Visualizing enzyme intermediates using fast diffraction and reaction trapping methods: isocitrate dehydrogenase

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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 1980s that such data can be used to observe the accumulation of intermediate states with extremely long chemical lifetimes [1,2].

Time-resolved crystallography uses a group of related techniques for reaction trapping and data collection. The experiments are designed to accumulate a specific catalytic intermediate throughout the crystal for a short period of time, during which diffraction data is collected [3–6]. In this context, three parameters can be defined: the method used to isolate the intermediate, the longest exposure time allowed for data collection (which is limited either by the lifetime of the intermediate or by the lifetime of the crystal during the experiment), and the method of data collection. At one end of the spectrum is a single-turnover experiment, triggered by a photochemical event, in which the lifetime of the rate-limited species is short, necessitating rapid data collection. At the other extreme is a reaction intermediate trapped by chemical and physical techniques, usually visualized by the use of a slower method of data collection.

The techniques used to extend the lifetime

Abbreviations used: IDH, isocitrate dehydrogenase; OSA, oxalosuccinate; ES, enzyme–substrate.
of an intermediate species fall into two broad categories, physical and chemical trapping [7].

Physical trapping
The rate constant for a single step in a reaction decreases at lower temperatures, with the magnitude of the decrease dependent on the absolute activation energy. By judicious lowering of the temperature of the crystal during turnover, an investigator might trap and observe a rate-limited species. The obvious advantage to the crystallographer is an increase in the lifetime of the intermediate of interest (which may, under appropriate conditions, become virtually infinite), allowing X-ray strategies requiring longer exposures. Under these conditions, protein structures experience a lowering of mobility and flexibility similar to a phase transition [8]. Two different protocols may be considered. In the first, the crystal is cooled during turnover, followed by data collection (‘freeze-trap’). Alternatively, investigators may accumulate and trap a catalytic intermediate and then flash-cool the crystals (‘trap-freeze’). In such experiments the intermediate species accumulate at a physiologically relevant temperature in response to natural rate barriers; the reaction of the turnover event is then rapidly quenched to cryological temperatures for data collection [9]. By using both strategies, entirely different structural intermediates may be isolated for structure determination. Such experiments may be performed under multi- or single-turnover conditions.

Chemical trapping
A different strategy may also be considered, either separately or in conjunction with low temperature. The relative occupancy of a specific catalytic intermediate may be elevated, and its structure determined, by adjusting reaction conditions so that a particular intermediate has a lower free energy than any other state. In essence, the kinetic profile of the catalytic reaction is altered and exploited to impose a novel rate-limit or a thermodynamic dead-end. Such techniques can be used to isolate an intermediate within the context of a single-turnover experiment, or as a high-occupancy, steady-state complex. These experiments may employ a significant change in the pH of the reaction or a perturbed, or even non-aqueous, mother liquor [10]. Alternatively, enzymes that catalyse single-substrate/single-product reactions (or that proceed through separable half-reactions) may be studied under conditions of thermodynamic equilibrium that favour a single predominant catalytic species. Another method of chemical trapping is the use of site-directed mutagenesis to create a system with enhanced lifetime and occupancy of a specific catalytic intermediate [11].

The enzyme isocitrate dehydrogenase (IDH) provides an excellent example of the challenges of time-resolved studies. This enzyme catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate and CO₂, via formation of an oxaloacetate (OSA) intermediate. IDH exhibits a catalytic mechanism with several rapid steps before the rate-limiting dissociation of products (Figure 1). The enzyme is fully reactive in the crystal [12], and under typical conditions (pH 7.5, 22°C) the enzyme turnover rate (60 to 70 s⁻¹) corresponds to a half-life for the bound product complex of approximately 10 ms, which can be extended to 40–50 ms by moderately lowering either the temperature to 4°C or the pH to 6.5. Initial structures of IDH apo- and phospho-enzyme, along with two stable binary substrate complexes, were solved by traditional monochromatic diffraction methods [13–16].

Multi-turnover experiments and the Michaelis complex: structural factors influencing hydride transfer rates
In order to accumulate the initial enzyme–substrate (ES) complex and the subsequent OSA intermediate, two separate site-directed mutants were used to impose specific kinetic barriers along the reaction coordinate, which resulted in half-lives for specific intermediate species of the order of tens of seconds. These mutants allowed steady-state accumulation of the rate-limited species in the crystal to be combined with Laue data collection [11] and molecular dynamics simulations [17] to describe the structures of two intermediates: the ES complex before hydride transfer, and the OSA intermediate formed before decarboxylation. In the initial ES complex, the most striking structural features suggested by these studies are substrate-dependent ordering of the NADP nicotinamide and ribose rings. This leads to (i) positioning of the nicotinamide ring so that its amide group interacts with the α-carboxyl of isocitrate and the carboxyl of Glu-336, and its carbonyl oxygen interacts with the amino group of Lys-100, and (ii) interactions between the side chain of Asn-115 and both the γ-carboxyl of isocitrate and the amino group of
Figure I

General kinetic mechanism of isocitrate dehydrogenase

The enzyme proceeds through a multi-step reaction pathway, ultimately converting isocitrate and NAD(P) to α-ketoglutarate (KG), CO2, and NAD(P)H. Random binding of substrate and cofactor (2,3) leads to formation of the initial ordered ES Michaelis complex (4). The nicotinamide ring in this complex is structurally ordered primarily through electrostatic and van der Waals contacts with the bound isocitrate. Three subsequent, sequential reaction steps proceed rapidly: reductive dehydrogenation to form oxaloacetate and NADPH (5), decarboxylation to form α-ketoglutarate (6) and dissociation of CO2 to produce the rate-limiting product complex (7). Intermediates 5, 6 and 7 all contribute to the absorbance signal from reduced NADPH cofactor.

Lys-100. These transient interactions produce a distance between the hydride donor and acceptor atoms of 3.7 ± 0.5 Å.

More recently, cryo-trapping methods were employed to further study the factors that contribute to an efficient hydride transfer reaction [18]. This method relies on trapping intermediates with greatly extended half-lives, then flash-cooling (‘trap-freeze’). The reaction trajectory of IDH at this step was modified by (i) changing the adenine moiety of NADP to a hypoxanthine ring (changing the 6-amino group to a 6-hydroryl) and (ii) replacing Mg2+, which has six co-ordinating ligands, with Ca2+, which has eight. Both changes produce large (10−1−10−5) changes in hydride transfer velocity, through small changes in the position of the nicotinamide ring and in the co-ordination and geometry of the metal ligand respectively. The structures of two additional rate-limited ES complexes, perturbed at these different positions in the substrate complex and therefore unable to catalyse hydride transfer efficiently, were compared with the previous ES complex structure of the Y160F enzyme mutant, and the effect of specific structural perturbations on the reaction was assessed. Analysis of these structures indicates that several different geometric aspects of the ES complex are important for efficient turnover: proximity between reactive groups (increased by the NADP to NHDP substitution), angle of hydride transfer (decreased by the Y160F enzyme mutation) and the interaction of substrate with the bound catalytic metal and waters (perturbed by the Mg2+ to Ca2+ substitution).

The enzyme–product complex

Multi-turnover trapping strategies are not easily used to visualize the IDH–product complex, because no readily available techniques have been identified that significantly increase the lifetime of that species. Specifically, no protein mutations are known that specifically reduce the rate of product release. Similarly, conducting steady-state reactions in the crystal at lower temperatures would require non-viscous cryobuffers that are not well characterized kinetically, and further reductions in pH alter the rate-limiting step of the reaction. We have therefore chosen a strategy that relies on a single-turnover Laue experiment with wild-type enzyme at 4°C. In order to conduct this experiment, we have designed and implemented a series of experimental photocaging strategies to synchronize single initial turnover cycles in the enzyme crystal, and used those strategies in conjunction with Laue diffraction. Comparison of the relative effectiveness of each strategy is of general applicability for time-resolved studies on other enzyme systems.

Photolytic liberation of either caged isocitrate or caged NADP (Table 1) and Laue X-ray data collection were used to visualize the complex, which has a minimum half-life of approximately 10 ms. The experiment was conducted with three different photoreactive compounds,
each possessing a unique mechanism leading to the formation of the ES complex. Photoreaction efficiency and subsequent substrate affinities and binding rates in the crystal are critical parameters for these experiments. The structure suggests that CO₂ dissociation is a rapid event that may help drive product formation, and that small conformational changes may contribute to slow product release. A small number of localized differences in the active site side chains and backbone positions, and along the adjoining areas of the dimer interface, are observed for the product complex. These movements are not observed in the structures of IDH in binary complexes with

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<th>Table 1</th>
<th>Chemical structures and photolytic characterization of caged substrate and cofactor analogues</th>
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<tr>
<td>Compound</td>
<td>( k_{\text{on}} ) (product release) ( t_{1/2} )</td>
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<tr>
<td>1. Caged isocitrate</td>
<td>234 s⁻¹ (pH 6.0)</td>
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<tr>
<td>1-(2-nitrophenyl)ethyl-1-hydroxy-1,2-dicarboxy-3-propanecarboxylate (NPE-isocitrate)</td>
<td>10 s⁻¹ (pH 8.0)</td>
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<tr>
<td>2. Affinity caged NADP</td>
<td>13 000 s⁻¹ (pH 7.0)</td>
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<tr>
<td>P^5-[1-(4,5-dimethoxy-2-nitrophenyl)-ethyl] NADP (DMNPE-NADP)</td>
<td></td>
</tr>
<tr>
<td>3. Catalytically caged NADP</td>
<td>42 s⁻¹ (pH 6.0)</td>
</tr>
<tr>
<td>N-(2-carboxy-2-nitrobenzyl)-NADP (CNB-NADP)</td>
<td>30 s⁻¹ (pH 7.0)</td>
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Apart from the inherent advantage of having the isocitrate or with NADP alone [14,16], or in ternary ES or enzyme-inhibitor complexes with isocitrate or oxalosuccinate [11]. This result appears to be consistent with the rate limit observed for product release, which might be coupled to small conformational movements that provide a significant energy barrier to turnover. This result is also consistent with the observation that multiple turnover events in IDH crystals, or direct soaking of product molecules into apo-enzyme crystals, cause significant deterioration in diffraction strength [11,19]. The largest structural difference observed in the active site of IDH between the complexes of isocitrate and α-ketoglutarate involves tyrosine 160, one of two residues involved in an interaction with the labile carboxy group of isocitrate which is lost upon conversion to α-ketoglutarate. Upon hydride transfer, decarboxylation and formation of α-ketoglutarate, this tyrosine side chain and its associated backbone move by over 1 Å and approach the non-bonded side-chain oxygen of Asp-307, a residue which is also involved in metal binding. In addition, movements of 0.5 to 1 Å are observed in the protein backbone of large areas of secondary structure flanking the dimer interface and the active site, including residues 230 to 290 (a region of mixed β-sheet and α-helix), which contain a pair of residues involved in substrate and metal binding (Lys 230', which complexes the labile carboxy group of isocitrate, and Asp 283', which participates in metal binding). This alteration in the packing of secondary structure is manifested to the greatest degree in a movement of the protein backbone of residues 267 to 271. The net effect of these movements appears to be a closure of the active site structure around the decarboxylated, α-keto- glutarate product which now contains a planar, sp²-hybridized C2 carbon and a polar C2 carbonyl group.

Rapid Laue studies have recently been reported on structural intermediates formed during two separate photoreaction processes: CO–myoglobin photolysis and the photocycle of a bacterial phototaxis protein (PYP) [20–24]. Apart from the inherent advantage of having the photoexcitable molecule bound to the protein at virtually 100% occupancy before reaction initiation, these studies were greatly facilitated by efficient triggering kinetics and by well-characterized, separable spectroscopic signals for each reaction intermediate [21,25–27]. This allowed nanosecond time resolution of early events in these reactions. These studies demonstrated that polychromatic X-ray data may be collected and processed to give very complete, highly redundant data sets, and that this data can be used to resolve extremely short-lived species. Additional time-resolved studies on both systems using low-temperature trapping strategies have also been described that provide many additional details on the mechanism of these reactions [28,29].

Compared with time-resolved studies of photoreactions, most enzyme catalysts offer several additional challenges that conspire against the accumulation of homogeneous intermediate populations and their visualization using single-turnover techniques. Photocaged substrates, when used as reaction triggers, are usually not bound to the enzyme before photolysis; diffusion and binding of the released molecule is sometimes slower than the rate of photolysis. Additionally, the photochemical rate and efficiency of many synthetic caging groups are significantly lower than those of natural protein-bound chromophores [30,31]. These effects can greatly diminish the synchronicity of a turnover cycle throughout the crystal. Finally, while many photoreaction processes can be repeatedly or continuously 'pumped' by a light source (allowing the crystallographer to take multiple exposures from a single crystal), similar experiments on enzyme catalysts, using irreversible release of photocaged substrates, usually involve single exposures for each crystal before and after the reaction initiation. This necessitates the use of multiple crystals to produce complete data sets, and long enough exposure times to ensure sufficient diffraction signal.

**Conclusions**

It is clear, based on a large number of studies from several laboratories, that time-resolved crystallographic methods offer an attractive experimental route towards detailed studies of structural enzymic mechanisms. Early work using the Laue data collection has been criticized for several shortcomings in relation to monochromatic data collection [32]. These problems mostly involved incomplete data sets (particularly evident in the loss of low-resolution terms as a result of harmonic overlap), the need to correct for wavelength-dependent flux and polarization, and the challenge of accurately measuring weak reflections in data sets that are usually collected over short exposure times. However, recent studies demonstrate that these
problems are now thoroughly addressed through the use of new, powerful polychromatic data reduction algorithms such as LAUEVIEW and LEAP and through scrupulous attention to experimental detail [5,33].

Similarly, methods of physical and chemical trapping are being exploited with increasing frequency. Such strategies may provide structural information for species that are not rate-limited in the wild-type reaction pathway. The key to these experiments, regardless of which specific trapping protocol is used, is a clear demonstration that while the free energy profile of the reaction is altered, causing a change in a specific rate constant in the kinetic mechanism, the overall chemical mechanism is not perturbed. This aspect of the experimental protocol can usually be directly examined by independent spectroscopic and kinetic methods, as shown for most of the studies summarized above.

Finally, recently published results dramatically demonstrate the importance of conducting time-resolved studies using more than one method for reaction trapping, in order to fully resolve and describe differences between closely related intermediates. Clearly, the energetic profiles of many reactions are often more complex than simple models describe. Separate data sets, collected at different temperatures or at different time-points after triggering, can visualize measurably different, but closely related intermediate species. The quality of the crystallographic data for these experiments (with $d_{min}$ ranging from 2.3 to 0.85 Å resolution for many of the currently reported systems) is allowing these differences to be exploited through increasingly detailed descriptions of structural mechanism. The challenge to crystallographers, apart from mastering details of data collection and processing, may be the a priori design of trapping protocols specifically tailored to isolate discrete intermediates formed during a generalized reaction step.

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DNA gyrase as a drug target
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Introduction
DNA topoisomerases are enzymes which control the topological state of DNA in cells [1,2]. They are found in all cell types and are essential for cell viability. All topoisomerases are able to relax supercoiled DNA but only bacterial DNA gyrase is able to also introduce supercoils into DNA in a reaction coupled to the hydrolysis of ATP. Gyrase consists of two subunits, GyrA and GyrB, the active enzyme being an A₂B₂ complex [3]. Mechanistic studies have revealed the steps associated with the supercoiling reaction. This process involves the wrapping of DNA (≈130 bp) around the A₂B₂ complex, cleavage of this DNA in both strands (involving the formation of DNA–protein covalent bonds) and passage of a segment of DNA through this double-stranded break. Resealing of the break results in the introduction of two negative supercoils. Catalytic supercoiling requires the hydrolysis of ATP, but limited supercoiling can be achieved in the presence of a non-hydrolysable ATP analogue. As shown in Figure 1, the two gyrase proteins are known to comprise distinct domains whose function in the supercoiling reaction has been established. The N-terminal domain of GyrA is involved in the DNA breakage–resealing reactions of supercoiling while the C-terminal domain is concerned with DNA wrapping. The N-terminal domain of GyrB catalyses ATP hydrolysis while the C-terminal domain interacts with GyrA and DNA.

Abbreviations used: GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein.

Gyrase is an essential enzyme in all bacteria but is not found in eukaryotes and is therefore a good target for antibacterial agents [4]. A number of gyrase-specific agents are currently known, the best studied being the coumarin and quinolone drugs. In this article the current status of our knowledge of the action of coumarins and quinolones on gyrase will be reviewed. It is hoped that an understanding of the mode of action of these compounds at the molecular level will potentiate the design of newer, more effective agents.

Coumarin drugs
Coumarin antibiotics (e.g. novobiocin and coumermycin; Figure 2) are Streptomyces products which target the B protein of DNA gyrase and inhibit gyrase-catalysed DNA supercoiling in vitro [4]. More specifically, the drugs are competitive inhibitors of the ATPase reaction catalysed by GyrB [5,6]. Given the lack of structural resemblance between coumarins and ATP (Figure 2), this result is somewhat surprising. Coumarins are potent inhibitors with $K_v$ values of $\approx 10^{-6}\text{M}$ [6]. However, they have low activity against Gram-negative bacteria (resulting from poor permeability), show toxicity in eukaryotes and are relatively insoluble in water, such that they have not been very successful as clinical drugs. Despite this, our understanding of the molecular basis of the action of coumarins on gyrase is quite advanced.

Analysis of coumarin-resistant bacterial strains from a number of species has identified point mutations to coumarin resistance that map