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[13] Conventional and time-resolved ribozyme X-ray crystallography

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phasing structure factors and obtaining high-quality electron density maps. Crystallographic experiments coupled with biochemical tests have allowed the functional interpretation of RNA structures. As occurred in the protein field, the number of RNA crystal structures solved per year is likely to grow exponentially.

### Acknowledgment

We thank Adrian Ferré-D'Amaré for helpful discussions about modeling ribose puckers in electron density maps. J.H.C. is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Research Fund; J.A.D. is an assistant investigator of the Howard Hughes Medical Institute, a Searle Scholar, a Beckman Young Investigator, and a Fellow of the David and Lucile Packard Foundation; this work was supported in part by a grant from the NIH.

## [13] Conventional and Time-Resolved Ribozyme X-Ray Crystallography

By WILLIAM G. SCOTT and JAMES B. MURRAY

### Introduction

Can X-ray crystallography help us to understand how ribozymes work? Although a number of biochemical techniques, perhaps most notably *in vitro* selection methods, have provided much needed insight into the nature and potentially broad spectrum of the catalytic capabilities of RNA, the problem of deducing the correlations between RNA three-dimensional structure and enzymatic activity remains a formidable one. Recent advances in crystallization methodologies and in RNA synthetic strategies have enabled us to begin to address this problem. In particular, the hammerhead ribozyme, hepatitis delta virus ribozyme, group I intron, hairpin ribozyme, RNase P, and other catalytic RNAs have been crystallized, and crystal structures of the first three have now been published. In addition, application of time-resolved crystallographic techniques to the hammerhead ribozyme allows us to begin to correlate the structure of a ribozyme directly with the chemistry of its catalysis. Here we discuss the methods used for both conventional and time-resolved crystallographic analyses of the hammerhead ribozyme. This ribozyme serves as a prototype for other analyses, because the techniques employed should be fairly generalizable for future work on other ribozyme systems.

## RNA Crystallizations

In general, the problem of crystallizing nucleic acids is more difficult than that of crystallizing proteins. The relative homogeneity of the phosphate backbone in nucleic acids and establishing helical end contacts between molecules make forming a crystal lattice difficult. Unlike protein crystallization, where a large number of structurally and chemically unique regions of surface are potentially available for the formation of crystal lattice contacts, RNA crystallization involves a much more limited repertoire of potential lattice-forming contacts. In crystals of simple helices, one often finds that two of the three or more contacts required to form a crystal lattice are composed of helical end contacts that establish formation of a quasi-continuous helix throughout the lattice. The base-stacking continues uninterrupted, but the phosphate backbone, which is not directly involved in such a contact, does not. (Minor variations on this theme, involving base triples at the lattice contacts, as well as 5' to 5' and 3' to 3' stacking arrangements, have also been observed, both in the context of DNA-protein complexes and in ribozyme crystallizations.)

The most definitive treatment of this problem is detailed in a crystallization paper of the CAP-DNA complex by Schultz, Shields, and Steitz.<sup>1</sup> Although the context is that of DNA-protein complex crystallizations, the principles are completely generalizable to RNA and RNA-protein complex crystallizations. The essential lesson from this treatment and from subsequent attempts at ribozyme crystallizations<sup>2-5</sup> is that one must often actively tamper with the nonessential sequences found at the helical termini until one finds, by trial and error, not only a sequence that is crystallizable, but one that produces crystals having suitable diffraction characteristics. This commonly involves synthesizing 10 or more trial sequence variants until one obtains the desired crystals. Rapid RNA chemical synthesis techniques make this approach much more tractable, at least in the case of relatively small RNAs that can be assembled from strands that are not longer than about 50 nucleotides.<sup>3</sup>

In addition to altering the sequences found at the ends of helices, it is generally helpful to experiment with nonessential regions within the molecule, especially to create potentially specific crystal packing contacts be-

<sup>1</sup> S. C. Schultz, G. C. Shields, and T. A. Steitz, *J. Mol. Biol.* **213**, 159 (1990).

<sup>2</sup> H. W. Pley, D. S. Lindes, C. DeLuca-Flaherty, and D. B. McKay, *J. Biol. Chem.* **268**, 19656 (1993).

<sup>3</sup> W. G. Scott, J. T. Finch, R. Grenfell, J. Fogg, T. Smith, M. J. Gait, and A. Klug, *J. Mol. Biol.* **250**, 327 (1995).

<sup>4</sup> B. L. Golden, E. R. Podell, A. R. Gooding, and T. R. Cech, *J. Mol. Biol.* **270**, 711 (1997).

<sup>5</sup> A. R. Ferre-D'Amare, K. Zhou, and J. A. Doudna, *J. Mol. Biol.* **279**, 621 (1998).

tween two elements of secondary structure, such as a GNRA tetraloop and an appropriate receptor sequence. Such contacts were found in both structures of the hammerhead ribozyme, although they were not planned. Interestingly, in some cases in which such contacts were deliberately designed into RNA molecules, crystals formed more readily but their diffraction properties were poor.<sup>5</sup> A subsequent variation on this theme, in which a loop that binds to an RNA-binding protein domain was grafted onto a nonessential region of the structure and the protein was employed in a cocrystallization, was much more successful at producing diffraction-quality crystals.<sup>6</sup> This success is probably due to the fact that potential protein–protein contacts are much more plentiful, and specific contacts did not have to be designed.

In essence, the difficulties in crystallizing nucleic acids arise from establishing good lattice contacts that allow the molecules to arrange themselves in a regular three-dimensional array. Molecules having only two separate helical ends still have a problem of establishing (at least) one more specific lattice contact, and the potential for forming such a third lattice contact may be enhanced by including a GNRA tetraloop along with known receptor sequences in the molecule, or alternately by including a bound protein that has the luxury of forming many different potential lattice contacts.

Several different “sparse matrix” crystallization screens have appeared recently that allow one to search for crystallization conditions efficiently.<sup>3,4,7</sup> Although these differ in detail, the differences are probably less important to successful crystallizations than the process of varying sequences to improve crystallizability.<sup>3</sup> Some general features for successful crystallizations of RNA, however, have emerged: whereas crystallizations of small duplexes and tRNA have relied on small organic molecules, particularly 2-propanol and MDP as precipitating agents, high concentrations of lithium and ammonium sulfate, and various molecular weights of PEGs and their derivatives in conjunction with 100 mM to 1 M monovalent salts, has proven to be particularly effective. The range of pH values employed for RNA crystallizations is generally more restricted than those used for protein crystallizations, because outside of the range of about pH 5–8.5, significant ionization of base functional groups may take place. Inclusion of  $Mg^{2+}$  or other divalent cations, such as  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ , and  $Zn^{2+}$ , at concentrations between 10 and 100 mM, is also worthwhile, especially if these cations are known to be required for biological activity. (Most of the softer metal ions form insoluble oxides at pH levels greater than 7.0 over the course of hours or

<sup>6</sup> A. R. Ferre-D'Amare, K. Zhou, and J. A. Doudna, *Nature* **395**, 6702 (1998).

<sup>7</sup> J. A. Doudna, C. Grosshans, A. Gooding, and C. E. Kundrot, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7829 (1993).

days, and therefore should be avoided at high pH). Inclusion of spermine or other such amines, while crucial for tRNA crystallization, does not in general appear to be a requirement for crystallization of most other RNA sequences (although its use as a nonspecific precipitation agent is sometimes incorporated in a crystallization condition). Within such parameters, it is generally most rewarding, and perhaps more rigorous in terms of reproducibility, to develop one's own sparse matrix screen tailored to the requirements of one's specific RNA, rather than to adhere slavishly to previously published conditions.

### Structural Determination by Isomorphous Replacement

From the standpoint of macromolecular crystallography, solving an RNA structure by isomorphous replacement is essentially identical to solving a protein structure; the physical principles of data collection and solving the phase problem are identical in each case. These are discussed extensively in *Methods in Enzymology*, volumes 114, 115, 276, and 277. What differs in the case of RNA is the likelihood of success in various different approaches for forming heavy atom derivatives. Although finding conditions in which a heavy atom binds with high affinity to one or a few sites on an RNA molecule may be more difficult than with protein crystals, crystals of nucleic acids also present some advantages. If some or all of the RNA used in crystallizations has been obtained by chemical synthesis, 5-bromouridine and other brominated and iodinated phosphoroamidites, which are commercially available, can be used to incorporate covalently bound isomorphous heavy atom derivatives having complete specificity, to a predetermined number of sites on the RNA, and to full occupancy.<sup>3</sup> The ability to produce heavy atom derivatives having these characteristics is often crucial to the successful solution of a macromolecular crystal structure in a straightforward manner. The modified nucleotide in many cases need not be a ribonucleotide; often, the less expensive deoxy versions of these derivatives will do just as well.<sup>3</sup>

For small RNAs, covalently bound heavy atom derivatives can be incorporated without difficulty using standard RNA synthetic techniques. For larger RNAs that cannot be synthesized by standard chemical means, one proven approach is to make the large RNA in two parts, where the large and small parts can associate via conventional base-pairing interactions. The larger part, containing a terminal deletion of about 30 residues or less, can be transcribed from a template derived from the original full-length template, and the missing RNA sequence can then be synthesized chemically, allowing for incorporation of covalently attached heavy atoms. Although the two strands can then be ligated to form a single covalently

linked RNA strand, this may not be required in practice for the formation of a stable complex, as was the case for a domain of the group I intron.<sup>8</sup>

In addition to brominated and iodinated phosphoroamidites, covalently bound heavy atoms may be incorporated into synthetic RNA using other commercially available phosphoroamidites, such as deoxyphosphorothioates that can be used to incorporate potential Hg binding sites in the RNA. (It is important to employ the deoxyamidite to prevent Hg-induced RNA strand cleavage involving the ribose adjacent to the modified phosphate.)

Data collection is typically carried out on area detector systems, as is the case with other macromolecular crystallography. However, nucleic acid X-ray diffraction data typically differ from protein diffraction data in that the lattice diffraction pattern is convoluted with the fiber-like diffraction that is the manifestation of the helical transform of RNA; i.e., the regular spacing of base pairs modulates the intensities of the Bragg reflections. As a consequence, the intensities typically do not tail off smoothly as a function of increasing resolution. Rather, the intensity modulation is periodic, and the reflections along the helical axes around 3.4 Å tend to be especially strong, while those perpendicular tend to be weaker. This gives rise both to anisotropy of the data as well as to very intense data, which contain much of the contribution to the electron density of the bases, that is quite difficult to process accurately. Conventional data processing in MOSFLM<sup>9</sup> or DENZO<sup>10</sup> involves defining an average spot profile; these reflections are often rejected due to poor profile fit unless the program is “tricked” into processing these very intense and seemingly highly mosaic reflections, either by relaxing the rejection criteria, or by processing the data a second time with the intent of only measuring these spots accurately, and then merging them with the conventionally processed data set after applying appropriate scaling correlations. These procedures are *ad hoc* and not particularly satisfactory, but have the merit of increasing the quality of the phases and therefore the electron density maps, at least in the case of the hammerhead RNA.

Cryoprotection of RNA crystals is often found to be essential, as most appear to be radiation sensitive. Cryoprotection of crystals for the purpose of multiwavelength anomalous diffraction (MAD) data collection is especially recommended. (The apparent radiation sensitivity of RNA crystals may be correlated with the frequent use of cacodylate buffers in RNA

<sup>8</sup> B. L. Golden, A. R. Gooding, E. R. Podell, and T. R. Cech, *RNA* **2**, 1295 (1996).

<sup>9</sup> A. G. W. Leslie, “MOSFLM Users Guide.” MRC Laboratory of Molecular Biology, Cambridge, UK, 1998.

<sup>10</sup> Z. Otwinowski and V. Minor, *Methods Enzymol.* **276**, 307 (1997).

crystallizations; merely changing the identity of a buffer has been shown to reduce dramatically the radiation sensitivity in the case of the hammerhead RNA.) The software used for isomorphous replacement phase calculation and refinement is identical to that commonly employed for protein crystallography, and the choices are generally a matter of personal preference. Maximum likelihood phasing algorithms such as MLPHARE<sup>11</sup> (distributed with the CCP4 crystallographic program suite, Ref. 12) or SHARP<sup>13</sup> have the advantage of not overestimating figures of merit and are thus more amenable to further improvement using solvent-flattening techniques. As with other macromolecular structures, noncrystallographic symmetry averaging can greatly improve the quality of initial electron density maps and structural refinement in cases where there are similar molecules in the asymmetric unit of the crystal. One must, however, be aware of the high degree of pseudosymmetry found in RNA molecules, because this can compound the problem of locating noncrystallographic symmetry axes. (It also makes the solution of RNA structures by molecular replacement quite difficult.)

Fitting a model of RNA to experimentally determined electron density can be rather more difficult than it is with protein structures, because one can generally distinguish side chains only as purines or pyrimidines unless the data are of high resolution. It is therefore extremely helpful to have both prior knowledge of the RNA secondary structure and convenient landmarks in the electron density. The latter can be obtained by using heavy atom derivatives that are covalently bound to known positions in the RNA sequence. An additional aid to the eye in terms of tracing the backbone of the RNA can be obtained by contouring the electron density map at about 2.0 to 3.0 times the rmsd (root mean square deviation) of the map; this procedure generally reveals the location of the phosphates unambiguously. These phosphate locations can often be verified using an anomalous differences Fourier map because the phosphorous atoms have detectable X-ray absorption even at the remote wavelengths of X rays typically employed for macromolecular structural determination. Elimination of (presumably spurious) anomalous differences greater than 3 times the rmsd tend to improve such maps dramatically.

Refinement of RNA structures again proceeds along the lines of the procedures typically used for protein crystallography. XPLOR<sup>14</sup> and its

<sup>11</sup> Z. Otwinowski, in "Isomorphous Replacement and Anomalous Scattering, Proceedings of Daresbury Study Weekend," pp. 80–85. SERC Daresbury Laboratory, Warrington, U. K., 1991.

<sup>12</sup> Collaborative Computational Project, N. 4, *Acta Crystallogr.* **D50**, 760 (CCP4, 1994).

<sup>13</sup> E. De La Fortelle and G. Bricongne, *Methods Enzymol.* **276**, 472 (1997).

<sup>14</sup> A. T. Brünger and L. M. Rice, *Methods Enzymol.* **277**, 243 (1997).

successor, CNS,<sup>15</sup> have proven to be successful in RNA structural refinement, especially when used with the new nucleic acid parameter library produced by Helen Berman and co-workers from the Nucleic Acids Structural Database.<sup>16</sup> It is generally recommended that the charges on the phosphates be reduced or set to zero during simulated annealing molecular dynamics refinement, because the counterion screening environment that neutralizes the RNA cannot be modeled readily. In practice, we have found that this does not seem to make an appreciable difference in the course of refinement, perhaps because the constraint of the electron density overcomes the potential electrostatic repulsion of the phosphate backbone in the model structure.

### From Static RNA Structures to Reaction Dynamics

Although conventional X-ray crystallography is perhaps unparalleled as a physical technique for elucidating RNA structure, it is limited in that it only provides a static representation of a molecule in a single state. In the case of the three currently elucidated ribozyme crystal structures, these states include the initial enzyme–substrate<sup>17</sup> or enzyme–inhibitor<sup>18,19</sup> complex, the free enzyme,<sup>20</sup> or the enzyme–product complex,<sup>6</sup> respectively. Although these structures provide starting points for inferring ribozyme mechanisms, their usefulness is inherently limited by the fact that they constitute single, and extremal, points in the reaction pathways of these ribozymes. However, reliable time-resolved crystallographic methods have now been developed in the context of protein enzymes that allow transient intermediates in the enzyme-catalyzed reaction that accumulate under steady-state conditions to high occupancy simultaneously throughout the crystal lattice to be observed rapidly (using Laue crystallography as a fast data collection technique) or trapped (either chemically or physically) and examined using monochromatic X-ray crystallography.<sup>21–23</sup> Because the

<sup>15</sup> A. T. Brünger, P. D. Adams, M. G. Clore, W. L. DeLano, P. Gross, R. W. Grosse-Kunstleve, R. W. Jiang, J.-S. Kuszewski, J. Nilges, N. S. Parru, R. J. Read, L. M. Rice, T. Simonson, and G. L. Warren, *Acta Crystallogr.* **D54**, 905 (1998).

<sup>16</sup> G. Parkinson, J. Vojtechovsky, L. Clowney, A. T. Brünger, and H. M. Berman, *Acta Crystallogr.* **D52**, 57 (1996).

<sup>17</sup> W. G. Scott, J. B. Murray, J. R. P. Arnold, B. L. Stoddard, and A. Klug, *Science* **274**, 2065 (1996).

<sup>18</sup> H. W. Pley, K. M. Flaherty, and D. B. McKay, *Nature* **372**, 68 (1994).

<sup>19</sup> W. G. Scott, J. T. Finch, and A. Klug, *Cell* **81**, 991 (1995).

<sup>20</sup> B. L. Golden, A. R. Gooding, E. R. Podell, and T. R. Cech, *Science* **282**, 259 (1998).

<sup>21</sup> D. W. J. Cruickshank, J. R. Helliwell, and L. N. Johnson, "Time-Resolved Macromolecular Crystallography." The Royal Society, Oxford Science Publications, 1992.

<sup>22</sup> K. Moffat and R. Henderson, *Curr. Opin. Struct. Biol.* **5**, 656 (1995).

<sup>23</sup> K. Moffat, *Methods Enzymol.* **277**, 433 (1997).



field of ribozyme crystallography is new, the techniques of time-resolved crystallography are only now beginning to be applied to the problem of ribozyme catalysis. For that reason we describe in some detail the methods that have proven successful in our laboratory in the context of the hammerhead ribozyme system with the hope that the lessons we have learned will be generalizable to other systems that have not yet been investigated.

### Initiation of Ribozyme Catalysis in Crystals

A successful time-resolved crystallography experiment requires that the reaction catalyzed by a crystallized enzyme be initiated simultaneously throughout the crystal lattice so that the intermediate species that is to be observed can accumulate to high or full occupancy throughout the crystal simultaneously. This enables us to observe the intermediate by fast data collection techniques or to trap it physically. In the case of the hammerhead ribozyme sequence that we have crystallized, the catalytic turnover rate in the crystal is approximately 0.4 molecule/min when crystals grown at pH 5–6 are activated with the addition of a mother liquor solution buffered at pH 8.5 and containing 50–100 mM divalent metal ion. The time it takes for a fairly complex substrate (NADP) to diffuse into a crystal of isocitrate dehydrogenase measuring 0.5 mm in each dimension and to saturate the enzyme's active sites is approximately 10 sec when measured directly by video absorbance spectroscopy.<sup>24</sup> The corresponding time it takes a much smaller divalent metal ion to diffuse into and saturate the considerably smaller hammerhead ribozyme crystals ( $0.3 \times 0.25 \times 0.25$  mm or smaller) is unlikely to be longer. Therefore, the diffusion time is sufficiently fast compared to the turnover rate to allow approximately synchronous initiation of the hammerhead ribozyme cleavage reaction throughout the crystal.

A hammerhead ribozyme cleavage reaction in the crystal therefore can be initiated by removing a crystal from the drop in which it has grown, using a small (approximately 0.4-mm in diameter) rayon loop mounted on a wire. The crystal is then immediately immersed into an artificial mother liquor solution containing divalent metal ions at a higher pH (typically 1.8 M  $\text{Li}_2\text{SO}_4$ , 50–100 mM divalent metal ion, and 50 mM Tris, pH 8.5, augmented with 20% glycerol as a cryoprotectant). The artificial mother liquor should be prepared immediately before use using freshly dissolved components. (This is especially critical when using the softer divalent metal ions because they slowly form insoluble metal oxides under even mildly basic conditions.) The crystal is placed into approximately 500  $\mu\text{l}$  of the artificial mother liquor in a glass spot plate and observed under a polarizing microscope while being slowly wafted through the solution to aid in mixing at

<sup>24</sup> P. O'Hara, P. Goodwin, and B. L. Stoddard, *J. Appl. Crystallogr.* **28**, 829 (1995).

the solution–crystal interface. The crystal is subsequently removed from the solution in the same manner as before at the preordained time and is flash frozen in liquid nitrogen or liquid propane to trap any accumulated intermediate state. Monochromatic X-ray data can then be collected in the usual manner for cryoprotected crystals. The crystal may be removed from the X-ray source subsequent to data collection and stored frozen in liquid nitrogen until such time that assay of the extent of cleavage is convenient.

The relatively slow reaction rate of the hammerhead ribozyme<sup>25</sup> allows us to use this very simple method of freeze trapping. Depending on the turnover rate of other ribozymes whose reactions might be examined in the crystal [such as hepatitis delta virus (HDV), group I intron, or RNase P], it may be necessary to employ a flow cell to deliver a carefully timed pulse of substrate or divalent metal ion cofactor, or even to use a photochemically activatable “caged” substrate precursor in order to initiate the reaction rapidly throughout the crystal lattice.<sup>26</sup> Constructing a flow cell need not be a laborious process. A simple procedure that makes use of a conventional crystallographer’s capillary connected to a syringe pump has been described previously.<sup>27</sup> Possible photoactivatable triggers include photolabile chelators for divalent cations and RNA substrates that are caged with a photolabile *o*-nitrobenzyl moiety attached to the 2'-oxygen of the active site ribose. The latter has already been accomplished for the hammerhead ribozyme<sup>28</sup> and crystallization of the “caged” hammerhead ribozyme is currently under way in our laboratory.

### Use of “Kinetic Bottleneck” Modifications to Trap Intermediates Chemically

To observe structural or chemical intermediates in crystallized enzymes, it is necessary for the intermediate to accumulate to high occupancy simultaneously throughout the crystal lattice. Otherwise, spatial and temporal averaging will make observation of the intermediate impossible, because the electron density will represent the entire ensemble of structures at a given time point. In practice, this means that there must be a rate-limiting step in the reaction scheme corresponding to the decomposition of the intermediate; i.e., a kinetic bottleneck in the reaction pathway must either

<sup>25</sup> T. K. Stage-Zimmermann and O. C. Uhlenbeck, *RNA* **4**, 875 (1998).

<sup>26</sup> I. Schlichting and R. S. Goody, *Methods Enzymol.* **277**, 467 (1997).

<sup>27</sup> G. A. Petsko, *Methods Enzymol.* **114**, 141 (1985).

<sup>28</sup> S. G. Chaulk and A. J. MacMillan, *Nucleic Acids Res.* **26**, 3173 (1998).

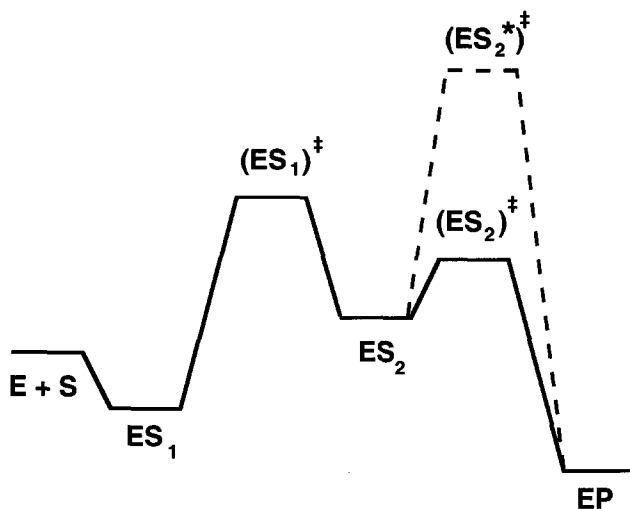


FIG. 1. An idealized potential energy diagram for an enzyme whose mechanism involves an intermediate species. The diagram depicts the relative energies of the free enzyme and substrate, the initial enzyme–substrate complex ( $ES_1$ ), the enzyme–intermediate complex ( $ES_2$ ), their corresponding transition states ( $ES_1^\ddagger$ ) and ( $ES_2^\ddagger$ ), and the enzyme–product complex (EP). A cross section of the potential energy surface along the reaction coordinate for the unmodified enzyme is shown as a solid line, and that for the idealized “kinetic bottleneck” mutant enzyme, where different, is shown as a dashed line. Note that for the unmodified enzyme, formation of the intermediate is the rate-limiting step, thus making observation of the intermediate species difficult. The ideal kinetic bottleneck modification designed for observing the intermediate will raise the energy of the transition state that follows formation of the intermediate ( $ES_2^*\ddagger$ ), in such a way that decomposition of the intermediate now becomes rate limiting, but with minimal perturbation of the reaction equilibrium (i.e., the relative energy levels of  $ES_1$ ,  $ES_2$ , and EP). In such a case the enzyme–intermediate complex ( $ES_2$ ) will accumulate transiently during the course of a single-turnover reaction even though the equilibrium remains unchanged, and can then be observed using the techniques of time-resolved crystallography.

exist naturally, or it must be created through alteration of the reaction conditions or of the enzyme itself (Figs. 1 and 2).

Following the example of a time-resolved crystallography experiment with isocitrate dehydrogenase, in which observation of the chemical intermediate oxalosuccinate was made possible using a kinetic bottleneck that causes the decarboxylation step of the reaction to be rate limiting,<sup>29,30</sup> we

<sup>29</sup> B. L. Stoddard, B. E. Cohen, M. Brubaker, A. D. Mesecar, and D. E. Koshland, Jr., *Nature Struct. Biol.* **5**, 89 (1998).

<sup>30</sup> J. M. Bolduc, D. H. Dyer, W. G. Scott, P. Singer, R. M. Sweet, D. E. Koshland, Jr., and B. L. Stoddard, *Science* **268**, 1312 (1995).

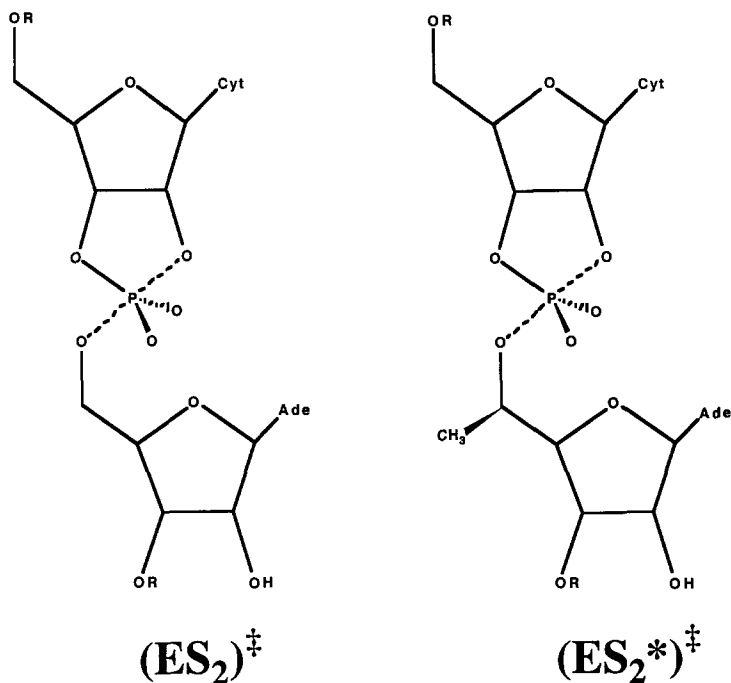


FIG. 2. Illustrations of normal (left) and kinetic bottleneck-modified (right) transition states of the hammerhead ribozyme cleavage reaction, corresponding to Fig. 1. Use of the methyl group adjacent to the leaving-group 5'-oxygen causes the bond-breaking step of the reaction to become rate limiting, enabling the conformationally changed structure,  $ES_2$  of Fig. 1 to be trapped and observed crystallographically.

modified our hammerhead ribozyme sequence to make the bond cleavage step of the reaction rate limiting. On ionization of the 2'-hydroxyl at the cleavage site ribose of the natural hammerhead ribozyme, the charged oxygen initiates nucleophilic attack at the 3'-scissile phosphate. As the bond between the 2'-oxygen and the phosphorus forms, the bond between the phosphorus and the 5'-oxygen of the adjacent ribose breaks in a manner consistent with an  $S_N2(P)$  mechanism. Though it is unclear whether this process is concerted or sequential, it is recognized that a conformational change must take place in the vicinity of the scissile phosphate (relative to the initial structure observed in hammerhead ribozyme crystals) in order for the chemical step or steps of the reaction to be initiated.<sup>17-20</sup> Therefore, the conformational intermediate state that is compatible with subsequent formation of an in-line transition state or chemical intermediate is of significant interest to those hoping to understand the stereochemical mechanism

of hammerhead ribozyme catalysis. If the bond-breaking step of the reaction were to be made rate limiting, this would permit the transient accumulation of the conformational intermediate required for initiation of the reaction chemistry. By altering the properties of the 5'-leaving group, one could in principle engineer a ribozyme having a kinetic bottleneck at the bond-breaking step of the reaction. This has in fact been realized in the form of a hammerhead ribozyme that has an extra methyl group appended to the 5'-carbon adjacent to the leaving-group oxygen; such a ribozyme still exhibits the normal pH versus rate profile of the hammerhead ribozyme (suggesting that the reaction pathway has not been substantially altered), but cleavage is slowed by at least two orders of magnitude in the crystal. Use of this sort of modified RNA substrate at the active site of the ribozyme in single-turnover reactions enabled us to visualize the conformational change to a structure compatible with subsequent formation of an in-line transition state.<sup>31</sup> In this case (unlike isocitrate dehydrogenase) the lifetime of the intermediate was sufficiently long to enable conventional monochromatic X-ray data to be collected on a frozen crystal.

The particular form of the kinetic bottleneck modification used will be dependent on the reaction chemistry that is catalyzed by the ribozyme. However, all of the naturally occurring ribozymes catalyze phosphate chemistry in which either the 5'-oxygen or the 3'-oxygen is a leaving group, suggesting that modifications similar to the one employed with the hammerhead ribozyme should be useful in principle, though their usefulness in practice must of course be tested experimentally. Again, the main characteristics of a useful kinetic bottleneck modification are (1) that it creates a rate-limiting step corresponding to the breakdown of the intermediate that is to be observed; (2) that it alters the kinetic properties of the ribozyme without grossly perturbing the thermodynamics of the reaction (and therefore its equilibrium); and (3) that creation of the kinetic bottleneck does not cause the reaction to proceed via a different (aberrant) chemical mechanism.

### Use of Cryocrystallographic Techniques to Trap Intermediates Physically

If the lifetime of a ribozyme intermediate species is on the order of seconds or more, physical trapping techniques can be used to further stabilize or immortalize the intermediate for the purpose of data collection.<sup>22</sup> The most straightforward approach is to simply flash freeze the crystal in

<sup>31</sup> J. B. Murray, D. P. Terwey, L. Maloney, A. Karpeisky, N. Usman, L. Beigelman, and W. G. Scott, *Cell* **92**, 665 (1998).

liquid propane or liquid nitrogen as one might normally do to collect data on a cryoprotected and frozen crystal.<sup>32</sup> Because one has normally determined the crystal structure of some initial state of an enzyme prior to embarking on a time-resolved crystallography experiment, it is likely that a set of conditions for cryoprotecting a crystal has already been found. In general, these conditions should be applicable to the case of freeze trapping without further alteration. In the case of the hammerhead ribozyme, simply augmenting the artificial mother liquor or the reaction mixture with 20% glycerol was sufficient for cryoprotection. Immersion of the crystal in the cryoprotectant solution for 15 sec or more was generally all that was required for subsequent flash freezing. Subsequent to flash freezing, the crystals were maintained at 100 K throughout data collection using an Oxford Cryostream system (Oxford Cryosystems, Oxford, UK). Further details on crystallographic freeze-trapping techniques and low-temperature data collection have been described elsewhere.<sup>22,32</sup>

#### Use of Polychromatic or Laue X-Ray Crystallography for Rapid Observation of Transient Intermediates

In the case of a ribozyme (such as RNase P, HDV ribozyme, or a group I intron) whose intermediate has a lifetime of less than 1 sec or so, it is unlikely that conventional physical trapping techniques such as flash freezing alone will allow crystallographic observation using conventional monochromatic data collection. Instead, fast data collection techniques such as Laue crystallography are required to observe more evanescent species as they accumulate transiently in the crystal subsequent to reaction initiation. In the case of Laue crystallography, polychromatic X rays generated from an intense synchrotron source may be used to collect a fairly complete data set in a single snapshot of picosecond to millisecond duration. Assuming a crystal can withstand repeated exposure to the intense X-ray beam, a series of time points may be obtained from a single crystal over the course of a reaction. If the lifetime of the intermediate that is to be observed is as long as, or is at least comparable to, the exposure duration of the X-ray snapshot, and if the homogenous synchronized accumulation of that intermediate can occur throughout the crystal at the time of the X-ray exposure, the transient intermediate should be observable, as has been shown to be the case for several protein enzyme crystals.

In addition to the requirements for synchronous accumulation of the intermediate to high occupancy throughout the crystal, the reaction must be initiated on a timescale that is fast compared to the lifetime of the

<sup>32</sup> D. W. Rogers, *Methods Enzymol.* **276**, 183 (1997).

intermediate, and the crystal must diffract sufficiently well throughout the course of the experiment in order to obtain useful data. Useful Laue data can be obtained only from crystals that are inherently well ordered and that are robust with respect to X-ray radiation damage. Lattice imperfections and even modest mosaicity can make such experiments impossible in practice. In the case of the hammerhead ribozyme, the crystals were not sufficiently perfect to allow processible Laue diffraction data to be collected. For that reason, we refer the interested reader directly to two comprehensive *Methods in Enzymology* discussions of Laue crystallography.<sup>22,33</sup> Hopefully crystals of other ribozymes, especially those having a much faster turnover rate, will be of sufficient quality to allow useful Laue data to be collected.

### Analysis of Results of Time-Resolved Ribozyme Crystallography

Although detection of a conformational change or chemical intermediate using X-ray crystallography is fairly straightforward, it is important to eliminate possible sources of bias and to perform the proper experimental controls to prove that the crystal whose structure is being modeled does indeed contain the intermediate state one purports to observe. The first dictum is addressed here; the second is the subject of the next section, in which an assay for ribozyme catalysis in the crystal is described.

A chemical or conformational intermediate structure can be observed crystallographically using difference Fourier techniques. If, for example, a conformational change is to be observed, it is quite possible that simply re-refining the initial structure against the new data set will reveal the conformational change with no apparent ambiguity. Even if this is the case, it is extremely important that the parts of the structure that appear to be involved in the conformational change be omitted, and that the refinement then be repeated, beginning again with the initial-state structure. The phases generated from the omit-refinement should not contain any model bias based on the presumed conformational change. Omit difference Fouriers based on Sim-weighted or Sigma-A weighted coefficients will further reduce model bias,<sup>34</sup> and difference Fouriers having coefficients of the form  $[F_{\text{obs}}^{(\text{intermediate})} - F_{\text{obs}}^{(\text{initial state})}] \exp\{i\Phi^{(\text{initial state})}\}$  should be essentially free of bias with respect to the model of the intermediate state. The clarity of the difference electron density, however, may be severely compromised, depending on how much the true phases deviate from the calculated phases of the initial structure. Sigma-A weighted maps should improve this situa-

<sup>33</sup> I. J. Clifton, E. M. H. Duke, S. Wakatsuki, and Z. Ren, *Methods Enzymol.* **277**, 448 (1997).

<sup>34</sup> R. J. Read, *Acta Crystallogr.* **A42**, 140 (1986).

tion as well, and recently developed “holographic” methods for reconstructing electron density using real-space methods hold much promise for dramatically improving the quality of such electron density maps without introducing phase biases from either the initial structure or from models of the intermediate.<sup>35</sup> Finally, if some sort of landmark feature can be detected reliably in the new electron density, such as the location of a phosphate group, further confidence in a modeled conformational change can be obtained. In the case of modeling a chemical reaction intermediate, the above difference Fourier methods can be employed, and these are especially powerful if one wishes to locate functional groups that appear or disappear in the course of the reaction that forms the chemical intermediate, using maps of the form  $\pm [F_{\text{obs}}^{(\text{intermediate})} - F_{\text{obs}}^{(\text{initial state})}] \exp\{i\Phi^{(\text{initial state})}\}$ , respectively.

### Development of Assay for Crystalline Ribozyme Catalysis

It is also imperative that the reaction being monitored crystallographically be characterized using biochemically based enzymatic assays, such that the time-resolved crystallographic experiments can be calibrated correctly. In the case of the hammerhead ribozyme, cleavage in the crystal has been monitored using an assay developed specifically for such reactions. Conventionally, kinetics assays are performed on the hammerhead ribozyme using <sup>32</sup>P-labeled substrate-strand molecules in a cleavage reaction. The cleavage products can be separated unambiguously from reactants using polyacrylamide gel electrophoresis under denaturing conditions similar to those used for nucleic acid sequencing. The relative amounts of substrate and product can then be quantified using autoradiography. This procedure is somewhat cumbersome for assaying cleavage within crystals of RNA because it would require phosphorylation of the substrate either prior to crystallization or subsequent to dissolving the crystals; the former procedure introduces complications for purifying and crystallizing the RNA, and the latter introduces potentially large systematic errors because cleavage of the RNA may continue in solution under conditions that are amenable to phosphorylating the reaction mixture.

Alternatively, one can use gel electrophoresis combined with a sensitive RNA staining procedure such as silver staining or staining with 0.5% toluidine blue solution (followed by destaining with hot water) to assay cleavage in the crystal. This procedure has the advantage of avoiding the problems associated with radiolabeling the RNA in the crystal or after dissolution of the crystal, but accurate quantitation of the RNA stained on a gel is much

<sup>35</sup> A. Szoke, *Acta Crystallogr.* **A54**, 543 (1998).



more difficult than with autoradiography. In addition, dissolved crystal solutions contain high concentrations of EDTA to quench cleavage (see later section) as well as other salts. Samples of high ionic strength tend to cause severe compressions in nucleic acid sequencing gels, making separation of product from substrate strands more difficult.

For these reasons, as well as convenience, we developed a high-performance liquid chromatography (HPLC)-based assay for crystallized hammerhead ribozyme self-cleavage (Fig. 3). This procedure has the advantages of (1) speed and convenience, (2) being immune to the effects of having a high concentration of EDTA and other salts and buffers in the sample being assayed, and (3) providing output in a form that is readily quantifiable (i.e., the absorbance profiles of the eluates can be integrated to provide an accurate assay of RNA cleavage). The one disadvantage of HPLC analyses is that they require a higher concentration of RNA than do autoradiographic procedures. However, this is not a problem when dealing with crystallized

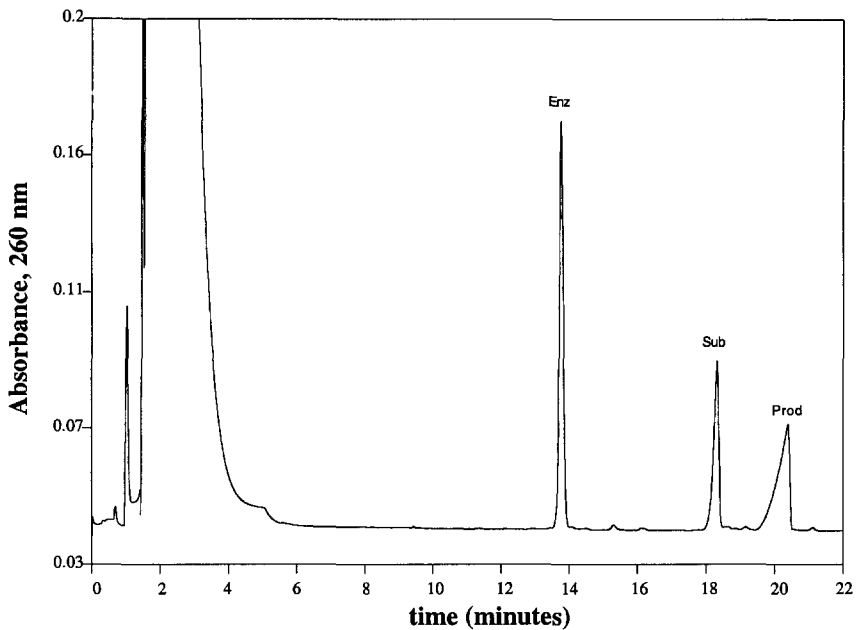


FIG. 3. A representative ion-exchange HPLC trace of a hammerhead ribozyme cleavage reaction monitored in the crystal, subsequent to quenching the reaction in 0.5 *M* EDTA as described in the text. The 16-mer enzyme strand elutes at about 14 min, the uncleaved (25-mer) at about 18 min, and the 20-mer cleavage product elutes later, at about 20 min, perhaps due to the greater charge density (see text). A large breakthrough peak is present that corresponds to the EDTA, buffers, and other salts in solution.

RNA; the RNA consumed in a typical crystallization experiment will drive most molecular biologists to tears. The use of HPLC to follow hammerhead ribozyme reactions in solution has previously been described.<sup>36,37</sup>

### Termination of Ribozyme Catalysis in Crystals

The hammerhead cleavage reaction was initiated in the crystal as described earlier. The cleavage reaction can be arrested indefinitely by flash freezing, making observation of a trapped intermediate state possible. However, to assay the extent of cleavage in the crystal, the crystallized RNA must be dissolved. To obtain a reliable estimate of the extent of cleavage in the crystal, it is imperative that the RNA not be allowed to react further once it has been dissolved.

We established a procedure, based on trial and error, that terminates RNA cleavage in the crystal prior to dissolution and also prevents further reaction from taking place in solution. The most reliable procedure is to remove the crystal from the reaction solution (described earlier) using a rayon loop, and immediately transfer it to a 0.5-ml drop of 0.5 M EDTA at pH 6.5. In our experience, the crystal is relatively stable in such a solution for over half an hour, although some cracks in the crystal develop. The EDTA quickly enters the crystal and quenches the reaction. We have observed that concentrations of EDTA much less than 0.5 M are not reliable for quenching the reaction completely. Once the crystal has been allowed to soak for 30 min or more, it can be dissolved in the EDTA solution by manually disrupting the crystal with a micropipette. Once the crystal has been observed to have dissolved completely (making use of a polarizing microscope to detect small shards of undissolved crystal), the solution can then be analyzed directly by ion-exchange HPLC.

### Analysis of Extent of Ribozyme Cleavage in Crystal by HPLC

The problems faced with in characterizing the amount of each RNA species in the dissolved crystals are large sample volumes (0.05–0.5 ml), high salt concentrations (0.5 M EDTA), and accurate quantification of the various RNA components. Ion-exchange HPLC coupled with UV absorbance detection is an ideal technique for dealing with these problems. Samples can be loaded in any size volume, which allows us to dilute the salt concentration to less than 100 mM. Quantification is straightforward, the area of each peak corresponds to the integrated absorbance intensity,

<sup>36</sup> L. Citti, L. Boldrini, S. Nevischi, L. Mariani, and G. Rainaldi, *Biotechniques* **23**, 898 (1997).

<sup>37</sup> R. Vinayak, A. Andrus, N. D. Sinha, and A. Hampel, *Anal. Biochem.* **232**, 204 (1995).

and therefore the concentration of RNA, assuming the molar extinction coefficient can be calculated accurately. The ion-exchange column of choice in many RNA synthesis laboratories is the Dionex DNA-PAC. For the hammerhead ribozyme experiments, we have used a resolving gradient of 0.35–0.65  $M$   $NH_4Cl$  in 22 min. This permits a sample to be analyzed every 40 min. The column should be maintained at 50°. This gives superior resolution relative to room temperature, allowing us to resolve the enzyme (16-mer), substrate (25-mer), and the 2',3'-cyclic-phosphate product (20-mer) strands from one another. [The 5'-OH product (5-mer) can be observed by starting the gradient at lower  $NH_4Cl$  concentrations and by using larger loading volumes.] We found that the 20-mer product strand eluted at higher salt concentrations than did the 25-mer substrate strand. (The identities of the eluates were independently characterized subsequent to chromatographic separation by denaturing PAGE.) It is possible that the reversal of the expected order of elution is due to the fact that length to charge ratio of the product strand is less than that for the substrate strand, i.e., the 20-mer has 20 phosphates and the 25-mer has 24 phosphates, due to the presence of the 2',3'-cyclic phosphate on the 3' end of the 20-mer.

The proportion of substrate remaining was calculated from  $A_{sub}/(A_{sub} + 1.25 A_{prod})$ . The 1.25 coefficient corrects for the differences in the extinction

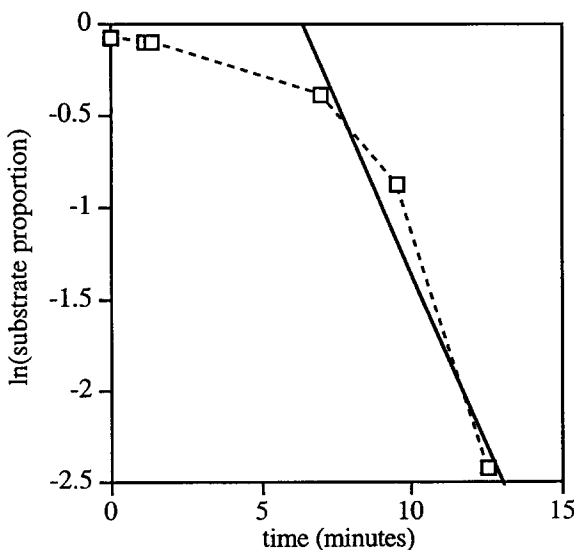


FIG. 4. A semilog plot of substrate proportion versus time. The solid line represents the best-fit linear slope following the characteristic lag time in the crystalline hammerhead ribozyme reaction, as measured by the HPLC assay.

coefficients between the substrate and product strands. The area of the substrate peaks serves as an internal control so that the  $(A_{\text{sub}} + 1.25 A_{\text{prod}})$  to  $A_{\text{sub}}$  ratio should remain constant. In our crystallized hammerhead ribozyme cleavage experiments, the ratio did in fact remain at  $1.51 \pm 0.03$ . A semilog plot of product production versus time reveals that a small proportion of the crystallized hammerhead RNA (about 7%) had cleaved during crystallization or subsequent storage and manipulation. Then after an initial lag phase of approximately 6 or 7 min, the remaining hammerhead RNA in the crystal undergoes cleavage at a rate of  $0.4 \text{ min}^{-1}$  (Fig. 4). The cause of the initial lag phase is unclear. Because the cleavage reaction is initiated by elevation of pH, and because the lag time increases with experiments done at lower pH values, it may be that the pH within the crystal is raised more slowly than would be expected based on the simple diffusion considerations described earlier, or it may reflect an activating conformational change that appears to take place in a concerted manner throughout the crystal lattice instead of in the stochastic manner one would expect from solution studies. These peculiarities, in addition to the fact that this particular RNA sequence actually cleaves several times *faster* in the crystalline lattice than it does in solution, illustrate the need to characterize the kinetics of the ribozyme cleavage reaction *in the crystal* when undertaking time-resolved crystallographic studies.

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## [14] Nuclear Magnetic Resonance Methods to Study RNA-Protein Complexes

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### Introduction

Structural studies of RNA-binding proteins and their RNA complexes are critical to our understanding of specificity in RNA-protein recognition and therefore how gene expression is regulated.<sup>1,2</sup> Considerable efforts have therefore been directed toward studying the structural principles underlying RNA-protein recognition, using both X-ray crystallography<sup>3-5</sup> and nuclear

<sup>1</sup> G. Varani, *Acc. Chem. Res.* **30**, 189 (1997).

<sup>2</sup> G. Varani and K. Nagai, *Ann. Rev. Biophys. Biomol. Struct.* **27**, 407 (1998).

<sup>3</sup> K. Nagai, *Curr. Op. Struct. Biol.* **6**, 53 (1996).

<sup>4</sup> C. Oubridge, N. Ito, P. R. Evans, C.-H. Teo, and K. Nagai, *Nature* **372**, 432 (1994).

<sup>5</sup> S. R. Price, P. R. Evans, and K. Nagai, *Nature* **394**, 645 (1998).