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Visualizing the structure and mechanism of a small nucleolytic ribozyme

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Abstract

Time-resolved crystallography has recently evolved into a powerful and invaluable technique for observing conformational and chemical intermediate states at or near atomic resolution in protein enzymes. The application of monochromatic time-resolved X-ray crystallographic freeze-trapping experiments to an RNA enzyme, the hammerhead ribozyme, is outlined here.

1. Introduction

Time-resolved crystallography enables the direct visualization of a conformational or chemical intermediate state in an enzyme's reaction pathway that either by nature or through trapping strategies accumulates at least transiently to high occupancy throughout the crystal lattice in a synchronized manner. For this to happen, the enzymatic state that one hopes to observe must be a true intermediate that corresponds to a well-defined local minimum on the potential energy surface that includes the reaction coordinate. For this reason a transition state, which occupies a saddle-point on the potential energy surface, cannot be observed directly.

Two strategies have been employed to do this. They rely on two very different approaches to X-ray data collection. One is Laue time-resolved crystallography, which makes use of a highly intense polychromatic X-ray pulse of picosecond to millisecond duration to obtain a diffraction pattern "snapshot" of an intermediate whose lifetime is of a corresponding duration. Its success relies heavily on synchronizing reaction initiation with data collection to a high degree of accuracy. Hence the use of photochemical triggers designed using photolabile caged substrate precursors that liberate active substrate on flash photolysis has proven invaluable in the development of such techniques. These experiments are also only possible using a high-intensity Laue X-ray source at one of several dedicated synchrotron beamlines [1].

The other strategy, most practical with systems that either by nature or with modification have a rate-limiting step of seconds or longer, involves trapping a transiently accumulated conformational or reactive intermediate simply by flash-freezing a crystal in liquid nitrogen or propane in a manner essentially identical to that routinely employed for conventional X-ray data collection. The intermediate state, thus physically trapped in a vitreous ice and maintained at 100 K, is essentially immortalized for purposes of data collection, which is then carried out in the conventional manner using monochromatic X-rays [2].

2. Ribozymes and crystallographic freeze-trapping experiments

Compared to protein enzymes, ribozymes have quite slow turnover rates, at least under standard in vitro conditions [3]. (The peptidyltransferase of the ribosome is the major exception, but its chemistry is also rather different from all of the other ribozymes in other respects, most notably in that it does something other than phosphodiester isomerization or hydrolysis.) This means many of the technical difficulties inherent to Laue data collection, including those mentioned above and the exacting requirement for crystals of very low mosaicity,

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can be avoided. For small, self-cleaving ribozymes such as the hammerhead ribozyme, typical in vitro cleavage rates of well-behaved ribozymes are on the order of one turnover per minute [4]. The turnover rate, which is highly sequence dependent, also can be modulated using pH as a controlling variable. Hence the turnover rate can often be adjusted for the convenience of the experimental design rather than the opposite, making time-resolved monochromatic X-ray freeze-trapping experiments an ideal choice for capturing and observing intermediate states along the reaction coordinate of ribozyme catalysis [5].

3. Characterization of the ribozyme reaction critical to experimental design

By the time that the first ribozyme structures began to emerge, RNA catalysis had been well characterized for many years. The hammerhead ribozyme and several others had been observed to have a pH dependence of the reaction rate that varied log linearly; in the case of the hammerhead ribozyme, it does so with a slope of 0.7 [6]. The simplest interpretation of this is that a single proton transfer occurs in the rate-limiting step of the hammerhead ribozyme reaction, and the degree of proton dissociation in the transition state is 70%. These analyses also showed that the rate tended to plateau at higher pH values, leading to an apparent pK_a of the reaction, using conventional extrapolation methods [7], of about 8.5 in the presence of Mg^{2+} and of about 8.0 in the presence of Cd^{2+} and Co^{2+} [6].

The cleavage reaction was also observed to be divalent metal ion dependent, and although the natural divalent metal ion is assumed to be Mg^{2+}, it was found that many other divalent metal ions, including Mn^{2+}, Co^{2+}, and Cd^{2+}, actually enabled the ribozyme to cleave itself faster [6].

Both observations proved to be invaluable for carrying out cleavage reactions in the crystal. Unmodified hammerhead RNA crystals produced at pH 5 cleave very slowly if at all in the absence of divalent metal ions. These crystals were obtained in 1.8 M Li_2SO_4 and 50 mM Mes or cacodylate buffer with 1–5 mM EDTA added to sequester any divalent or trivalent metal ions present [5].

The cleavage reaction could be initiated simply by soaking the crystals in a large excess of an artificial mother liquor that contained 50 mM of one of the active divalent metal ions, 1.8 M Li_2SO_4, and 50 mM Tris, pH 8.5 [5,8]. Divalent metal ions other than Mg^{2+} have the advantage of being more easily detected in an X-ray diffraction experiment. Raising the pH to a value at or above the apparent pK_a of the rate-limiting step of the reaction helps to maximize the probability that a significant fraction of the molecules populating the crystal lattice will be activated for catalysis.

4. Use of a modification that creates a kinetic bottleneck

The chemical step of a self-cleaving RNA reaction is of course the one in which the phosphodiester bond breaks. In the case of the hammerhead ribozyme, previous experiments had identified a modified ribose having an extra *talo*-methylation at the 5′-carbon adjacent to the leaving-group oxygen as one that creates a bottleneck specifically at the bond-breaking step of the cleavage reaction. This modification slows the cleavage reaction about 20-fold in solution and at least 100-fold in the crystal [8]. The reaction can be initiated normally because the 2′-OH attacking nucleophile is unaltered and obeys the usual pH dependence.

Used in conjunction with crystallographic freeze-trapping, this modified RNA, whose initial-state structure was shown to be identical to that of the unmodified hammerhead ribozyme, facilitated observing a precatalytic conformational change that accumulated to apparent full occupancy in the crystal lattice prior to cleavage. The conformational change involved rotation of the cleavage-site base and the adjacent ribose as a single rigid body about two pivot points. One of these is the β-angle of the cleavage-site ribose–phosphate linkage, and the other is the β-angle of the ribose–phosphate between the first and the second nucleotides in Stem I, just 3′ to the cleavage site. The net result is that the 2′-oxygen becomes positioned for an in-line attack, the known mechanism for hammerhead ribozyme self-cleavage. Based on this result, we concluded that such a conformational change constituted at least a subset of the movements required for hammerhead ribozyme catalysis [8].

The use of active-site mutations in protein enzymes has proven invaluable for creating kinetic bottlenecks that facilitate observation of chemical intermediates [9,10]. This approach was modified only slightly in that the substrate, rather than the enzyme, was modified in the case of the hammerhead ribozyme, and a conformational, rather than a chemical, intermediate was trapped and observed.

5. Control experiments useful for correlating observed structures with enzymatic states

Enzyme kinetics and mechanistic studies provide a necessary framework for understanding how an enzyme works. Each enzyme–substrate, enzyme–intermediate, or enzyme–product complex must correspond to a unique chemical or conformational intermediate along the reaction pathway. However, the difficulty is in assigning an observed structure to one of the known (or possibly unknown) enzymatic states. Enzymes such as bacteriorhodopsin that have unique spectroscopic signatures make this task relatively straightforward, as the

spectrum of the protein in the crystal may be recorded at the same time as X-ray data are collected [10]. RNA, however, has no such convenient spectroscopic handle, so the task of assigning a structure to a unique kinetic state is more difficult.

In the case of the hammerhead ribozyme, the biochemical characterization of the reaction is somewhat self-contradictory. Essentially all of the enzymology experiments are interpreted in terms of a simple Michaelis–Menten mechanism in which only one intermediate, the enzyme–substrate complex, is invoked, and a simple turnover scheme in which k_2 is identified with k_{cat} or the chemical step of the reaction is almost always assumed. The more thorough analyses [4,11] take into account enzyme–product complexes in which either or both of the product strands are associated, but these states may generally be neglected in the realm of single-turnover reactions. Because k_2 is so slow, ($\sim$1/min), K_m is identified with substrate binding and k_2 is identified with the chemical transition state in the self-cleavage reaction.

With hammerhead ribozyme structures from two research groups [12,13] showing the need for a conformational change of some sort to reach the known in-line transition state of the chemical step of the reaction, it has generally been assumed that there must exist an additional intermediate. (Alternatively, it has been asserted that bonding interactions absent in the ground state appear only in the transition state, but this violates the assumption that a transition state is a saddle-point on a potential energy surface; i.e., a transition state is not a species in which unique bonding interactions can exist.) That additional intermediate has so far escaped detection by enzymologists [14].

The primary control experiment for assessing the nature of an observed intermediate therefore is limited to an assay for phosphodiester bond breakage. Both gel electrophoresis and HPLC provide the means for assessing this but lack the advantage of immediacy that a spectroscopic probe might allow. For that reason a protocol to assure that the RNA is maintained in the same state in the crystal between data collection and HPLC assay has been developed [15]. This assay enables us to assign the RNA as ''cleaved'' or ''uncleaved,'' but it is incapable of telling us which precatalytic intermediate has been observed or, indeed, whether the ''intermediate'' observed is on the reaction pathway. The best that can be hoped for under such conditions is that the inference that a conformational change that occurs under conditions in which the reaction takes place with high efficiency (i.e., at or above the pK_a) and which involves major movement at the site of catalysis in a direction consistent with the known mechanism of catalysis must be relevant to catalysis is valid. This inference, though seemingly conservative, has met with skepticism [16]. Therefore observation of an identical or at least a similar conformational change using a different experimental approach is probably the best approach to minimizing the chances that a trapped intermediate is an off-pathway artifact.

The HPLC assay in addition enabled us to conduct another experimental control—measuring the rate of catalysis and extent of cleavage of the RNA within the crystal. The rate of cleavage in the crystal in the presence of Mg^{2+} was found to have a log-linear dependence upon pH essentially identical to that in solution, indicating that the same rate-limiting step was being observed in both instances. Moreover the cleavage rate in the crystal was found to be five times faster than that in solution under otherwise identical conditions, and the extent of cleavage in the crystal was essentially complete, whereas the extent of cleavage of the RNA in solution was only about 75%. These controls permitted us to suggest that the crystal lattice apparently did more to assist the reaction than to inhibit it [17].

6. Previous assumptions may be wrong

Skepticism in the opposite direction may also be warranted. Two assumptions about RNA catalysis have dominated most researcher's thinking and therefore experimental design and interpretation. The first of these is that ribozymes are metalloenzymes, relying on divalent metal ions such as Mg^{2+} for both their folding and their catalysis [18,19]. This assumption is based upon the observation that, stripped of the proteins that often are associated with ribozymes in vivo, a dilute electrolyte solution containing RNA requires the presence of approximately 10–100 mM Mg^{2+} for catalytic activity. The second is that an observed pH dependence of the rate-limiting step of a ribozyme reaction entails that the rate-limiting step of the reaction is the chemical step. This assumption appears justified because proton transfer most likely occurs in the chemical step, and nucleotide bases are not normally ionizable between about pH 4 and 9 [4].

Additional control experiments in which the pH was elevated in the crystal as was done to initiate catalysis, but in the absence of divalent metal ions and in the presence of potent chelating agents, demonstrated that divalent metal ions were not required for hammerhead ribozyme cleavage in the crystal. Subsequently it was demonstrated that if enough monovalent salt, including ammonium salts, were present, then no divalent cations were required in solution. This proved to be the case for the hammerhead, hairpin, and VS ribozymes, all of which were previously thought to require divalent metal ions for catalysis [20].

The significance of this observation is that the RNA itself, rather than acting as a passive scaffold for metal ions that bind specifically and cleave the RNA, must be

an active participant in the chemistry of catalysis. Hence this control compels us to identify functional groups within the RNA molecule that are at least in part responsible for mediating catalysis.

A careful set of crystallographic titrations has established that the apparent pK_a of the observed conformational change is approximately pH 8.5 [17]. Much below pH 8.5, the conformational change does not accumulate to high occupancy. Therefore it appears that higher pH selectively stabilizes (and therefore populates) an intermediate state that is higher in potential energy (and thus lower in occupancy) under "standard" pH 7 assay conditions. The simplest interpretation of the similarity between the conformational change apparent pK_a of 8.5 and the kinetic apparent pK_a of 8.5 is that they are identical. If this is the case, it entails that the rate-limiting step is the precatalytic conformational change rather than the chemical step and that the chemical step is significantly faster. Further controls in which the active-site 2′-OH is substituted with 2′-F (isoelectronic to an ionized 2′-O but uncharged) or 2′-OMe (containing the 2′-O but unionizable) indicate that these substitutions inhibit the conformational change even above pH 8.5, a further indication that the conformational change that moves the 2′-O into an attacking position is characterized by k_2 [17].

7. Interpretation of electron density maps

Large-scale conformational changes or structural changes of a chemical intermediate require calculation of electron density maps having the least possible model bias. Two such approaches have proven very useful for generating maps having minimal bias. Both rely on omitting whatever portion of the structure that one suspects might have changed.

A real-space electron density refinement and optimization algorithm called EDEN (for electron density) relies on a formal analogy between the reference wave of a holographic reconstruction and the contribution of the known or partial model of the structure to help to phase the unknown contribution to the X-ray scattering generated by the omitted (unknown) structure, whose scattering is analogous to the object wave in a holography experiment [21]. Using positivity of the electron density as a constraint, this approach has sufficient power to resolve interpretable electron density for a molecule in which at least two thirds of the structure is unknown [22].

A small price to be paid is that the data must be "apodized" (as with NMR data), resulting in an apparent small loss of resolution compared to a conventional $3F_{obs}–2F_{calc}$ map. However, the density is rather more continuous (and thus more easily interpreted). Recent advances in maximum-likelihood refinement combined with sigma-A map calculations as implemented in CNS 1.1 minimize these differences [23,24]; the analogous test case with this more conventional approach yielded a $3F_{obs}–2F_{calc}$ omit map in which density for the two thirds of the molecule omitted was also of surprisingly high quality. In both cases, the calculations were done in the absence of a solvent mask to avoid "clipping" the density of the missing portion of the molecule (see Fig. 1).

Minimally biased electron density maps are thus powerfully equipped to enable detection of even large-scale conformational changes. EDEN possesses the added advantage of having two calculations modes. The "completion" mode does best at problems in which part of the molecule is omitted or unknown. The "correction" mode takes advantage of EDEN's ability to minimize the effects of model bias from structural errors, thus further increasing the reliability of the electron density.

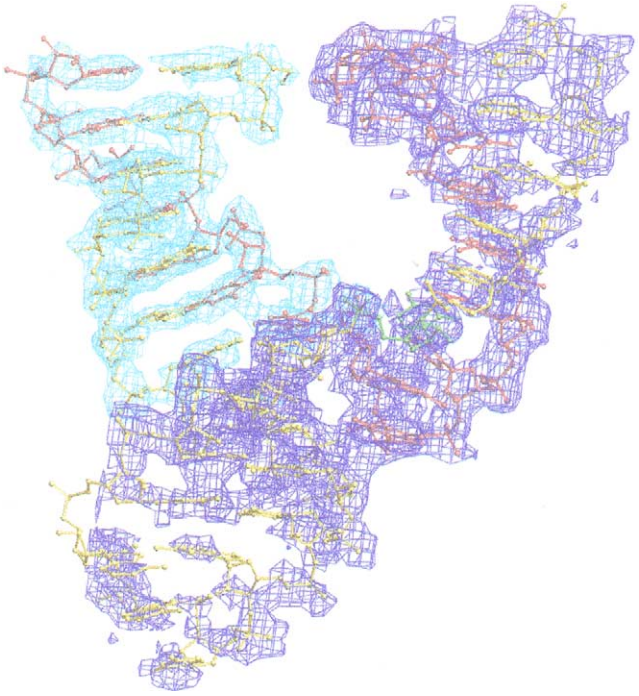

Fig. 1. X-ray holographic reconstruction in EDEN in which only one-third of the molecule (corresponding to the green portion of the map) was used as a partial model to calculate electron density for the omitted region (corresponding to the blue portion of the map). EDEN uses real-space model-independent electron density refinement to produce an F_{obs} map on an absolute scale; F_{calc} values are not determined. Hence the blue portion of the map is contoured at 0.45 while the green portion is contoured at 1.3 to produce the functional equivalent of a $3F_{obs}–2F_{calc}$ map. Such maps are thus ideal for locating large-scale structural changes in a minimally biased manner when only a comparatively small portion of the structure may be trusted to remain static.

8. Representing results: making movies

The ultimate goal of both Laue and monochromatic time-resolved crystallography is to obtain a series of snapshots along the enzyme reaction coordinate. Conventional PDB files represent each individual snapshot faithfully, assuming that the structure was solved correctly. However, a sense of movement can be properly obtained only when the series of snapshots are strung together as individual frames in a motion picture. There is currently no similar standard for representing molecular motion, although a rather comprehensive Database of Macromolecular Movements [25] has been established at http://molmovdb.mbb.yale.edu/MolMovDB/, and a gallery of movies, many augmented using morphing software at http://molmovdb.mbb.yale.edu/MoIMovDB/morph/, has been established at this site.

The development of Quicktime-based or animated gif-based movies from a series of still-image renderings of PDB files is exceptionally straightforward on Mac- and Unix-based platforms, and presentation of time-resolved crystallographic results using such technologies is increasingly commonplace. (Microsoft movie formats tend to present more difficulties.) In addition to being aesthetically appealing, it is much easier for the human eye (and brain) to appreciate and analyze global movements in movie presentations than it is when observing a series of still images displayed simultaneously adjacent to one another. This in turn is crucial for assessing the catalytic relevance of a series of conformational movements and perhaps in gaining new insight into what occurs between snapshots, most notably at the elusive chemical transition state. Implementation of such an analysis gives rise to a new appreciation for the dynamics associated with RNA catalysis [26].

A major improvement to conventional movie-players that display a series of still images of different conformational states of a macromolecule has recently been developed, called PyMol [27]. PyMOL is a molecular graphics visualization free software program based on the Python scripting language that allows rapid assembly and alignment of several pdb files (usually representing distinct conformational states of a given macromolecule) into a single object that can then be displayed as a movie. To my knowledge, it is the only molecular graphics display package specifically designed for the purpose of movie display and making. Display parameters such as playback speed and view are fully configurable and infinitely customizable using commands similar to those used in other crystallographic programs or python commands. This permits a movie of molecular motions to be viewed from a variety of viewpoints simply by moving the molecule around the screen via standard mouse manipulations. Pymol movies can also be saved as a series of png still images thay may subsequently be assembled into Quicktime, avi, or animated gif movies. Because PyMOL is open-source, free software and is based on a portable scripting language, it is easily ported to any modern computer operating system (Mac OSX, Windows, Linux, various Unix flavors, *etc*.) and is thus emerging as a standard for representing macro-molecular motions.

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