Reports

Single Crystals of Transfer RNA: An X-Ray Diffraction Study

Abstract. *Large single crystals of formylmethionyl transfer RNA have been prepared. An x-ray diffraction study shows that the material crystallizes in a hexagonal lattice with* a *equal to 170 angstroms,* c *equal to 234 angstroms. The diffraction pattern extends to spacings just under 20 angstroms at present. The crystals are heavily hydrated, containing 88 percent water.*

Transfer RNA (tRNA) is one of the central molecules of molecular biology. It functions at the point where the genetic information of the nucleic acids is being expressed through the synthesis of proteins. It has a specific role in protein synthesis, serving as an adaptor for fitting specific amino acids onto specific points along the polynucleotide messenger RNA (mRNA) when it is engaged in a ribosome. A great deal of effort has been spent in the isolation of specific tRNA's pure enough for determining the primary nucleotide sequence. The work of Holley and others has clearly indicated that these molecules have common features in their sequence of 70 to 80 nucleotides (*1*). Thus it is believed that the molecule can adopt a double-stranded configuration over selected regions; this has given rise to a variety of proposals concerning ways in which the molecule may fold to make a more globular unit. Although a number of physical chemical studies have been carried out on tRNA, it is clear that an x-ray diffraction investigation is necessary to determine the three-dimensional configuration of the molecule. Accordingly, several investigators have attempted to crystallize purified species of tRNA. Clark *et al.* (*2*) recently reported that they obtained an x-ray powder pattern from a microcrystalline preparation of *Escherichia coli* formylmethionyl tRNA. Our own efforts, independently initiated, have resulted in the production of large single crystals of *E. coli* formylmethionyl tRNA which are big enough for single-crystal x-ray diffraction investigations. We report now the results of this preliminary crystallographic investigation and describe the methods for preparing the crystals. These methods have resulted in the preparation of two different types of crystalline tRNA and may, therefore, be of more general interest.

Our investigation began approximately a year ago when B. Vold, using a column method (*3*), purified some yeast phenylalanyl tRNA. A number of attempts were made to obtain crystals from this material. We believed that although tRNA contained several regular double-helical regions, it was likely that there was considerable conformational variability in the molecule because of the existence of large regions which are single stranded, as, for example, in nonhelical loops, or in the central part of the conventional "cloverleaf" model of the molecule. Our attempts at crystallization were directed toward adding components to the medium which might tend to stabilize the configuration of the single-stranded portions of the molecule. Initial experiments carried out by Vold (*4*) began to appear successful in that single crystals of yeast phenylalanyl tRNA were obtained two times from a water-chloroform system. These experiments were generally carried out with low concentrations of the magnesium salt at neutral *p*H. The crystallization was effected by allowing the solution to concentrate in a desiccator over a drying agent. These initial crystals were somewhat unstable, and will be described more fully (*5*).

Somewhat better results were obtained, however, while working with purified formylmethionyl tRNA from *E. coli* B. We were fortunate to have obtained a sample of the material produced at Oak Ridge National Laboratory in a project jointly sponsored by them and the National Institutes of Health. This material was purified on reversed-phase column chromatography (*6*). Our sample was purified to the following extent; per absorbance unit at 260 nm, it had a methionine acceptance of 1328 pmole, a formyl acceptance of 1328 pmole, and 1333 pmole of terminal adenosine (*7*). The original starting solution had a tRNA concentration of 1.7 mg/ml in a mixture containing 0.6*M* NaCl, 0.01*M* $MgCl_2$, 0.01*M* tris (*p*H 7.0), and 0.002*M* $Na_2S_2O_3$. This solution was dialyzed at 5°C against a large volume of $7 \times 10^{-3}M$ $MgCl_2$. The tRNA solution in the dialysis bag was then concentrated sixfold by evaporation to a final concentration of approximately 10 mg/ml. The solution was then dialyzed against glass-distilled water for 6 hours, and then against $2 \times 10^{-3}M$ $MgCl_2$ overnight. Because of this extensive dialysis it is unlikely that cations other than magnesium were present. After this dialysis, a 0.2-ml sample was removed to a small glass test tube, and 0.2 ml of chloroform was added. This formed a two-phase system with chloroform at the bottom and an aqueous layer at the top. The test tube was placed in a desiccator containing a drying agent (calcium sulfate) and was allowed to stand in the cold room at 4°C for 2 weeks. This was very similar to the method first developed by Vold (*5*) for yeast phenylalanyl tRNA. The tubes were examined periodically and at the end of 2 weeks the chloroform phase had disappeared, and the remaining diminished aqueous phase was filled with a large number of clear crystals, easily visible to the naked eye. With a pocket magnifying glass it could be seen that the crystalline material was polyhedral, often taking the form of hexagonal prisms with pyramidal ends. The larger crystals were often 1.0 to 1.7 mm long and approximately 0.6 mm in width and thickness. The crystals are strongly birefringent and extinguish sharply (Fig. 1). During the 2-week period, the aqueous phase diminished considerably in volume, and the chloroform phase disappeared completely. Since crystal formation occurs subsequent to the departure of the chloroform phase, at the present time we do not know whether chloroform molecules are actually incorporated into the crystal lattice.

The process of crystal formation appears to be somewhat variable. In another preparation we observed that no crystals were present after the solution had been standing for 2 weeks. At that time, only the aqueous phase remained at a volume which had been reduced to less than a quarter of the initial volume. We estimate that the concentration of tRNA was then approximately 40 to 50 mg/ml (approximately $2 \times 10^{-3}M$ tRNA). However, on the

following day the material had crystallized, and the tube was now filled with a large number of well-formed crystals. In this instance it is likely that the solution had been supersaturated and that nucleation and crystallization had thus occurred very rapidly.

Our initial experiments were directed toward confirming that the crystals consisted of tRNA rather than magnesium chloride or other salts. The sheer volume of the crystalline preparation which tended to fill a large portion of the aqueous solution suggested that the material was indeed tRNA. Crystals were removed at 5°C with a transfer pipette and put into a hanging drop where they could be observed microscopically. Two such drops were observed to remain stable for a considerable period. An extremely small flake of lyophilized pancreatic ribonuclease was placed into one of these drops. Within 5 seconds the crystals within that droplet began to dissolve, whereas the untreated droplet was unchanged. Within a few minutes the treated crystals had completely disappeared, leaving a clear solution. This simple initial experiment convinced us that it was the RNA that had crystallized rather than an inorganic salt. This impression was reinforced by a study of other physical properties.

The crystals are extremely soft. During manipulation under the microscope, when we were planting crystals into capillaries, it was observed that the crystals readily deform. When poked with a small glass rod or even with a strip of filter paper, the crystals deformed and pieces were gouged out. This softness makes it unlikely that we were dealing with an inorganic salt.

The crystals are thermolabile. A crystal sitting in a droplet of mother liquor was placed inside a capillary and was taken out of the cold room and allowed to stand at room temperature. Within 10 to 20 minutes the crystal had dissolved. Because of this thermal sensitivity, the diffraction studies were carried out with samples maintained near 5°C, at which temperature they are stable. The temperature instability of the crystals could also be demonstrated in a graphic fashion. The test tube containing the crystals was placed on the stage of a microscope in order to obtain photographs similar to those shown in Fig. 1. If the test tube was held with the fingers for a prolonged period during the alignment of the microscope, the sharp corners of the crystals became round. When the crystals were allowed to cool overnight, they once again regained the sharp corners shown in the photographs.

The crystals are also sensitive to changes in *p*H. Crystals were obtained from a solution which was initially buffered to *p*H 7. However, if a crystal in a small droplet of mother liquor were placed in a glass capillary at 5°C, the crystal dissolved completely within a 1-hour period. We attributed this behavior to the alkalinity of the glass because the crystals were stable when placed in a quartz capillary. Similar behavior has been reported for some protein crystals as well as for crystals of poliomyelitis virus (*8*).

These tRNA crystals require mother liquor in order to maintain internal order. Thus, for example, when the crystal is allowed to dry in the air in the absence of mother liquor, the crystals change from being clear and become slightly cloudy and finally opaque. This transformation occurs even though the external morphology of the crystal does not change. However, x-ray diffraction examination of these crystals shows that they have become internally disordered in the absence of mother liquor.

A considerable period was spent becoming familiar with the physical properties of the tRNA crystals prior to attempting an x-ray diffraction investigation. For x-ray work, a crystal was placed in a thin-walled quartz capillary with a small droplet of mother liquor. After the capillary was sealed, the crystal was stable when maintained near 5°C. This temperature was maintained by placing the capillary in a brass holder mounted on the x-ray goniometer head which was cooled by a supply of circulating 5°C water. A Jarrell-Ash microfocus x-ray tube was used, and precession photographs were taken with a Supper precession camera having a 100-micron collimator. We used CuKα radiation, and the exposures ranged from 15 minutes to 2 hours. The crystals were sensitive to damage due to x-radiation. Small crystals with little mother liquor produced a diffraction pattern which decayed after 30 minutes in the beam. However, larger crystals with more mother liquor appeared to be considerably more stable.

Sample diffraction photographs of single crystals of formylmethionyl tRNA are illustrated in Figs. 2 to 4. The photographs have several features worth noting. The diffraction patterns extend out to a spacing just under 20 Å; reflections have not appeared at higher diffracting angles in photographs which have been exposed up to 2 hours. It is likely that more reflections will appear at somewhat smaller spacings for longer exposures, but it is clear that the diffraction patterns produced by these crystals do not have the high degree of order which has been observed in many protein crystals in which the resolution sometimes extends out to 1.5 Å. It is likely that this property is associated with the extremely high water content of the crystals. Analysis of the diffraction pattern shows that the tRNA molecules had crystallized in a hexagonal lattice with $a = 170$ Å and $c = 234$ Å. Absences occurred for the reflections $00l$ except where $l = 3n$. A diffraction photograph of one of the principal zones (Fig. 2) shows *mm* symmetry with mirror planes perpendicular to the a^* and the c^* axes. From these results we conclude that the space group is either $P6_2$ or $P6_222$ (or their enantiomorphs $P6_4$ and $P6_422$). The former space group has 6 asymmetric units in the unit cell, while the latter has 12. It is interesting that this unit cell and space group differ substantially from those reported as probable assignments by Clark *et al.* (*2*) for the microcrystalline spherulites prepared by dioxane precipitation which crystallized in an orthorhombic unit cell.

The density of the crystals was measured in a bromobenzene-xylene density gradient. The gradient was calibrated with droplets of cesium chloride solution of known densities. The measured crystal density was 1.123 g/cm^3. In calculating the volume fraction of water in the crystal we have used the partial specific volume of 0.505 cm^3/g which was measured for unfractionated yeast tRNA in a solution of *p*H 7.0 containing 5m*M* $MgCl_2$ (*9*). From this data we find that the crystal is 87.5 percent water by volume, which is considerably higher than the solvent content for most protein crystals which contain between 27 and 65 percent (*10*). An exception to this, however, is the highly hydrated crystal of tropomyosin which has a water content of 95 percent (*11*). It is our belief that the high content of water in these crystals is responsible both for their plasticity as well as for the fact that the diffraction pattern does not extend beyond 20 Å. It may also account for the lability of the crystals to both temperature and changes in their chemical environment.

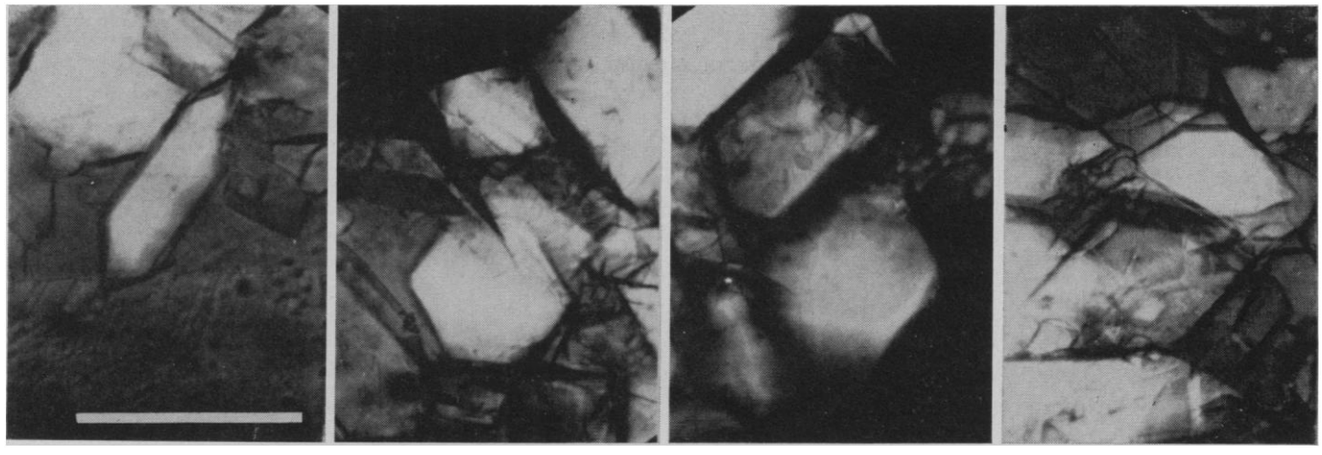

Fig. 1. Single crystals of formylmethionyl tRNA. The crystals were photographed while they were resting at the bottom of a test tube in mother liquor. The sample was at 5°C and between crossed polarizers of the microscope. The crystals are birefringent, which accounts for the differences in light intensity seen between different crystals. The scale (at the bottom left) is 0.5 mm.

From the density and partial specific volume we can also calculate the mass of tRNA in the crystal. There are approximately 33 molecules of the magnesium salt of formylmethionyl tRNA in the unit cell. If the space group is P_26, this suggests that five or six tRNA molecules are in the asymmetric unit. However, if the space group is $P6_222$, then it is likely that there are three tRNA molecules in the asymmetric unit in this space group with higher symmetry. In these calculations, we have assumed that there are no chloroform molecules in the lattice; if these are present they would modify the figures only slightly.

At the present time, these diffraction patterns are being analyzed further. However, it is worthwhile pointing out a few features. Along the *c* axis the reflection (003) is very intense, while the reflection (006) is quite weak. This suggests that a large part of the tRNA molecules may be organized in three layers separated by a distance of approximately 80 Å.

In the plane perpendicular to the *c*-axis the reflections (100) are weak compared to the reflections (110). There are several open lattice arrangements of diffracting molecules which could produce these differences. At the present time we feel that the crystal is held together by interactions between tRNA molecules organized in a somewhat extended configuration to form lattice channels which trap a large amount of water. The nature of these interactions between the tRNA molecules is completely unknown, but it is possible that these include hydrophobic interactions between the bases of single-stranded regions of different molecules, possibly with the participation of chloroform molecules. However, a detailed description of the lattice must await further analysis of the diffraction pattern.

Although the formation of heavily hydrated crystals of tRNA offers certain difficulties in terms of x-ray analysis, there may also be some intrinsic advantages. Thus, it is possible that the molecule has a configuration very close to that found in the native state because of the large amount of water. It is perhaps likely that there is a minimum amount of lattice distortion due to molecular interaction. The diffraction pattern starts to disappear at spacings be-

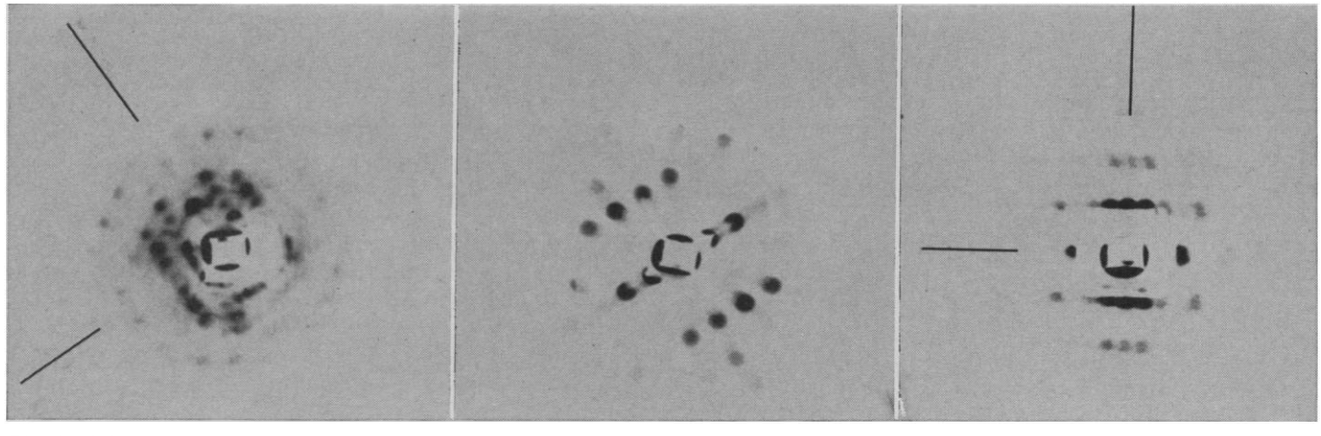

Fig. 2 (left). X-ray diffraction pattern of a principal zone, *h*0*l*, of a single crystal of formylmethionyl tRNA. The white rectangular area in the center is the shadow of the beam stop in the direct beam. The c^* axis is indicated by the line in the upper left; the a^* axis is in the lower left. The light area on the right is a shadow of the beam stop in the scattered x-ray beam. Fig. 3 (center). X-ray diffraction pattern of a zone in which the incident x-ray beam is perpendicular to a crystal face close to (101) of formylmethionyl tRNA. Fig. 4 (right). X-ray diffraction pattern of a zone showing the c^* axis indicated by the horizontal line, and the $(a^* + b^*)$ axis (110 direction) indicated by the vertical line. A shadow due to a portion of the beam stop obscures part of the diffraction pattern in the center and produces the straight edges which are visible on the first layer line.

low 20 Å, and this is probably related to the large amount of water and the ease of movement of portions of the molecule which are not restrained by lattice interactions. However, a Fourier analysis of these materials, even at a resolution of 20 Å, would be of great value because it would provide information which might lead us to infer the overall shape of the molecule. The double-stranded regions themselves have a diameter of 20 Å.

Transfer RNA molecules are the first pure nucleic acid molecules to form single crystals. This clearly indicates that these molecules have a definite and regular size and shape, such that they can be organized into a lattice array. The initial crystallization of proteins was of great importance in that it helped to focus the thinking of biochemists on a molecule with a fixed and definite configuration, one which many at the time believed to have a variety of shapes and forms. In a similar way the crystallization of tRNA and the recognition of a definite diffraction pattern leads us to conclude that these molecules also have a definite shape and form and one which is amenable to the same type of physical analysis which has proved so successful with the proteins. Unlike proteins (or most proteins), a unique feature of the tRNA molecule is the fact that it can be readily denatured but readily renatures again. In our investigation, for example, some of these preparations were dried completely and then rehydrated and subsequently crystallized. This behavior, unlike that observed with most proteins, suggests that the molecule has a configuration which is readily reversed once the appropriate hydrating medium is supplied.

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References and Notes

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12. We acknowledge the advice and assistance of Dr. D. L. D. Caspar who made available to us his x-ray diffraction equipment. We are also indebted to Dr. Fred Bergmann of the National Institute of General Medical Sciences and Dr. G. David Novelli of the Oak Ridge National Laboratories for their advice and assistance in obtaining the samples of purified formylmethionyl tRNA. The material was produced at the Oak Ridge National Laboratories under an interagency contract with the U.S. Atomic Energy Commission and the NIGMS. Supported by research grants from NIH and NSF.

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Single Crystals of Transfer RNA from Formylmethionine and Phenylalanine Transfer RNA's

Abstract. *Reproducible conditions have been developed for crystallization of transfer RNA. The conditions may be applicable to many pure transfer RNA species since identical procedures (except for initial transfer RNA concentration) yielded good crystals from both yeast and* Escherichia coli *transfer RNA. These crystals, which must be kept at temperatures below about 10°C and handled in vapor of controlled alcohol concentration, have been studied by x-ray crystallography. The availability of crystals of a nucleic acid opens a route for extending knowledge of the tertiary structure of transfer RNA and its relation to important biological functions.*

Techniques have been developed which permit growth of single crystals of transfer RNA (tRNA). The crystals are of sufficient size, stability, and order that good quality x-ray diffraction patterns were observed. Identical procedures were successfully used in producing crystals of phenylalanine tRNA ($tRNA^{Phe}$) and formylmethionine tRNA ($tRNA^{f\text{-}Met}$). The former was isolated from *Escherichia coli* and the latter from yeast, indicating considerable generality in this procedure for producing the crystals which are essential for determination of three-dimensional molecular structure by x-ray diffraction.

Transfer RNA molecules act as "Rosetta stones" by serving as adapter molecules between the nucleotide sequences of messenger RNA (mRNA) and the amino acid sequences of proteins. An unambiguous translation of mRNA to protein requires that each tRNA interact with an appropriate mRNA codon and also be specifically recognized by the corresponding aminoacyl tRNA synthetase. Genetic experiments have shown that this enzymic recognition does not involve the three anticodon bases in tRNA (*1*). The three-dimensional conformations of tRNA's must share in common the property of binding to ribosomes and being recognized by certain modifying enzymes (*2, 3*). In addition, certain tRNA molecules are recognized by methionyl tRNA formylating enzymes (*4*), methylating enzymes (*5*) and several other enzymes which introduce specific modifications, including the addition of the cell division hormones into tRNA (*6*).

It is known that tRNA folds into a compact structure in solution (*7*). The thermal stability of this structure and the reversible denaturation of biological activity indicate the importance of specific modes of folding of the polynucleotide chain (*8*). Comparison of the primary nucleotide sequence of various tRNA's (*3*) reveals several regions that are capable of forming Watson-Crick base pairs. A secondary structure in accord with these sequences may be an important factor in determining the conformation of tRNA, but an understanding of the basic mechanisms of tRNA function would be greatly enhanced by a detailed knowledge of the three-dimensional structure. By analogy with the recent advances made in the study of proteins (*9*), x-ray crystallographic analyses on single crystals of tRNA are expected to be valuable for this purpose. As the first phase of work in this direction, we report here a method for obtaining single crystals of (*E. coli*) $tRNA^{Phe}$ and (yeast) $tRNA^{f\text{-}Met}$, and conditions necessary for the stabilization of these crystals.

Techniques for purification of tRNA on the milligram to gram scale have been perfected, and several groups of investigators have purified tRNA to a stage approaching a single species (*10*). The Oak Ridge National Laboratories has a program, under sponsorship of the National Institutes of Health, which makes possible the distribution of useful amounts of purified tRNA's from *E. coli* (*11*).

After several pure species of tRNA became available, we began a systematic survey of conditions likely to promote the growth of single crystals.