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Marvels of enzyme catalysis at true atomic resolution: distortions, bond elongations, hidden flips, protonation states and atom identities

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Although general principles of enzyme catalysis are fairly well understood nowadays, many important details of how exactly the substrate is bound and processed in an enzyme remain often invisible and as such elusive. In fortunate cases, structural analysis of enzymes can be accomplished at true atomic resolution thus making possible to shed light on otherwise concealed fine-structural traits of bound substrates, intermediates, cofactors and protein groups. We highlight recent structural studies of enzymes using ultrahigh-resolution X-ray protein crystallography showcasing its enormous potential as a tool in the elucidation of enzymatic mechanisms and in unveiling fundamental principles of enzyme catalysis. We discuss the observation of seemingly hyper-reactive, physically distorted cofactors and intermediates with elongated scissile substrate bonds, the detection of 'hidden' conformational and chemical equilibria and the analysis of protonation states with surprising findings. In delicate cases, atomic resolution is required to unambiguously disclose the identity of atoms as demonstrated for the metal cluster in nitrogenase. In addition to the pivotal structural findings and the implications for our understanding of enzyme catalysis, we further provide a practical framework for resolution enhancement through optimized data acquisition and processing.

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Introduction

Over the past decades, protein crystallography has emerged as an indispensable tool for the elucidation of enzyme mechanisms. It is nowadays routinely used to derive structural information about the overall fold and the active site architecture of enzyme catalysts. When possible, structures are not only determined of the resting state enzyme but also of complexes with substrates, products, intermediates and inhibitors, obtained by either co-crystallization or soaking experiments and subsequent data collection, mostly at cryogenic temperatures [1]. At the typical resolution of 'conventional' protein crystallography of $\sim$2 Å, the substrate binding locus and mode can be unambiguously identified thus providing detailed insights into molecular interactions of substrate/intermediate/product with protein groups and with cofactors. This allows one to propose functional roles of protein or cofactor groups in discrete elementary steps of catalysis in the course of substrate binding, processing and product release, which can be tested by functional characterization. Even at 'conventional' (medium) resolution, the substrate locale can be defined with an accuracy sufficient to disclose differences in atomic positions smaller than 1 Å. Structural studies on enzymes using protein crystallography thus showcased that the protein very precisely aligns the substrate through exquisite interactions [2]. This mode of 'orbital steering' is highly sensitive to small structural changes, a dispositioning of the substrate by as small as 1 Å or a rotation of a reacting substrate moiety by just 20° away from a perfect 'maximum-overlap' conformation may result in dramatically reduced reaction rates (10^3–10^5 fold) [2,3]. Structural studies on enzymes were further highlighting that substrates are, at least in some cases, bound in higher-energy conformations, ones that would not be accumulated under equilibrium conditions outside the enzyme. This recurring feature of enzymes is termed ground-state destabilization and seems to be relevant for many enzyme families [4–7]. At resolutions well beyond 2 Å, electron densities around substrates or intermediates are starting to indicate that these could be distorted or strained, however, the discreteness of the assigned catalytic state *in crystallo* cannot be unambiguously proven (phase from other marginally accumulated chemical or conformational states with a potential impact on electron density), and the degree of a distortion, if so, cannot be reliably determined in quantitative terms unless true atomic resolution data ($\leq$1 Å) are obtained. Crystallographic analysis of enzymes proved to be also very informative regarding the detection of conformational flexibility of the protein itself (both large scale and locally), of involved cofactors and substrates. There

is an increasing body of evidence that enzymes sample a rugged landscape of conformations with different, often complementary catalytic 'competence', and that this landscape reorganizes upon substrate binding and catalysis also impacting the physicochemical properties of the different micro-cavities at the active site [8–10]. Large and small changes in the positions of active site lids, loops, side chains and even water molecules can be reliably disclosed at conventional medium resolution in protein crystallography. This raises the question, what remains invisible to our eyes at conventional resolution, if at all and how a structural analysis of enzymes at ultrahigh resolution is capable to yield additional structural and chemical information about an enzymatic reaction. Organized in different chapters, we will demonstrate the extraordinary potential of true atomic resolution X-ray crystallography for the analysis of enzyme mechanisms, and highlight which fine-structural and chemical details of enzymes are actually becoming visible compared to conventional resolution analysis, and how the structural observations made impact our current understanding of how these amazing biological catalysts work. We will specifically discuss the observation of physically distorted substrates, intermediates and cofactors, an ability that apparently permits enzymes to preserve high-energy states throughout the pathway for maximized throughput; and how enzymes productively utilize substrate binding energy to specifically destabilize scissile substrate bonds. We will further highlight that ultrahigh-resolution crystallography is capable to unveil the existence of 'hidden' multi-state conformational and chemical equilibria, which go completely undetected at lower resolution due to almost identical atomic positions of the involved states, through thorough analysis of anisotropic displacement (ADPs) parameters and detection of 'anomalies' in thermal motions. Next, we will summarize examples from the literature, where an analysis of protonation states of catalytically important protein groups, of cofactors and of intermediates revealed novel, important and sometimes quite surprising insights into some classic enzyme mechanisms. Lastly, we will briefly outline how ultra-high resolution X-ray crystallography has been utilized to identify the interstitial carbon atom of the metal cluster cofactor in nitrogenase.

We start by highlighting the methodological processes that are required for enhanced resolution by optimized data collection and processing.

Optimizing crystallographic data collection and processing

It is obvious that not all protein crystals have the property to diffract beyond 1 Å, many crystals possess a hard diffraction limit that *per se* precludes atomic resolution structural analysis. Nonetheless, iterated rounds of crystallization with consistently refined conditions including a systematic and subtle screening of chemical space (precipitants, additives, substrate analogs, inhibitors), thorough screening of cryo-protection conditions for flash-cooling, and testing many crystals may in some instances be successful for substantial resolution enhancement [11,12]. Once a crystal with good diffraction properties has been grown, it is important to employ an optimal strategy for data collection and processing to obtain a complete data set with high resolution.

Obtaining strong, high-resolution data from protein crystals is often hampered by low signal-to-noise ratio of weak, high-scattering angle reflections. On the basis of counting statistics and normal distribution describing measured diffraction intensities, increasing the number of counts (exposure time) by a factor of N increases the signal-to-noise ratio by $\sqrt{N}$. However, an increase in exposure time typically boosts intensities of strong reflections and diminishes medium or weak ones due to faster crystal decay caused by lowering crystal perfection and increasing disorder. These radiation-damage effects can be minimized by measuring several wedges (20–50°) from different positions of the crystal (Figure 1a). Those wedges, when merged together, can improve both redundancy and the signal-to-noise of weak reflections, for example high-scattering angle ones, which are more sensitive to radiation damage than the lower-resolution reflections. Optimization of signal-to-noise ratio of weak reflections should also include statistical noise reduction, which for photon-counting detectors, such as PILATUS, is mostly dominated by radiation contribution from crystal mounting materials (loop, cryo meniscus) and the air. Contribution of the non-air sources can be significantly reduced by exposing the part of the crystal outside the mounting loop or lowered by removing excess cryo/mother liquor from the mounting loop before flash-cooling (Figure 1b). A resolution enhancement of up to 0.15 Å can be reached when the data set is collected outside the mounting loop. The amount of background radiation can be also reduced by firstly, using a more brilliant beam (reducing the spot size reduces background, which is proportional to the detector area occupied by spot); secondly, setting the crystal-to-detector distance to maximum while still capturing all measurable reflections (shorter wavelength could be used to reduce air scattering), and finally using a proper oscillation range that is 1/3 to 1/2 of the mosaic spread.

For structural analysis of the enzymes pyruvate oxidase and transketolase, where true atomic resolution at ≤1 Å was accomplished (see following sections), the rod-like shaped crystals were fished co-axially with the loop axis with up to half of their length (or even more) sticking out of the loop (Figure 1b,c). Overlapping wedges of 20–65° were sequentially obtained by exposing fresh portions of the crystal along the rod axis, separated by at least 1.5–2 times the beam size (Figure 1c). After each translation the crystal was re-centered. Depending on beam size, crystal length and its symmetry, larger amount of short wedges,

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