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Crystallographic Structure Determination of Unstable Species

ILME SCHLICHTING*

Max Planck Institute for Molecular Physiology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany

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ABSTRACT

Crystal structures of reactive short-lived species, as occurring during chemical reactions, can be determined through time-resolved crystallography or trapping approaches. Prerequisite is the initiation and characterization of the reaction in the crystal. Ways to do this, recent results, caveats, and future prospects are discussed.

1. Introduction

Many (bio)chemical reactions are complex, often involving several reaction intermediates. To thoroughly understand the reaction mechanism, it is necessary to know all steps occurring along the reaction coordinate, the kinetics of their interconversion, their equilibrium constants, and their three-dimensional structures. Crystallography, NMR, and electron microscopy are excellent tools for determining three-dimensional structures to atomic resolution, but they are generally regarded as static methods, averaging over space and data collection time. Because data collection is generally lengthy and intermediates (see note below) are usually short-lived, their structure determination is not amenable to conventional approaches. Thus, transition-state or substrate analogues, inhibitors, and mutants are traditionally used to obtain mechanistic information. [Note: Here, "intermediate" is used synonymously for unstable complexes that can be appreciably populated under certain conditions. This definition includes relatively stable substrate or intermediate complexes of enzymes as well as very short-lived high-energy states, such as may be encountered in covalent bond rearrangements.]

In the past decade, however, crystallographic structure determination of species that are (normally) short-lived has become feasible[1] either on fast time scales ("time-resolved crystallography"; see note below) or via slowing the reaction by using low temperature, pH, and slow substrates ("trapping approach", sometimes referred to as "kinetic crystallography"). [Note: The term "time-resolved crystallography" is not well defined. Often no distinction is made between the trapping and time-resolved approach that is understood here as an experimental strategy in which the speed of the process to be studied dictates that of data collection and thus the data acquisition method (very often the Laue geometry). In the trapping approach one seeks to increase the peak concentration of an intermediate, which is often brought about by slowing its decay to experimentally convenient time scales. However, the two approaches are not exclusive; common is the study of productive catalytic intermediates.] Moreover, recent developments in instrumentation, such as third-generation synchrotron sources and fast efficient detectors, and in methodology, most notably in cryocrystallography,[2] have pushed the possibilities of determining structures of short-lived intermediates to new limits,[3−5] while making such studies more generally feasible.[6,7]

In this Account, the basis for the crystallographic structure determination of short-lived species is described, some recent results are alluded to, and an outlook to future developments is given. Comprehensive summaries of systems studied and intermediates visualized have been given recently.[6,7] Although macromolecules, and specifically mostly enzymes are treated explicitly, the principles for determining crystal structures of reaction intermediates are general and thus also applicable to small molecules.

2. Crystals

Before embarking on an experiment that seeks knowledge on the three-dimensional structure of an intermediate state, one has to make sure that the reaction does, indeed, take place in the crystal. Intuitively, this may seem unlikely. However, crystals of macromolecules contain typically 30−80% solvent, which is arranged in large, interstitial channels spanning the crystals. Moreover, the molecules are arranged in the lattice by relatively few weak interactions that allow for some mobility and often for catalytic activity.[8,9] Thus, the crystalline environment is less artificial than it may appear at first sight and resembles that of a solution—although a very concentrated one: the protein concentration in a crystal is typically 5−50 mM. Nevertheless, one has to be aware that the crystallization conditions (i.e., ionic strength, pH, solvents, etc.) or steric effects (such as blocked active sites or conformational restraints due to crystal contacts) may affect the kinetics of the system. This may be in terms of binding constants, rates (often slowed), the equilibrium distribution of catalytic intermediates, or even the catalytic mechanism itself.[9] Therefore, a detailed knowledge of the kinetics is required not only for the reaction as it takes place in solution but also in the crystal.[10] Obviously, in the ideal case the crystal lattice should not affect the reaction, but the inverse is also true, since meaningful measurements cannot be carried out otherwise.

Ilme Schlichting is a biophysicist trained in Kenneth C. Holmes's laboratory at the Max Planck Institute for Medical Research in Heidelberg. She started her scientific work with small-angle scattering studies, then switched to high-resolution NMR and subsequently to crystallography. After a postdoc with Gregory A. Petsko at Brandeis University, she became a group leader in the Department of Physical Biochemistry at the Max Planck Institute for Molecular Physiology in Dortmund. She is interested in understanding the structure−dynamics−function relationship on a molecular basis and tackles this problem by determining crystal structures of reaction intermediates.

* Phone: ++49(0)231-1332738. Fax: ++49(0)231-1332797.

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Activity monitoring within crystals is done most conveniently using a microspectrophotometer.[11,12] The advantage is that it is noninvasive and can be done in situ while the data are being collected. This minimizes unpleasant surprises such as low occupancy of the species in question due to incomplete reaction initiation (e.g., partial photolysis) or insufficient turnover the data collection. In addition, heating effects or X-ray-induced photoreduction can be detected while the experiment is being performed, and counter measures can be taken.

3. General Considerations

There are no general recipes for the structure determination of short-lived species. The characteristics of the system, especially the kinetics of the reaction to be studied, govern the experimental strategy. As with any direct method, intermediates can be detected crystallographically only if they are sufficiently occupied (detection limit typically 30%). This translates into the necessary but not sufficient requirement that the apparent rate constant for generation of the intermediate be higher than that of its disappearance. But occupancy is not the only issue for deciding beforehand whether one may actually "see" the intermediate—resolution of the diffraction data is equally critical. For instance, many reactions depend on protonation or ionization states, but in most cases (near) atomic resolution data are required to decide these issues.

Kinetic investigations cannot prove mechanisms; they can only disprove them, and in many cases several may explain the data. Fortunately, predictions can be made on the kind of intermediate that should be formed according to a specific mechanism. Since the intermediates often differ in their characteristics, one may devise experimental conditions specifically to look for one or the other.[13]

The structure determination of an intermediate is straightforward experimentally if it accumulates under steady-state conditions: either the reaction is allowed to proceed under steady-state conditions during X-ray data collection[14,15] or the steady-state distribution in the crystal is freeze trapped. The situation is completely different if the intermediate is (to be) studied under single-turnover conditions of the reaction (because, e.g., substrate binding or product release is rate limiting, or maintenance of the crystal lattice is an issue). In such cases, short-lived intermediates may be observed only if they live much longer than it takes to generate them and to collect the diffraction data. The latter is made possible by either decreasing the data collection time (using intense X-ray sources, such as synchrotrons and fast data collection geometries such as the Laue or Weissenberg technique)[16] or slowing the reaction, which is referred to as "trapping".[17] This is subdivided into chemical (unfavorable pH, substrates, or mutations) and physical (high-pressure or low-temperature[18]) trapping. Low temperature can be used not only to slow reactions[19] but also to separate steps in a reaction mechanism. This may be possible if the rate coefficients for the steps differ in temperature dependence. Cooling below the glass transition temperature will freeze out large anharmonic motions and may therefore be used to suppress certain reactions. This has been applied in studies on ligand binding to myoglobin,[20-22] on photoisomerization in the photoactive yellow protein,[23] and on catalysis in elastase.[24] In the latter, substrate was flowed into the crystal at a temperature below the glass transition temperature. Then the temperature was raised to allow the reaction to proceed until an acyl enzyme intermediate was formed. Subsequently, the temperature was dropped again below the glass transition temperature, thereby stabilizing the intermediate.

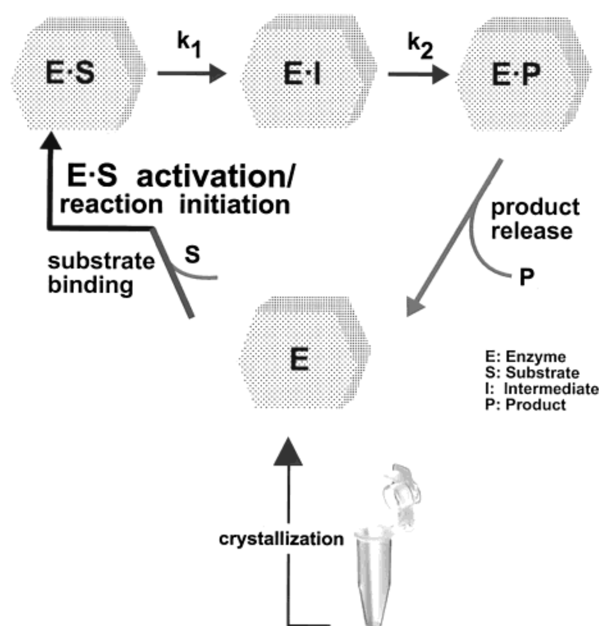

FIGURE 1. Crystallization is a lengthy process that prohibits crystallization of unstable species. Therefore, approaches to start the reaction in the crystal have to be developed. Depending on the kinetics of the system, structures of unstable species can be determined under single-turnover or multiturnover conditions of the reaction (see text).

Generally, studying a reaction under single-turnover conditions requires that the intermediate of interest can be generated with high efficiency and much faster than the reaction will proceed. This translates into finding a way to synchronize the reaction initiation of the 10^{13}–10^{15} molecules in a crystal.

4. Triggering Methods

Crystallization is a slow process, usually taking days to weeks before the crystals are big enough for data collection. Thus, it is not possible to cocrystallize, e.g., an enzyme–substrate complex; product would have formed long before data collection could even be started. It is therefore necessary to crystallize a stable, biochemically inert complex that can be activated immediately before data collection, a process often termed "triggering" (see Figure 1). Finding ways to initiate reactions in crystals rapidly, gently (i.e., without affecting the activity of the protein or quality of the crystal lattice), and uniformly is thus a major task in time-resolved and kinetic crystal-

lographic studies. As mentioned above, analysis methods need to be devised to follow the reaction as it proceeds in the crystal.

Reactions can be initiated by changing thermodynamic parameters such as temperature or pressure, by irradiating with light or other radiation, or by changing the concentration of substrates, cofactors, protons, and electrons.[25] Concentration jumps can be generated most easily by diffusion (see below), but fast reactions require other approaches. The influence of the rapidity of the trigger in relation to the time constants of the system on the detection of intermediates has been demonstrated exemplarily for the photoactive yellow protein,[7] which belongs to the systems with built-in triggers, as do light-sensitive carbonmonoxide complexes of heme proteins,[3,20–22] photosynthetic reaction centers,[26] and bacterio-rhodopsin.[27] Triggering enzymatic reactions is usually less straightforward and may require several strategies for capturing different steps.[28,29]

4.1 Photolysis. Reaction initiation with light is ideal since it may be achieved very rapidly depending on the respective (photo)chemistry. In addition, photolysis is broadly applicable experimentally—it can be used for crystals either mounted in a capillary at ambient temperatures or mounted in a loop at cryogenic temperatures.

Systems that are not inherently light sensitive can be rendered so by chemically attaching photosensitive, biochemically inactivating groups to, e.g., substrates, cofactors, or catalytically important residues of the protein.[30] Such "caged compounds" render the system light sensitive and biologically inert.[30] Commonly used "cage" groups are substituted 2-nitrobenzyls (Figure 2) such as 2-nitrophenylethyl (2NPE) that can be cleaved with light around 350 nm wavelength with concomitant production of a nitroso ketone. An important application of 2NPE groups is in the caging of nucleotides such as ATP and GTP.

The former was pioneered in solution studies of the muscle protein myosin, and much of the knowledge originating from those studies was used for the latter in a time-resolved Laue study on the Ras protein.[31,32] Since then, several time-resolved crystallographic studies using caged compounds have been performed.[33–37]

Despite its success, modification with a 2-nitrophenylethyl group is not ideal: First, it contains a chiral carbon atom, resulting in a mixture of stereoisomers that are difficult to separate. The pure enantiomorphs may have very different properties in terms of stability when complexed to a protein and in terms of the diffraction limit of the respective crystalline complexes.[38] Second, the liberation of the nucleotide is in the millisecond time range, and thus too slow for many processes. In addition, the cogenerated nitroso ketone is very reactive toward cysteine residues, requiring protection of the protein with high concentrations of scavenging thiol compounds such as dithiothreitol. However, not all proteins tolerate this.[37] Third, the nitroso ketone absorbs at roughly the same wavelength as the parent 2NPE-modified compound, rendering efficient photolysis of high concentrations of the caged compound difficult. Since one needs to study

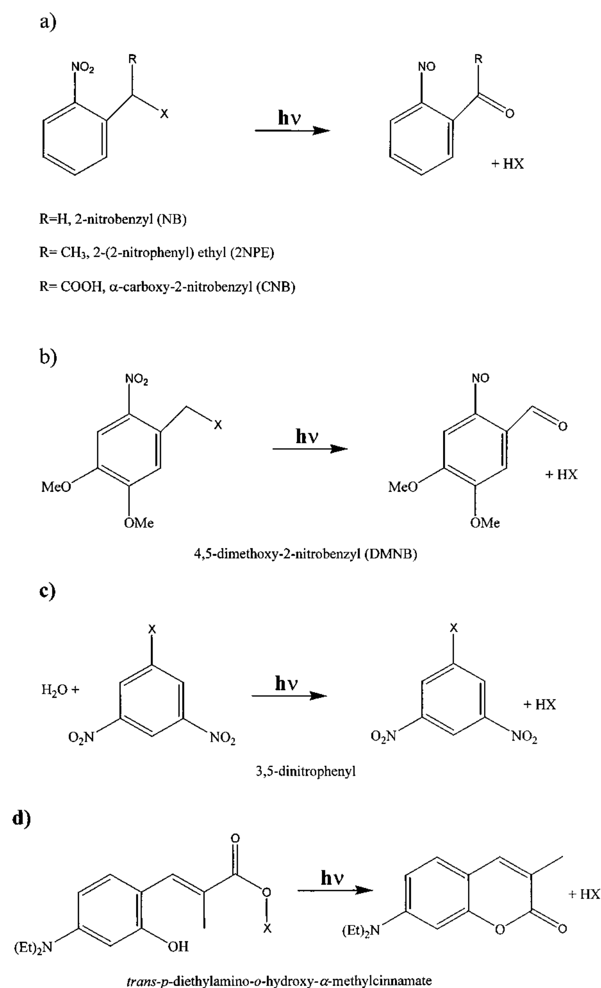

FIGURE 2. Cage groups used in crystallographic studies.

at least a 1:1 complex between protein and uncaged compound, the lower limit of the required concentration of the caged compound is given by the protein concentration in the crystal—usually in the tens of millimolar range—modulated by the affinity between protein and (un)caged compound.[37] For complete photolysis, several flashes with a laser or xenon flash lamp may be required that have to be balanced in terms of intensity and repetition rate against heating effects caused by absorption. Thus, in practice photolysis may take several seconds, despite the relatively high photolysis rate of caged compounds (10^1–10^5 s^{-1}). However, it is important to note that this limitation is not inherent in the technique itself; in the ideal case of a caged compound with high quantum yield and low extinction coefficient at the exciting wavelength, reaction initiation by a single flash of a powerful laser should be feasible. Reaction monitoring can be done either spectroscopically[39] or by HPLC analysis of dissolved crystals[32] if the reaction is slow enough.

Other cage groups used successfully in crystallographic studies (Figure 2) are 3,4-dinitrophenyl (attached to phosphate in a study[37] of glycogen phosphorylase b which is inactivated by dithiothreitol and the nitroso ketone metioned above) and (4,5-dimethoxy-2-nitrophenyl)ethyl or α-carboxy-2-nitrobenzyl attached to NADP in a study of isocitrate dehydrogenase.[40] *trans-p*-Diethylamino-*o*-

hydroxy-α-methylcinnamate was attached to the active site serine in chymotrypsin.[41] If one has a choice of ways to cage a system, one should choose caged compounds that bind to the active site,[40] in order to minimize diffusion times of the photoliberated compound to its business end.

Independent of whether a system is inherently light sensitive or has been rendered so by chemical modification, intense photolysis beams may cause heating of the crystal. This not only distorts the kinetics but very often results in a transient increase of the mosaicity (disorder) of the crystal lattice, which has a particularly detrimental effect on data quality when using the Laue technique. Therefore, photolysis is often performed at a wavelength away from the absorption maximum, and, if the kinetics of the system permits it, several shortly spaced light pulses are used to allow the system to thermally relax.[42] Photolysis by two-photon absorption[43] could be ideal to alleviate these problems, which are particularly pronounced in the ultrafast Laue experiments using single-bunch X-ray pulses.

4.2 Diffusion. Concentration jumps of substrates, cofactors, protons, etc. can also be achieved by diffusion, an experimentally straightforward approach. Because of the intrinsic generation of gradients and the competing effects of diffusion and catalysis, reaction initiation by diffusion is suitable only for very slow processes (half-lives of minutes for the rate-limited species). Typical diffusion times across 200-μm-thick crystals are seconds to minutes depending on the size of the compound and the solvent channels and the viscosity of the mother liquor.[44,22] pH changes—if tolerated by the crystal lattice—can be used to chemically trap intermediates[13] or to initiate single-turnover reactions to be followed by time-resolved crystallography[45] using a flow cell.[46] This setup can be also be used for the structure determination of intermediates accumulating under steady-state conditions of the reaction (turnover rates of up to 0.1 s^{-1}). Depending on the solvent (water versus, e.g., 70% methanol), flow cells can be used at ambient and cryogenic temperatures.[47] Reaction initiation by diffusion of substrate was used in time-resolved studies on cytochrome *c* peroxidase,[48] catalase,[49] and hydroxymethylbilane synthase.[50]

4.3 Radiolysis. The interaction of X-rays with matter depends strongly on the energy of the X-rays and thus their wavelength. Generally, when determining structures of intermediates, one tries to maximize elastic scattering, although there may be cases where anomalous effects are beneficial, especially if metal ions are involved. In most cases, however, absorption is undesired as it results, among other effects, in heating and radiation damage. The latter includes generation of photoelectrons that recombine with water to form hydrated electrons, leading to a range of subsequent radical reactions. X-ray induced reduction has been observed in many metal-containing systems[51] and has been used deliberately in experiments on cytochrome P450.[52] Since X-ray absorption and thus X-ray-induced reduction is strongly wavelength-dependent ($\sim\exp(\lambda^3)$), reduction can be minimized by collecting diffraction data using very short wavelength X-rays.

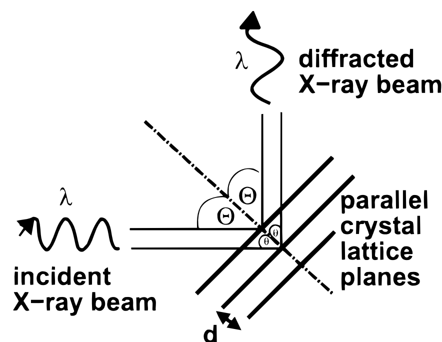

FIGURE 3. Bragg's law.

Increasing the wavelength can be done to generate photoelectrons that may reduce the system under study, thereby initiating a reaction.[52]

5. Mono- and Polychromatic Data Collection Methods

The Laue method, which has been mentioned in conjunction with very fast X-ray data collection, denotes the original geometric arrangement used by Max von Laue, Paul Knipping, and Walter Friedrich to collect diffraction data: a stationary crystal placed in a white X-ray beam. This allows sampling of a large fraction of reciprocal space (and thus of the diffraction data to be collected) in one exposure. One can rationalize this when inspecting Bragg's law: it describes X-ray diffraction as constructive interference of X-rays (wavelength λ) reflected by parallel lattice planes (distance d) of the crystal (Figure 3): $2d\sin\vartheta = n\lambda$ (n is an integer). There are two experimentally accessible variables in the equation, the wavelength λ and the angle ϑ between the X-ray beam and the lattice planes. Rotating the crystal with respect to the X-ray beam changes ϑ. In the case of monochromatic X-rays (Figure 4a,c), this translates to the rotation method, where the crystal is rotated incrementally and continuously over a wide range. This has been the commonly used X-ray data collection technique since the 1930s. In the Laue geometry, a polychromatic X-ray beam is used and the Bragg equation is fulfilled for many d-values simultaneously (Figure 4b). The Laue method[53] experienced a renaissance in the early 1980s[54,55] with the availability of strong continuous X-ray spectra provided by synchrotrons. These are electron or positron storage rings in which the particles are injected in "bunches" and kept in orbit by magnets. Due to the bunch distribution, the radiation is emitted not continuously but in pulses that last around 150 ps. Depending on the number and separation of the bunches in the storage ring, either the X-rays are available quasi continuously or, in the case of a single bunch filling mode, 150-ps flashes are generated that are separated by microseconds. The latter allows collection of X-ray data with 150-ps time resolution.[56,57] As mentioned above, the highest yield in terms of number of reflections per exposure is provided by the Laue method.[53] However, it also has disadvantages,[16,58] most notably concerning its sensitivity with respect to imperfections in the crystal lattice and high noise due to background scattering, but

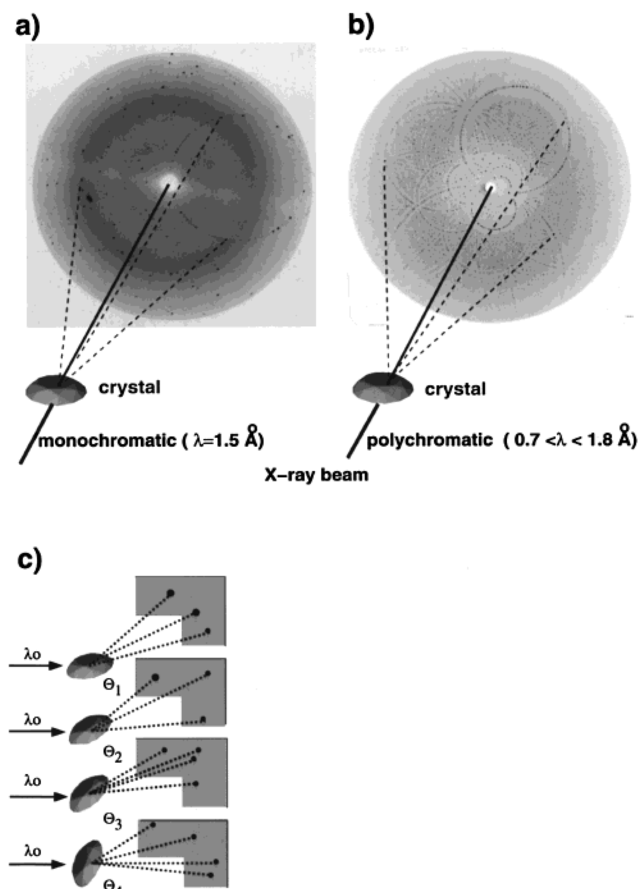

FIGURE 4. Mono- and polychromatic (Laue) data collection.

also in requiring wavelength normalization, deconvolution of harmonic and possibly spatial overlaps, increased radiation damage, etc.

6. Data Collection Strategies

For data acquisition one has to decide whether to collect the diffraction data after reaction initiation in real time as the reaction proceeds at ambient temperatures or to "trap" an intermediate by slowing its decay and to follow the reaction in a series of time-lapse snapshots at an experimentally convenient time scale. Both, the former "time-resolved" approach and the latter "trapping" or "kinetic" approach have advantages and drawbacks, as becomes apparent in experiments on myoglobin,[3,20–22] the photoactive yellow protein,[4,23] and the Ras protein,[33,59] all of which have been studied using both approaches.

In most cases, time-resolved crystallography requires use of the Laue method to allow data collection on the (typically fast) time scales set by reaction rates. Studies down to nanosecond time resolution are possible using single bunch exposures. Obviously, these ultrafast studies are technically very demanding[56] and need at least equally fast means to initiate the reaction,[57] which translates into laser-induced photolysis. In addition to being light-inducible, reactions to be studied on very fast time scales should be reversible. This allows averaging of exposures (with the crystal having the same orientation) to improve the signal-to-noise ratio of the data and to collect complete data sets (which requires in almost all cases collection of data from several orientations of the crystal in the X-ray beam) using the same crystal.[3,4] Following a reaction on fast time scales is much more difficult if the reaction is nonreversible. This is a fundamental difference between systems that are inherently light sensitive and those that have been rendered light sensitive by chemical modification. In the latter case, data are collected at the same time points from differently oriented crystals, which may introduce scaling problems and errors depending on how kinetically similar the crystals are at the various data collection time points, both resulting in poorer data quality. Therefore, when studying fast, nonreversible reactions, it may be worth considering alternative approaches to time-resolved crystallography, such as trapping by low temperature. This also applies if crystals are mosaic or become so during the reaction (initiation), since the Laue method is exquisitely sensitive toward imperfections in the crystal lattice. This was a severe problem in the time-resolved Laue experiments on the GTP complex of the Ras protein[33] and on ligand binding to glycogen phosphorylase b.[55] The latter study was successful only when monochromatic X-rays were used.[60]

7. Caveats

In general, the speed of structural changes and their magnitude are correlated—fast time scales permit only small motions. Unfortunately, in practice so far—and it is important to note that this is not an inherent limitation of the technique itself—the speed of data collection is inversely correlated to the resolution obtainable. This means that in cases where extremely high-resolution data are required to allow an unambiguous interpretation of the small conformational changes occurring on fast time scales, they cannot be obtained.

Both systems used for the nanosecond time-resolved experiments, carbonmonoxy myoglobin and the photoactive yellow protein, are exceptional in several respects: the photolysis reactions are reversible and can be initiated with high quantum yield. Moreover, the crystals are mechanically sturdy, rather insensitive toward photolysis and X-ray radiation or heating, have low mosaicity, and diffract monochromatic X-rays to at least 0.85 Å resolution. Nevertheless, in the nanosecond experiments[3,4] "only" data to 1.9 Å resolution could be obtained (likely due to the strong background caused by the white radiation which makes detection of weak (high-resolution) reflections difficult), challenging the interpretation of the small changes in electron density corresponding to partially occupied states. In the case of myoglobin, the latter appears mainly due to incomplete photolysis on very fast time scales.[3] Another problem is the heating of the system by the X-ray and photolysis beam and the associated uncertainties in the kinetics.

However, despite the significantly higher resolution of the intermediate structures obtained at cryogenic temperatures using monochromatic X-rays, there are problems too. Generally, the cryoprotectant needed for preventing ice formation in crystals upon freeze trapping may change the reaction characteristics, as may temperature-

induced changes in pH, dielectric constant, proton activity, etc. Moreover, the equilibrium distribution of the protein structure or spin distribution in heme proteins and the ratio of rate coefficients may change with temperature and may also depend critically on cooling rates. Changes with temperature observed specifically are slightly differing orientations of α-helices in the carbonmonoxy complex of myogobin,[61] slightly changed positions of catalytic water molecules in Ras,[59] and somewhat different kinetics in the case of the photoactive yellow protein.[4,23,62]

8. Future Developments

Obtaining well-diffracting crystals is the prerequisite in any crystallographic study, but it is only the beginning. If finding a heavy atom derivative is one of the major bottlenecks in determining the structure, identifying a suitable trigger method for starting the reaction is the equivalent in time-resolved crystallographic studies and those using trapping methods. Thus, more and better cage groups are needed that have faster apparent photolysis rates, higher quantum yields, and less absorption overlap between the caged compound and the photoliberated cage group (see above). Photolysis by two-photon absorption may help in some aspects, especially concerning heating effects, but has hardly been applied in crystallography[63] so far.

Especially in time-resolved experiments—in contrast to the trapping approach where one seeks to optimize the occupancy and to extend the lifetime of intermediates by changing the experimental conditions—methods are needed to "deconvolute" the electron densities of the various species occurring simultaneously. It has been suggested[64] to do this analogously to the decomposition of time-dependent optical spectra, which are also sums of exponential terms. It is likely that the resulting data will be noisy and, consequently, the interpretation of the electron density difficult. In a pioneering study,[65] this problem has been tackled by taking the predictions made by molecular dynamics (MD) calculations as guidelines for the interpretation of the electron density maps. MD calculations are also important for filling in the "gaps": time-resolved and kinetic crystallographic measurements provide snapshots of the system in the valleys of the energy landscape but do not show how the system crosses the barriers. This trajectory may be obtained by MD calculations based on the boundary values given by the structures.

The development of fourth-generation synchrotron sources, such as free electron lasers,[66] may make it possible to push the time resolution of time-resolved experiments into the femtosecond time range. While this may be applicable to only a few systems with rapidly switchable internal triggers, the mechanistic information that could arise from such studies is of fundamental importance.

9. Conclusions

Time-resolved crystallographic studies and those using trapping are interdisciplinary investigations on the structures of intermediates requiring intimate knowledge of the biochemistry and kinetics of the system under investigation. They provide unique insights into the system at work, and this at atomic resolution. Structures of intermediates have clear implications not only for understanding reaction mechanisms and protein dynamics but possibly also for structure-based drug design. Structures of proteins may change significantly along the reaction coordinate, as is most obvious for enzymes exhibiting an induced fit mechanism. Unfortunately, designing inhibitors based on the stable structures obtained by traditional approaches using substrate analogues or mutants may be inadequate. For optimizing the interactions of an inhibitor and thereby potentially increasing its specificity, one needs to know all structures occurring along the reaction coordinate.

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