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Visualizing enzyme and ribozyme intermediates using fast diffraction and reaction trapping methods

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Abstract: Using mutatagenesis, steady-state trapping, and photoactivation of caged substrates in two separate series of experiments, we have determined the structures of three sequential intermediates formed by the enzyme isocitrate dehydrogenase. Using pH triggering and physical trapping (flash-cooling), we have determined the structure of an unmodified, fully reactive hammerhead ribozyme construct at two separate stages of the autolytic cleavage reaction, and have proposed a structural mechanism for transition state formation.

Introduction

In order to collect complete diffraction data sets from crystals of macromolecules, the standard technique employed has been monochromatic oscillation photography, with the crystal irradiated using a single incident wavelength and simultaneously oscillated through a large rotational range in order to satisfy Bragg's law for the majority of reflections. Such data sets may be collected in as little as several minutes at a synchrotron with strongly diffracting crystals, and it was shown in the late 1980's that such data can be used to observe the accumulation of intermediate states with extremely long chemical lifetimes (1,2). We have recently reported time-resolved structures of discrete, transient intermediates formed during reactions catalyzed by the enzyme isocitrate dehydrogenase (IDH) and the RNA hammerhead ribozyme (3,4). These studies demonstrate the potential of applying standard enzymatic methods of reaction trapping and spectroscopic analysis to the protein crystal. When done in combination with a data collection strategy specifically tailored to match the lifetime of the intermediate of interest, the resulting time-resolved experiment can be truly illuminating.

Most catalytic and biological intermediates possess lifetimes much less than one second; macromolecular crystallographers have therefore historically sought to prevent enzymatic turnover while visualizing mechanistically relevant complexes, usually through the use of specific inhibitory compounds. IDH is an excellent example of this problem, displaying a turnover number of greater than 50 sec^{-1} per active site, qualifying it as a reasonably efficient enzymatic catalyst. For such a turnover rate, the approximate half-life of a single predominant rate-limited species would be less than 20 milliseconds. However, IDH is typical of most enzymes, in that it follows a catalytic mechanism involving a series of several intermediate states, none of which is uniquely rate-limiting. How then might a protein crystallographer visualize the individual intermediates formed during turnover?

When crystallographers think of the term "time-resolved" crystallography, the image is generally an experiment in which one or more data sets are collected extremely rapidly during the course of a single, efficiently triggered and synchronized turnover event or reaction cycle in the crystal. If the reaction proceeds via a kinetic mechanism with a unique rate-limiting step, then a specific

catalytic intermediate accumulates throughout the majority of the active sites in the crystal for a short period of time. In essence, the intrinsic free-energy profile of the reaction is exploited during such an experiment. The method of polychromatic "Laue" diffraction can be used to collect data on a time-scale equivalent to the lifetime of that intermediate, by using the tremendous flux of x-rays and the full spectral bandpass available at the synchrotron (5,6). Bragg's law is satisfied for the majority of possible unique reflections from a stationary crystal in the time necessary to collect a single x-ray exposure.

The potential difficulties of such an experiment are numerous. Rapid, efficient enzymes can be difficult to trigger uniformly throughout the crystal, may not display a unique rate-limited step, and may proceed through a pathway that produces a statistically complex mixture of intermediates or individual conformers that are difficult to precisely interpret. Additionally, the Laue diffraction geometry produces data that must be deconvoluted at low resolution, and must also be scaled and corrected as a function of the wavelength of the measured intensities. Although a large number of recent experiments (both published and unpublished) demonstrate that the limitations of the Laue geometry may be overcome experimentally, it is still a great challenge to isolate, even transiently, a homogenous population of a discrete reactive intermediate.

Biochemists have for years addressed this problem in solution through specific trapping protocols designed to either rapidly quench reactions at a specific reaction time point, or conversely to alter the free-energy profile of a reaction in a manner that allows the study of specific reaction step or intermediate species (7). A number of related techniques now show considerable promise in the crystal for providing detailed structural information of discrete intermediate states. When these techniques are combined to target a specific catalytic species, the high-resolution structure of that state can often be determined crystallographically. As described previously (8,9), the techniques used to extend the lifetime of a specific intermediate species fall into two broad categories, physical trapping and chemical trapping.

Physical trapping The rate constant for a single kinetic step in a reaction varies directly in a geometric relationship with the temperature, with the magnitude of the decrease in rate dependent on the absolute activation energy for the system. For a step with an activation energy barrier of 4 k_{cal} / mol, a rate of constant measured at 20 ° C is 38-fold lower at -80° C; for an energy barrier of 10 k_{cal} / mol, the same rate constant is reduced by over four orders of magnitude. Hence, by judicious lowering of the temperature of the crystal during turnover, an investigator might be able to trap and observe an accumulated rate-limited species. Using this approach, the free-energy profile of the reaction is presumably unaltered, while the total energy available to the system is decreased substantially. The obvious advantage to the crystallographer is a dramatic increase in the lifetime of the intermediate of interest (which may, under appropriate conditions, either become virtually immortal or at least longer than the time needed for data collection), allowing X-ray strategies requiring longer exposure times.

This method was initially used by maintaining the bulk solvent and channels in the enzyme crystal as a liquid at very low temperatures through the use of mixed cryosolvents, while introducing substrate directly to the crystal (10). However, several difficulties can arise as a result of low temperatures in crystallographic studies of enzyme-ligand complexes. The physical constants of various chemical groups in enzyme catalysts display a large temperature-dependence, including the pK_a's of ionizable groups which are often involved as critical residues in catalysis. The magnitude of the shift in pK_a as a function of temperature is itself dependent on the enthalpy of ionization for a specific group. This implies that the catalytic mechanism may be altered in an unexpected manner by cooling. In addition, the dielectric constant of the reaction medium can be affected by the change in temperature, as well as by the change in medium composition when cryosolvents are used.

Many of these problems can now be avoided to a certain extent by the use of rapid flash-cooling (commonly used to immortalize crystals for data collection) for intermediate trapping experiments (11). In such experiments the intermediate species accumulates at a physiologically relevant temperature during the experiment in response to natural rate barriers; the reaction or the turnover event is then rapidly quenched at liquid nitrogen temperatures for data collection. At these temperatures, protein structures experience a drastic lowering of mobility and flexibility similar in appearance to a phase transition. This effect is capable of hindering the dynamic freedom of the protein and the free exchange of solvent necessary for continued reactivity. Such a temperature effect has been noted for several crystal species and is probably independent of the nature of the macromolecule.

Chemical trapping. Rather than reducing the total energy available to the enzyme catalyst by flash-cooling (and thereby reducing the overall reaction rate), a different strategy may be considered, either separately or in conjunction with flash-cooling. The population and relative occupancy of a specific catalytic intermediate may be elevated, and its structure then determined crystallographically, by adjusting substrate concentrations or solvent conditions so that a particular intermediate has a lower free energy than any other state (12). In essence, the free energy profile of the catalytic reaction is altered and exploited to impose a novel kinetic rate-limit or a thermodynamic dead-end for the reaction. Such techniques can be used to isolate an intermediate within the context of a single-turnover experiment, or alternatively as a high-occupancy, steady-state complex during a multi-turnover protocol. These conditions may include a significant change in the pH of the reaction or conducting the reaction in a perturbed or even a non-aqueous mother liquor (13). Alternatively, enzymes that catalyze single-substrate/single-product reactions (or that proceed through separable half-reactions) may be studied under conditions of thermodynamic equilibrium that favor a single predominant catalytic species (14-17). Yet another method of chemical trapping, described here, is the use of site-directed mutagenesis to create a system with enhanced lifetime and occupancy of a specific catalytic intermediate state (3).

Therefore, a time-resolved crystallographic experiment may be defined, somewhat loosely, as an experiment designed to isolate and accumulate a productive catalytic intermediate throughout the crystal lattice for a well-defined period of time, during which diffraction data is collected in order to carry out a structure determination. In this context, three parameters can be defined: the method used to isolate the intermediate, longest potential time allowed for data collection (which is limited either by the lifetime of the intermediate or by the lifetime of the crystal lattice under the experimental conditions used to accumulate that intermediate), and the necessary method of data collection (which is dictated by the first two parameters). At one extreme end of this spectrum is a single-turnover experiment, triggered by a photolytic event, in which the lifetime of the rate-limited species is extremely short and dictated by the natural reaction profile of the system, necessitating extremely rapid data collection with the Laue method. At the other experimental extreme is a trapped reaction intermediate with a greatly extended lifetime in the crystal, visualized by the use of a slower (and therefore more standard) method of data collection.

Time-resolved studies of isocitrate dehydrogenase using mutagenesis, steady-state accumulation, and freeze-trapping.

We have demonstrated the utility of combing site-directed mutagenesis, kinetics, and fast diffraction to trap and visualize specific sequential intermediate states (3). Such studies, carried out using steady-state methods as described below in conjunction with careful single-turnover strategies, may prove to be the future of crystallographic studies of enzyme intermediates.

Isocitrate dehydrogenase (IDH) from *E. coli* [threo-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.42] catalyzes the conversion of isocitrate to a-ketoglutarate and CO_2. The enzyme is dependent on NADP and on bound metal (usually Mg^{2+}) and lies at an important branch point in carbohydrate metabolism. The enzyme is a dimer of 416 residues per subunit and contains a single catalytic metal per monomer which is tightly chelated by two conserved aspartate residues and by bound isocitrate. The substrate molecule is bound in the

active site primarily through interactions between its free carboxylate groups and several conserved polar residues.

The crystal structure of IDH bound with $NADP^+$ in the absence of substrate provides ordered density only for the adenosyl portion of the cofactor. This observation is attributed to interactions between bound substrate and the nicotinamide ring which are necessary in order to form a fully ordered complex with $NADP^+$. Additionally, in the absence of bound substrate the positively charged nicotinamide ring is subject to repulsion by positively charged residues in the binding site. Reduction of the cofactor to NADPH also leads to disorder of the nicotinamide ring even in the presence of bound substrate.

After formation of the initial ternary Michaelis complex, IDH catalyzes two sequential reactions: 2R-3S isocitrate is oxidized (by transfer of a hydride ion from C2 of isocitrate to the cofactor C4 carbon), forming an enol intermediate which is thought to be oxalosuccinate, followed by the rate-limiting decarboxylation of this species to α-ketoglutarate and CO_2. Since the position of the nicotinamide ring was undetermined in the binary complex of $NADP^+$, the precise interactions between substrate, cofactor, and enzyme side chains during hydride transfer were undetermined and represent an intermediate structural state of importance to the overall reaction mechanism. This transient structure represents a distinct structural intermediate that can be studied and compared to the known mechanism of regulation, substrate-assisted cofactor binding, and the stereochemistry of hydride transfer. Additionally, the formation of oxalosuccinate, while reasonable chemically and supported by isotope effect studies, is not unambiguously supported by rate studies with the actual proposed intermediate; therefore direct structural identification of this chemical species in the active site prior to decarboxylation is a second, independent time-resolved experimental goal.

In the steady-state study two mutants: (i) Y160F, in which Tyr^{160} was converted to Phe, and (ii) K230M, in which Lys^{230} was converted to methionine, were used to accumulate specific intermediates in the crystal by creating bottlenecks in the enzymatic reaction. The evidence that the mutants follow the same chemical mechanism as the wild type enzyme are: (1) the mutagenized proteins have the same structure as wild-type enzyme, (2) they bind substrate at the active site in a conformation and position identical to wild-type enzyme, (3) they exhibit the same $NADP^+$-dependent process of dehydrogenation as the wild-type enzyme, and (4) although for each mutant a unique step in the reaction pathway is slowed by the localized mutagenesis, their remaining individual catalytic steps proceed at rates comparable to the wild-type enzyme.

Polychromatic x-ray diffraction data were collected first from several crystals of each mutant before continuous presentation of saturating substrate with a flow cell, and then at two specific time-points after accumulation of the steady-state complex while continuing to apply substrate. The Laue method of data collection was used in order to collect complete data sets during the very short life-times of the crystals undergoing turnover. Multiple crystals were used for each mutant (rather than collecting several exposures from the same crystal in different orientations before and after substrate binding) because these crystals will not physically survive an extended presentation of substrate and slow turnover in addition to multiple polychromatic x-ray exposures.

Y160F allows visualization of the rate-limited ternary Michaelis complex. Kinetic experiments (see below) show that Y160F exhibits a large reduction in the rate of hydride transfer from isocitrate, and that there is no difference in apparent binding constants and on-rates, or in the rate of the decarboxylation step that directly follows the hydride transfer. A high percentage of the enzyme active sites in the crystal accumulate this productive complex during continuous saturation of the crystal with high concentrations of the substrates. The electron density from difference Fourier maps can be modeled by $NADP^+$ and isocitrate-Mg^{2+} by means of simple adjustments in torsion angles through the pyrophosphate backbone. The crystallographic electron density for the adenosyl portion of the cofactor and for the bound isocitrate molecule agree with that for previously solved binary complexes of IDH (Hurley *et al.* 1990b, 1991).

Isocitrate is closely associated with the face of the sp^2 prochiral C4 carbon of the nicotinamide ring, as expected for an A-type dehydrogenase such as IDH. This alignment allows hydride transfer from the C2 carbon of the substrate to C4 of the nicotinamide ring, with stereospecific incorporation of a hydrogen at that position. The distance from the hydride donor to acceptor is 3.0 Å, and the angle through the planar nicotinamide ring to the substrate hydride donor carbon is 110°. This distance and angle are consistent with other previously solved dehydrogenase ternary complexes, whose bond distances range from 2.3 to 3.9 Å and angles of 101° to 147°, respectively. A series of interactions between the cofactor and the enzyme appear to play a role in positioning the nicotinamide ring properly and in activating its C4 carbon for electrophilic substitution, thereby lowering the transition-state energy during dehydration. These contacts represent the most significant protein dynamic movements when the Michaelis complex is formed. The side chain of Thr^{104} moves by 2.5 Å to form a hydrogen bond to the 2'-hydroxyl oxygen of the nicotinamide ribose. The distance between oxygens in the complex is 2.7 Å. This movement appears to stabilize the sugar in the proper configuration necessary to bring the nicotinamide C4 carbon within distance for the hydride transfer. In addition, Asn^{115} moves by 1.0 Å to make a 2.9 Å contact with the cofactor at the carboxamide group. Finally, a pair of conserved residues (Lys^{100} and Glu^{336}), not previously assigned a role in turnover, both make specific interactions with the carboxyamide group of IDH and appear to orient the nicotinamide ring and facilitate hydride transfer through electron inductive effects.

K230M allows visualization of the subsequent oxalosuccinate complex. The lysine to methionine mutation at residue 230 causes a marked decrease in the rate of enzymatic decarboxylation of oxalosuccinate, but a much smaller reduction in the preceding rate of hydride transfer, causing the accumulation of an intermediate complex of oxalosuccinate, Mg^{2+}, and reduced cofactor (NADPH) in the crystal. Thus, an experiment similar to that performed for the Y160F mutant allows visualization of an intermediate in which oxalosuccinate is clearly visible.

The results of this steady-state Laue study are fully consistent with kinetic measurements of the enzyme. The overall forward rate constants of the wild-type enzyme and both mutants have been measured in the crystal. For both mutants, the maximum turnover rate was approximately 90% of the maximal rate in solution, whereas the apparent binding constants were all about 20 times higher because of competition with sulfate for binding. The site-directed mutants both turn over at a rate less than 1 sec^{-1} and form saturated, steady-state substrate complexes in the crystallographic flow cell. Calculations of the rate of formation of product in these experiments, compared to the number of enzyme molecules in the crystals, demonstrate that the enzyme is binding substrate and turning over throughout the body of the crystal rather than only at the surface. The K230M mutant is extremely slow to catalyze the elimination of the β-carboxyl. The ratio of the rate of formation to the rate of degradation of oxalosuccinate, which forms the partition driving the accumulation of this intermediate, is greater than 10^3, and the overall rate constant is 0.85 sec^{-1}. Conversely, the Y160F mutant displays a wild-type rate of decarboxylation, but is slowed during the initial hydride transfer and formation of oxalosuccinate by over 10^3, with an overall rate constant of 0.311 sec^{-1}.

Time-resolved studies of RNA strand cleavage by the hammerhead ribozyme using pH jump triggering and flash-cooling.

The structure of a catalytically reactive, unmodified hammerhead ribozyme was solved as a collaborative effort with the Klug group (MRC, Cambridge) (4). The reactivity and internal equilibrium of the ribozyme were measured in solution and in the crystal. The ribozyme cleaves itself efficiently in the crystal in a metal-dependent manner. In the crystal at neutral pH, the equilibrium strongly favors re-ligation of the free ends, whereas at pH 8.5 cleavage takes place with a rate constant of 0.14 min^{-1}. This biologically active RNA fold is very similar to those determined previously for two modified hammerhead ribozymes. Addition of divalent cations at low pH enables us to capture the uncleaved RNA in metal-bound form. We have also captured a conformational intermediate, with a new Mg(II) bound to the cleavage-site phosphate, by freeze-

trapping the RNA at an active pH prior to cleavage. The most significant conformational changes are limited to the active site of the ribozyme, and the new conformation requires only small additional movements to reach the transition-state.

Unlike the structures of the unmodified, metal-free hammerhead RNA and the Mn(II)-bound RNA at low pH, both of which are similar to the ribozyme-inhibitor structures, the higher pH structure reveals a significant conformational change in the region of the cleavage site, induced by the presence of 100 mM $MgSO_4$ at pH 8.5. The rest of the molecule, including the regions flanking the cleavage site and catalytic pocket, (*i.e.*, Stems I, II and III, and the augmenting region of Stem II), remains essentially unchanged. The conformational change is most pronounced in the substrate strand cleavage-site nucleotide (C-17) phosphodiester backbone and base, as well as in the backbone of the residue immediately 3' to the cleavage site (A-1.1). This results in a localized "upward" translation of the substrate strand relative to the catalytic pocket. The relevance of the location of this conformational change is immediately apparent, as it is the scissile phosphodiester bond, located between C-17 and A-1.1, which moves the most (2.9 Å). The averaged movement of the ribose and phosphate between C-17 and A-1.1 is 2.0 Å, and of the ribose and phosphate 5' to C-17 is 1.8 Å.

In addition to the backbone conformational change in the substrate strand, the base and sugar of C-17 move relative to the catalytic pocket residues. C-17 is no longer within good hydrogen bonding distance of C-3, moving from 3.2 Å to 3.65 Å apart. In addition, the furanose oxygen ($O_{4'}$) of the ribose of C-17 no longer stacks directly upon the aromatic base of A-6 in the catalytic pocket, but rather the nucleotide as a whole has pivoted "outward" such that a fairly good base-stacking interaction between C-17 and A-6 is achieved. G-5 and A-6 in turn pivot slightly upward either to accommodate C-17 in this new conformation or perhaps to induce it. The freeze-trapped conformational intermediate is stabilized primarily by a new hydrogen bond which forms between the furanose oxygen of C-17 and the 2'-hydroxyl of U-16.1. In addition, the hydrogen bonding distance between the endocyclic nitrogen N_3 of A-6 and the 2'-hydroxyl of U-16.1 is now decreased from 3.1 Å to 2.6 Å. These hydrogen bonds perhaps compensate for the loss of the hydrogen bond between C-17 and C-3 caused by the conformational change. Both hydrogen bond interactions are consistent with the observation that replacing U-16.1 with deoxy-T significantly reduces the activity of the hammerhead RNA[25]. It is quite possible that elimination of the potential for hydrogen bond formation between the 2'-hydroxyl of U-16.1 and the furanose oxygen of C-17 would destabilize the structure of the conformational intermediate, thus accounting for the observed 10-fold diminished activity in such a ribozyme.

In light of the new structure, it appears that a new Mg(II) ion, at Site 6, binds to the *pro*-R phosphate oxygen at the cleavage site before the RNA adopts a conformation compatible with in-line attack. Several possibilities thus emerge for the mechanism of cleavage. One possibility is that two Mg(II) ions are required for initiating cleavage; one bound to the *pro*-R phosphate oxygen at Site 6, and the other (possibly originating at Site 3) in the form of a metal hydroxide which then attacks the cleavage-site 2'-hydroxyl upon further RNA conformational change. Another possibility, which we favor in view of its simplicity and explanatory power, is that a single Mg(II) binds first to the *pro*-R phosphate oxygen at Site 6, and subsequently induces the conformational change required for in-line attack at the cleavage-site 2'-hydroxyl moiety. In this case the same metal that binds to the *pro*-R phosphate oxygen also provides the hydroxide which initiates the base-catalyzed step of the cleavage reaction. The phosphate oxygen-metal complex may alter favorably the effective pK_a of the metal hydroxide, thus activating the cleavage reaction.

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