still the resolution was no better than 7 Å (Brown *et al* 1972). We were therefore depressed to find that Kim and Rich had been able to obtain X-ray reflections to 3 Å resolution from yeast tRNAPhe bought from Boehringer Mannheim (Kim *et al* 1971).

We had also bought yeast $tRNA^{Phe}$ from Boehringer, which Ladner co-crystallized with *E. coli* $tRNA^{Val}$ to check whether tRNAs with complementary anticodons would crystallize better (figure 2). She obtained some extremely thin crystals by vapour diffusion in sitting drops. Despite their appearance, these crystals (X-rayed in 1972 by Brown) gave X-ray diffraction patterns that extended beyond 3 Å. It turned out that only the y $tRNA^{Phe}$ was present in the crystal. As these crystals possessed a smaller unit cell than that published by the MIT group, it gave us confidence that our crystals would ultimately give better diffraction data. Hence,

Figure 2. Large (~1 mm long), flat crystals of yeast RNA^{Phe}.

we switched to working with y tRNA^{Phe} and the race was really on (Ladner *et al* 1972).

In 1972, the solution of the y tRNA^{Phe} structure was still a tough problem. The y tRNA^{Phe} crystals that diffracted well were thin and twinned (i.e. composed of two intergrown crystals), making good data collection very difficult. Large crystals were grown by dialysis as used successfully in Ieuan Harris's group by a postdoctoral worker for *Bacillus stearothermophilus* protein crystallization. Indeed, Rhodes used this method to grow most of the large y tRNA^{Phe} crystals for data collection. Out of thousands of crystals few were suitable. Thus Finch and Brown took close to 500 precession photographs to find heavy atom derivatives.

Certainly, at the beginning of the race the MIT group had a short lead, particularly when they published in *Science* (Kim *et al* 1973) a 4 Å resolution structure of yeast tRNA^{Phe} with a general shape they called an 'L', which has stuck over the years. We went straight for a 3 Å resolution structure published in 1974 (Robertus *et al* 1974a). Ladner, Robertus and Finch were mainly involved with the data collection, and Rhodes and Brown with the production of the necessary crystals. My group thus concentrated on growing crystals and searching for compounds suitable for heavy atom derivatives. We also embarked on a study using chemical probing to show that the three-dimensional structure of y tRNA^{Phe} was the same in solution as in the crystal (Robertus *et al* 1974b). In particular, Rhodes and Brown were leading players for our part of the work, which exemplified a complementary integration of the work of Klug's crystallography group and my biochemistry group (figure 3).

The 3D structure of y tRNA^{Phe} in the monoclinic crystal form was solved to 3 Å resolution in Cambridge using isomorphous replacement with five heavy atom derivatives (Robertus *et al* 1974a). One of these, Pt, was located by chemical methods to a site in the anticodon loop and served to assign unambiguously residues in the electron density map (Rhodes *et al* 1974).

Why did the bitterness between MIT and Cambridge occur? As I mentioned previously, we were disheartened when the MIT group published in *Science* a 4 Å resolution structure that looked very similar to our structure with respect to the backbone tracing. Our horizon then brightened when

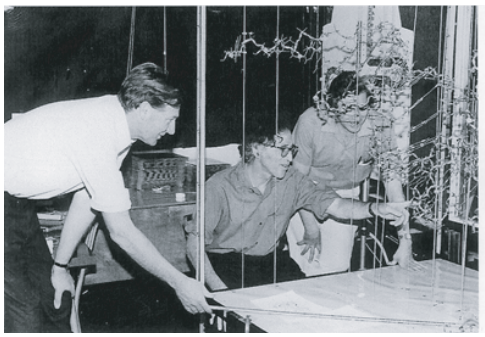

Figure 3. Aaron Klug, John Finch and Brian Clark discussing finer points of the skeletal model. built in Cambridge, UK, to represent the 3D structure of yeast tRNA^{phe}.

they published a 3 Å resolution structure in *Nature* (Suddath *et al* 1974) in the spring of 1974. At this time we were just finishing our structure determination and were astonished to find that, according to our structure, the MIT group had made a serious mistake in tracing the polynucleotide chain. We sailed on and wrote the paper for *Nature*, submitting it in June 1974. In addition, Robertus and I gave talks on the new structure at a Steenbock Symposium in Madison, also in June 1974.

At this time we reported detailed information on our structure. We called our structure a T because of the two long helices forming a T junction. These helices were composed of the acceptor stem that was stacked on the T$'\Psi$C-stem, and the anticodon stem stacked on the D-stem. We also described, for the first time, several base triplets that helped hold the structure together as well as some crucial non Watson-Crick base pairs that had not been presented by the MIT group. Our 'T' for tRNA structure did not catch on. Imagine our chagrin when the MIT group who had attended at the Steenbock Symposium wrote a revised paper on the 3 Å structure and submitted it to *Science*. This was published so quickly (within one month) that it came out two weeks before our *Nature* paper in August 1974.

6. Postscript

Further work by both the Cambridge and MIT groups pushed the resolution to 2.5 Å resolution and refined the structure. Interestingly, many investigations of tRNA structure in which the tRNA is either alone (naked) or in complexes with aminoacyl-tRNA synthetases and translational elongation factors, have encountered difficulties in reaching a higher resolution for tRNA structures. However, with modern technology, Rhodes, still at the LMB, and Peter Moore's group at Yale, gave recently re-examined y tRNA^{Phe} crystals (Rhodes' were ~20 years old) and obtained resolution structures better than 2 Å (figure 4), so it could be worthwhile to return to investigating the tRNAs we studied before.

Figure 4. My thanks are due to Daniel Rhodes for producing this 2 Å sturcture of $tRNA^{Phe}$ including ions and water.

Elucidation of the y $tRNA^{Phe}$ structure was a milestone in the development of our knowledge about the molecular mechanisms governing protein biosynthesis. It enabled various functional parts of the structure to be assigned and gave a framework for later work in the tRNA field, for example, determination of interaction sites between tRNA and aminoacyl-tRNA synthetases and elongation factors, and assignment of tRNA binding sites on the ribosomal subparticles. Moreover, the y $tRNA^{Phe}$ structure was the first nucleic acid structure to be solved to 3 Å and gave a picture of the ordered complexity of a folded RNA molecule – a complexity as great as that of a protein – and also gave details of metal ion binding sites. The types of nucleotide conformation, bonding and interactions, including metal-binding sites seen in the folded $tRNA^{Phe}$, have been shining examples for other RNA structures. An interesting by-product of the $tRNA^{Phe}$ structure was the first detailed chemical picture of a G–U base pair in a double helical stem, in which the pairing was that predicted by Crick's 'Wobble' hypothesis. It was also satisfying that our group was the first to confirm that the y $tRNA^{Phe}$ structure was the same in solution as in the crystal (Robertus *et al* 1974).

So, the 3D structural determination of a tRNA did not gain anyone a Nobel Prize, possibly because too many people were involved and the methods used turned out to be relatively standard, but it gave us enormous satisfaction at the time. And, I am happy to add that Aaron Klug, who led the crystallographic analysis at the LMB, did win the Nobel Prize in 1982 for many other important, innovative contributions to structural biology.

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