It's show time

New crystallographic techniques make it possible to observe directly all of the intermediates in an enzymatic reaction. Such a series of structures can be combined to create a detailed movie of enzymatic catalysis.

The goal of any enzymologist is a complete understanding of how an enzyme catalyzes a chemical reaction. Until recently, this aspiration was satisfied by determining both a kinetic and a chemical mechanism. The kinetic mechanism for an enzyme is complete when the order of events and the rates of all of those events have been determined for one catalytic cycle. The chemical mechanism is complete when the structures of all of the intermediates and transition states between those intermediates have been described. Figuring out these mechanisms usually requires a variety of techniques, which commonly include steady-state and pre-steady-state kinetic analyses, inhibition studies and determining of the effects of isotopes on the reaction.

What is generally missing from these studies is a description of all of the interactions formed between the enzyme and the intermediates on the reaction pathway, including all of the motions of the protein necessary to form these transient bonds. The important residues in the active site of an enzyme can often be identified — for example, by chemical modification studies, pH profiles, site-directed mutagenesis and sequence comparisons — but these studies fall far short of explaining how the key residues are used during catalysis. What enzymologists would really like to have is a series of structures at all of the important points of the reaction. When done with true substrates, such a molecular movie would constitute a new type of mechanism in enzymology — the structural mechanism.

X-ray crystallography is the obvious tool to use to determine the structural mechanism of an enzyme. Unfortunately (as collaborators around the world can attest), crystallography is slow and enzymes are very fast. Even under the best circumstances, monochromatic data collection takes hours, whereas enzymes usually take only milliseconds to catalyze a reaction. During the long time necessary to collect a useful X-ray diffraction data set, the enzyme cycles through many catalytic events. Under normal conditions, the structures determined from such

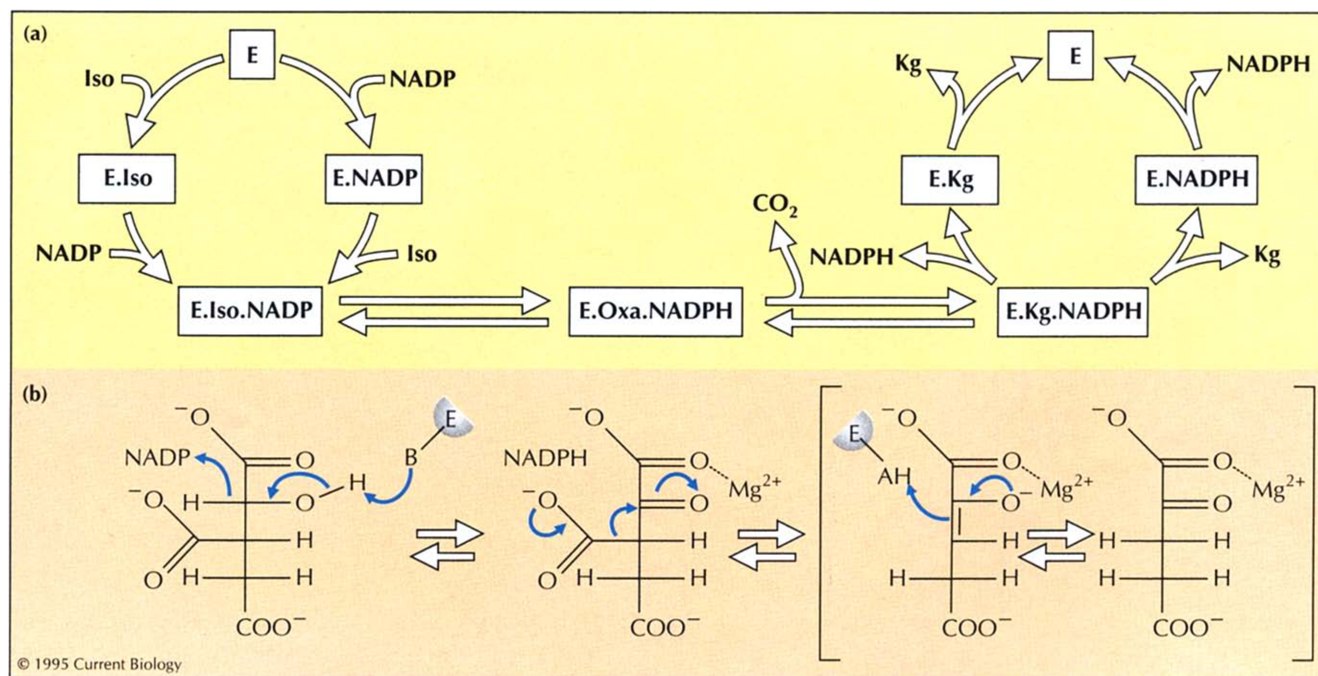
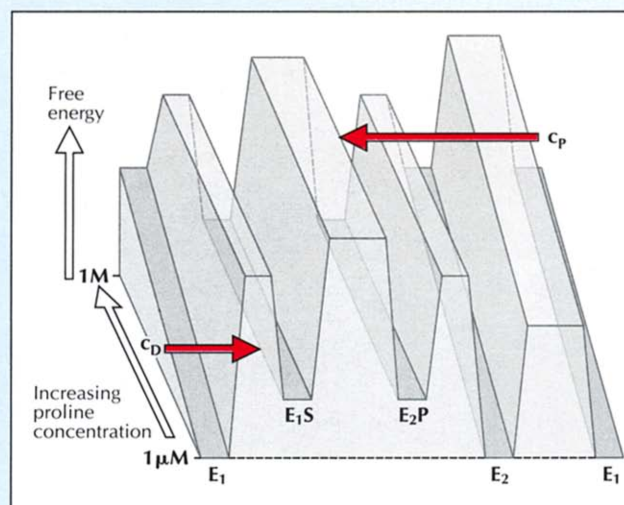


Fig. 1. (a) The kinetic mechanism of isocitrate dehydrogenase [11,12]. Either NADP or isocitrate (Iso) can bind to the apo enzyme (E). Once both molecules are bound, the first of the ternary complexes (E·Iso·NADP) is formed. During the first chemical step, the isocitrate is converted to oxalosuccinate (Oxa). The oxalosuccinate intermediate (E·Oxa·NADP) then decarboxylates, leaving a final ternary complex of α-ketoglutarate (Kg) and NADPH (E·Kg·NADPH); the two products dissociate in a random fashion, leaving the apo enzyme ready for another round of catalysis. **(b)** The chemical mechanism of isocitrate dehydrogenase. The first panel corresponds to the ternary complex E·Iso·NADP. The enzymatic base (B) is probably Asp 283 [9]. In the second step, the essential metal ion stabilizes the anion generated after oxalosuccinate decarboxylates. Finally, an enzymatic acid (AH) donates a proton to form α-ketoglutarate. The identity of this acid is not yet known.

Trapping enzyme-bound intermediates

The key advance that has made it possible to observe many different enzymatic intermediates in a crystal is the realization that altering substrate concentrations changes the free-energy profile for a reaction. Consider the free-energy profile for the reaction catalyzed by proline racemase (see diagram) [13]. There are four different intermediates of interest in this reaction. From left to right in the diagram, these are the native enzyme (E_1), the complex of the native enzyme with L-proline (E_1S), the complex of the native enzyme with D-proline (E_2P) and a second isomer of the native enzyme (E_2). The two forms of the enzyme differ from each other in the protonation state of an active-site residue.



At the front of the free-energy profile, the proline concentration is low (1 μM). If 1 μM proline were added to crystals of proline racemase, the resulting structure would show a mixture of E_1 and E_2. At the concentration marked c_D, the free energies of the native enzyme and the intermediates with proline are the same. Crystal structures determined above this substrate concentration would be dominated by the intermediates E_1S and E_2P.

In contrast to equilibrium experiments, the use of a flow cell allows the single intermediate preceding a rate-determining step to be trapped. In the case of proline racemase, the rate-determining step for the reaction changes at the concentration marked c_P. If substrate (S) were flowed through the crystal at a concentration less than c_P, the intermediate E_1S would be observed; at substrate concentrations higher than c_P, E_2 would be trapped.

For isocitrate dehydrogenase, site-directed mutants were used by Bolduc *et al.* [7] to make particular steps in the reaction rate limiting. A very high concentration of substrate was then flowed through the crystal to populate the intermediate preceding the rate-determining step. The Y160F mutant made the conversion of isocitrate into oxalosuccinate the slow step in the reaction. Measurement of the kinetics of the other steps in the reaction indicated that they were unaffected by the mutation. When a high concentration of isocitrate was flowed through the crystal, the enzyme–isocitrate–NADP intermediate was trapped. In principle, this intermediate should be stable, and the data could have been collected using conventional data collection techniques. However, crystals of isocitrate dehydrogenase are not stable for very long periods in the presence of high concentrations of substrates, so the Laue method was used to collect the data before the crystal shattered. The enzyme–oxalosuccinate–NADPH intermediate was trapped using the same data collection strategy with a second mutant (K230M).

diffraction data show an empty active site, as the apo (unbound) enzyme has lower free energy than any of the bound intermediates.

These problems in the construction of a structural mechanism can be overcome in two very different ways. By altering the substrate and product concentrations in the crystal lattice, it is sometimes possible to make one enzyme-bound intermediate have a lower free energy than all the others (see box). Some of the intermediates that occur during the reaction catalyzed by chymotrypsin have been trapped in this way [1,2]. The other solution is to collect X-ray data sets on the enzymatic timescale. Using the polychromatic Laue method of data collection at a synchrotron X-ray source, it is now possible to obtain complete data sets on a millisecond timescale [3].

The Laue method would seem, on the surface, to solve most of the problems in determining a structural mechanism. But although the Laue method solves one problem, it creates another. In order for the Laue method to work, some way must be found to initiate the reaction so that all of the molecules in the lattice are at the same state of the reaction simultaneously. Various ways of achieving this requisite synchronicity have been tried. One is to induce a rapid change in a macroscopic parameter (such as the pH) to take the enzyme from an inactive to an active state [4]. Photolabile substrate precursors have been used to deliver substrate to all active sites at the same time by irradiating the crystal with light and then collecting Laue data sets [5]. A related triggering mechanism inactivates the enzyme with a photolabile inhibitor, and then uncages the enzyme with light [6]. All of these techniques have the problem that the synchronization of the reaction is lost rather quickly. The successful Laue experiments reported so far all have trapped only the first enzymatic intermediate in a reaction, and these intermediates have all been rather long lived.

Recent experiments of Bolduc *et al.* [7] have combined site-directed mutagenesis with X-ray crystallography to provide a new method for trap intermediates that occur far along an enzymatic reaction. Bolduc *et al.* studied the reaction catalyzed by isocitrate dehydrogenase. As Figure 1 shows, there are seven intermediates in this reaction. Structures of three of the four binary complexes (those with NADP, isocitrate and α-ketoglutarate) have already been reported [8–10]. In the new work, the structures have been determined of the ternary complex with NADP and isocitrate, as well as of the ternary complex with oxalosuccinate and NADPH. These five available structures give a very clear picture of exactly how isocitrate dehydrogenase works. A sketch of the active site is shown in Figure 2. When isocitrate binds in the active site, both Arg 119 and Arg 129 move to lock the substrate in place [8,9]. These side chains move by as much as 2.1 Å towards isocitrate, and are clearly responsible for making isocitrate a sticky substrate.

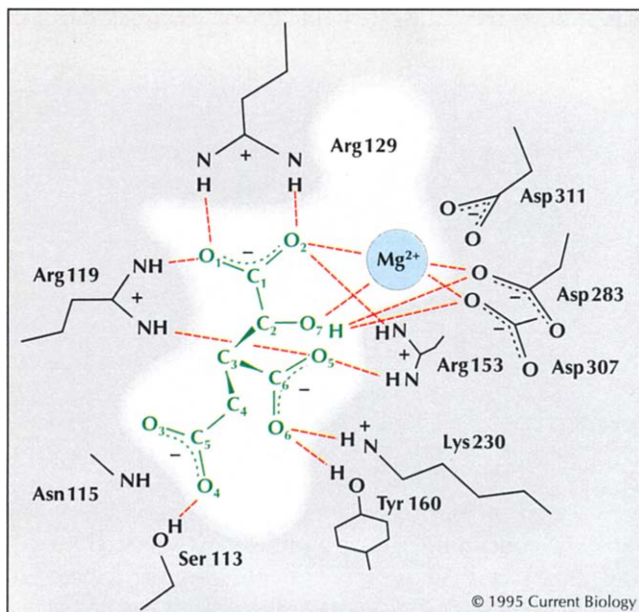


Fig. 2. The active site of isocitrate dehydrogenase. Arg 119 and Arg 129 are the clamps which hold the substrate in place. Lys 230 and Tyr 160 were the sites of the mutations used by Bolduc *et al.* [7]to trap some of the ternary complexes. Ser 113 is the site of regulatory phosphorylation [8].

Although the binding site for isocitrate in isocitrate dehydrogenase is clear from the structure of the binary complex, the exact location of the other substrate, NADP, is not revealed in the structure of its binary complex [9]. NADP is a large molecule with two distinct parts; the reaction chemistry occurs in the nicotinamide ring portion of the molecule. This ring is disordered and unobservable in the binary complex. The other part of NADP, the adenosine 2′,5′-diphosphate, makes a number of good hydrogen-bond interactions with the protein and is clearly observed in the binary complex.

Bolduc *et al.* [7] used site-directed mutagenesis to substitute tyrosine residue 160 of isocitrate dehydrogenase by phenylalanine. When they adjusted the substrate concentrations to high levels and collected data on this Y160F mutant, Bolduc *et al.* were able to trap the ternary complex between the enzyme, isocitrate and NADP. The electron density in the active site of the mutant enzyme reveals the missing portion of the NADP cofactor. The nicotinamide ring was induced to assume a single conformation by the motion of two side chains: Thr 104 moved by 2.5 Å to form a new hydrogen bond to the cofactor, and Asn 115 moved by 1.0 Å to position the nicotinamide cofactor in exactly the right orientation to accept the hydride from isocitrate. These motions were the most significant changes in the active site. The presence of negatively charged isocitrate close to the positively charged nicotinamide ring also helped to stabilize the ring.

In order to observe the next intermediate on the pathway, a second mutant, with lysine residue 230 replaced by methionine, was used. With this K230M mutant, the rate-determining step in the reaction occurs between the intermediate with oxalosuccinate and the intermediate with α-ketoglutarate. There has been some question as to whether oxalosuccinate is a true intermediate in the reaction. In order to settle this controversy, isocitrate and α-ketoglutarate were both refined against the data. Difference maps clearly show that the intermediate in the active site of the Laue experiment is oxalosuccinate. Interestingly, in this intermediate, both Thr 104 and Asn 115 have returned to their normal positions, and as a result, the nicotinamide portion of the NADPH cofactor is once again disordered.

This set of structures presents an almost complete view of how isocitrate dehydrogenase works. The local motions which are necessary for catalysis could not have been guessed from the structure of the native enzyme alone or even from the structures of the binary complexes. However, the structural mechanism for isocitrate dehydrogenase is not yet complete. The identity of the enzyme bound acid (AH in Fig. 1b) is still not clear. Presumably this character will be introduced in the final frames of the movie (the binary complex with NADPH and the ternary complex of NADPH with α-ketoglutarate). The isocitrate dehydrogenase movie has clearly become a good detective story, and like all good mysteries the culprit will not be revealed until the end of the show.

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