RNA (1995), *1*:4–6. Cambridge University Press. Printed in the USA.

PERSPECTIVE

Keeping RNA happy

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A basic tenet of the emerging RNA religion is that most, if not all, RNA molecules fold into well-defined three-dimensional structures. Just as with proteins, a given RNA sequence in a given functional state has a unique conformation where each molecule is identical to every other molecule. We also believe that the folded structure of RNA is critical for its biological function, whether it is to bind proteins, participate in catalysis, or simply serve as a template. These statements of faith are supported by abundant experimental evidence for many RNAs and future pages of this journal will document even more evidence. The purpose of this commentary is to point out that some of our most cherished biochemical procedures can potentially get us into a lot of trouble.

Standard methods of purifying RNAs from cells or from in vitro transcription reactions involve denaturants. Proteins are removed by extraction with phenol and chloroform, sometimes with the aid of even more powerful chaotropic agents, such as guanidinium isothiocyanate. RNA is then concentrated by precipitating in high concentrations of ethanol and sometimes even completely dehydrated. RNA samples are then often heated in urea or formamide and fractionated on denaturing acrylamide gels. After elution from the gel, RNAs are usually stored in low ionic strength buffers containing chelators to remove all traces of divalent ions that could potentially stabilize their structure. When an RNA molecule is to be assayed, salts are added, a perfunctory "renaturation" step is performed and the reaction is initiated. The molecule is expected to quickly and accurately return to the conformation that it had before its traumatic experience. In other words, virtually all RNA biochemistry is done with renatured RNAs. No wonder our protein-centric colleagues think we're a bit misguided. Imagine purifying enzymes with SDS gels.

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How likely is it that an RNA molecule will renature into a unique conformation and how likely is it that this conformation will be the biologically relevant species? Perhaps RNA is different from proteins and can easily return to its native state. Although the answer will obviously depend upon the individual RNA sequence and the details of the renaturation process, I contend that in most cases it is highly unlikely that all of the molecules will renature to a single conformation. Indeed, if anything, a fully successful RNA renaturation is less likely than a fully successful protein renaturation. Instead, a renatured RNA sample will generally contain a mixture of conformations which, if one is lucky, will contain some that are biochemically active.

The underlying reason for the above conclusion is the extraordinarily high stability of RNA structure in physiological buffers. In contrast to proteins, where the total free energy of folding rarely exceeds 15 kcal/mol, even small RNA hairpins can have folding free energies of this magnitude, and the total free energy of folding a large RNA can be more than 100 kcal/mol (Turner et al., 1988). This impressive stability is primarily the result of the high favorable free energy of stacking of the nucleotide bases in the folded structure and the ability of counterions to effectively neutralize phosphate repulsion. However, the downside of a very stable native structure is that non-native states of RNA are often also very stable. As anyone who has played with RNA folding programs knows, a given RNA sequence can potentially form a large number of alternative secondary structures that are often predicted to be as stable as the native secondary structure. Although some RNAs may have stabilizing tertiary interactions unique to the native structure, the very stable alternative forms must be unfolded to reach the native state. These formidable energy barriers result in a serious kinetic folding problem for RNAs that have been denatured.

Experimental evidence for stable denatured conformers of RNA has been available for as long as quantitative measurements of RNA activity have been made. An elegant example from the 1960s is yeast $tRNA_3^{leu}$

where a single denatured conformer was identified by its lack of aminoacylation activity and its altered chromatographic properties (Fresco et al., 1966). Although the denatured form was thermodynamically less stable than the native molecule, the energy barrier was so high that interconversion took weeks under native conditions and could only be achieved at high temperatures in certain buffers. A similar story with *Escherichia coli* 5S RNA in the 1970s involved a deprotonation between native and denatured states (Kao & Crothers, 1980). However, it was not until the 1980s that the extent of the problem became evident. Although many examples can be cited, the struggles of ribozymologists to obtain RNAs that had uniform kinetic properties are typical (Altman & Guerrier-Takada, 1986; Fedor & Uhlenbeck, 1990; Herschlag & Cech, 1990; Walstrum & Uhlenbeck, 1990; Franzen et al., 1993; Thill et al., 1993). Unless great care is taken, ribozyme preparations are often only partially active and their cleavage properties can be difficult to reproduce. For example, efforts to find kinetically well-behaved hammerheads were (and continue to be) complicated by the ability of RNA fragments as short as 15 nucleotides to form alternative conformations and various intermolecular complexes in every buffer tested. The freezers in our laboratory are full of such unanalyzable hammerheads, trapped in "alternative conformer hell."

Those working with complex systems that are not as amenable to quantitation could well ask whether working with a conformationally heterogeneous population of molecules is such a great disadvantage. Indeed, it is sometimes argued that a mixture of conformations may improve one's chances for a successful assay. It is therefore worth enumerating the many benefits that result from obtaining an active RNA preparation that has a single conformation. First, because all the molecules are active, much less RNA has to be added to get a signal. This helps avoid the nasty possibility that some essential factor is unavailable to react because it is all bound to an inactive RNA conformation. Second, because all the molecules are the same, the kinetic and thermodynamic properties of the assay are more likely to be homogeneous. Thus, slow kinetic steps due to isomerization between inactive and active RNA conformations can be minimized. Third, structure–function studies are infinitely easier because the sample can be used directly to study the structure of the RNA. If only a small fraction of the molecules are active, the structure determined for the RNA sample as a whole will reflect a mixture of the native and non-native forms. This, in turn, will complicate the design and analysis of mutants.

So what can one do to achieve RNA conformational nirvana? One approach is to continue to use traditional methods of RNA purification and develop an appropriate renaturation protocol. The challenge is to allow the RNA to "find" its most stable (presumably native) state without getting trapped in one of the energetically stable alternative states. Such protocols generally involve heating the RNA to a specific elevated temperature in an appropriate stabilizing buffer and cooling at a defined rate to the assay temperature. Finding a successful protocol can be quite tedious and is generally idiosyncratic for the particular RNA (and RNA scientist). Moreover, as is always the case in science, we only hear about the successes. How many failed experiments are due to an unsuccessful RNA renaturation protocol? To pull a skeleton from our laboratory closet, we have struggled for years to obtain a fully active sample of unmodified *E. coli* $tRNA^{Phe}$. This annoying molecule has an alternative, inactive conformation that has a free energy of folding that is very similar to the native. Despite many attempts, we have never been able to find a protocol that gave fully active RNA once denaturation had occurred. To make matters even more irritating, the energy barrier between the two forms was low enough that virtually every denaturant (phenol, ethanol, distilled water) and even slow freezing produced a mixture of structures. Many labs have similar horror stories.

In some cases, a good way to avoid alternative conformer hell is to follow the lead of protein chemists and avoid denaturing the RNA during purification. Avoiding denaturants is relatively easy when purifying RNAs from in vitro transcription reactions. The high affinity of T7 RNA polymerase to phosphocellulose makes removal of protein straightforward. Unreacted NTPs and abortive initiation products can be separated from larger RNAs on a DEAE-cellulose column using an NaCl gradient to elute the RNA and including $MgCl_2$ to help maintain the native structure. Buffer changes and sample concentration can be achieved using the dialysis and gel filtration procedures favored by protein chemists. Because it is generally believed that RNA folding occurs during transcription, this approach is an attractive one and solved our problem with *E. coli* $tRNA^{Phe}$. However, not all RNAs are fully active as in vitro transcripts (Emerick & Woodson, 1993), reminding us that an in vitro transcription reaction only distantly resembles native conditions.

Although rarely attempted and undoubtedly difficult, developing methods for purifying RNA from cells without the use of denaturants is an important goal. The challenge is to remove the numerous proteins generally found associated with RNA and replace them with appropriate divalent ions or polyamines such that the intrinsically stable RNA secondary and tertiary structure is maintained. This is tricky because as proteins are removed, naked RNAs are susceptible to the numerous potent degradative ribonucleases that seem to frequent all cell lysates. Furthermore, currently available methods for fractionating RNAs under native conditions are not nearly as effective as denaturing polyacrylamide gels. Despite these obstacles, gentle

RNA purification methods can give samples with very different properties than those purified by more traditional methods. A good example comes from those studying ribosomal RNA. Using a combination of proteases, SDS, and a stabilizing buffer, virtually all the ribosomal proteins can be removed without greatly disturbing rRNA structure or biochemical activity (Noller et al., 1992). Some variations of these methods may well work with other RNAs.

Even if one has reached the happy situation of an active RNA in a single conformation, it remains to be shown that the conformation is the biologically relevant one. Certain RNAs may need a specific binding protein to maintain its active conformation. When the protein is removed, the most stable conformation may be quite different. Thus, it is critical to have independent genetic or phylogenetic data that support the biochemical structure. For example, genetic evidence for a base pair classically requires a mutation that disrupts the pair and a second site reversion that restores it. If the same mutations result in loss and gain of biochemical function and the appropriate change in the structure can be detected, one can rest easy about the RNA preparation.

In the end, then, if we really believe that RNA is a "nucleic acid trying to be like a protein," we should treat it with the same care that protein chemists treat their molecule. Just as with proteins, once an RNA is denatured, it may be difficult to coax it back to its native state. Thus, it is worth considering methods that avoid denaturants. With kind treatment, I am sure that our favorite macromolecule will continue to perform feats that puzzle and amaze us for years to come.

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