Time-Resolved Biochemical Crystallography: A Mechanistic Perspective

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I. Introduction

A. Crystallography, Crystals, and Mechanism

X-ray crystallography is generally thought of as exploring structure, not mechanism. The essence of a chemical or biological mechanism is time-dependent change in structure, not static structure, yet the essence of a crystal is time-independent, spatially periodic order: a crystal exhibits translational symmetry. At first glance, these features are incompatible. How can crystallography contribute to a study of structural change? Can very fast structural transitions be produced in the crystalline state, let alone directly observed? Can the structures of transient intermediates and the pathways by which they interconvert be identified? That is, can mechanism be directly investigated by structural means? This article reviews a crystallographic approach to these questions with an emphasis on the application of time-resolved crystallographic techniques to problems in biochemistry. Koshland et al.[1] describe the more widely used, time-independent approaches, again from a chemical and biochemical perspective.[5]

There is a major difference between crystals of small molecule, organic and inorganic species, on the one hand, and of macromolecules, on the other, that hinders the application of time-resolved crystallography and direct, mechanistic studies to the former and conversely underlies its effective application to the latter (ref 2; see also Ohashi[3]). Crystals of small organic and inorganic species generally contain little or no bulk solvent and are stabilized by strong intermolecular or interatomic interactions: they exemplify hard condensed matter. Any prospective structural transition in such crystals is greatly affected by the presence of the crystal lattice. The transition may not occur at all, or if it does, the transition enforces a phase change in the crystal that alters the cell dimensions, the space group, or both. Reactivity in the solid state then may differ both qualitatively and quantitatively from reactivity in dilute solution or in the gas phase. Crystallographic studies are likely to be limited to a study of the reactant and product phases. The structures of any reaction intermediates are likely to be obscured by the extensive disorder that accompanies the phase change, and crystallographic techniques cannot probe such structures directly. The reaction pathway in the solid state may differ qualitatively from that in solution with different reaction intermediate and

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even product structures. Of course, it may be precisely the properties of the solid state that are of interest, as for example in the ultrafast studies of surface and bulk melting and recrystallization after illumination by an intense laser pulse.[4] Examination of these properties may not require retention of extensive crystallinity. It may be sufficient, for example, to probe the time evolution of the X-ray structure amplitudes of one or a small number of Bragg reflections or of the continuous, non-Bragg scattering.

In contrast, crystals of macromolecules contain extensive solvent whose physicochemical properties are indistinguishable from those of the bulk. The intermolecular contacts that form the crystal lattice are only weakly stabilized: such crystals exemplify soft (often, very soft) condensed matter. The large solvent channels permit the ready diffusion of small, reactant-sized molecules through the crystal lattice, their binding to the active site of the macromolecules, and their transformation. For example, enzyme molecules embedded in a crystal lattice may readily catalyze the conversion of substrate to product. Indeed, crystals of macromolecules more closely resemble very concentrated solutions with active site concentrations in the tens of millimolar range.[5]

As might be expected, the above is an idealized picture. The reactivity of macromolecules in the crystalline state may be affected by the unusual solvent from which the crystals were grown and which permeates the mature crystals. Binding of reactants at the active site may be subject to steric hindrance from adjacent molecules in the crystal lattice, and essential conformational changes may be hindered or qualitatively modified in nature. The changes may even be incompatible with retention of a well-ordered crystal lattice throughout the reaction. These solvent and lattice effects can nevertheless be explored experimentally, for example by comparing the reaction in dilute solution, in a polycrystalline slurry of microcrystals for which diffusion effects have been minimized, and in a single, macroscopic crystal of the dimensions typically used for X-ray analysis, i.e., 100 μm in average dimension. In those (admittedly few) cases in which such a quantitative analysis has been conducted, reactivity and mechanism in the crystal closely resemble that in dilute solution.[5] Were this not the case, then the usefulness of all crystallographic approaches to mechanism could be questioned.

The focus of this review is on time-resolved crystallographic investigation of the mechanism in biochemical systems. The important question as to whether a chemical kinetic mechanism exists or whether the reaction is more complex will be considered further below. If such a mechanism does exist, an overall reaction can be expressed as the interconversion of a set of M structurally distinct states or intermediates A_i, $i = 1$ to M. The goal is to identify these states or intermediates, the pathways and rate coefficients by which they interconvert, and ultimately to explain them in chemical and energetic terms.

B. Reaction Intermediates: Trapping and Time-Resolved Approaches

Reaction intermediates are often very short-lived with lifetimes ranging from femtoseconds[6] to seconds or longer. A widely used crystallographic approach has sought both to prolong the lifetime of such intermediates into a more experimentally convenient range and to maximize their peak population by manipulation of the chemistry of the system or the temperature or both. That is, the goal is to trap authentic intermediates.[7] One difficulty here hinges on ensuring that a candidate intermediate is both homogeneous and authentic and can be accumulated to high concentration. Suppose that a structural experiment can be conveniently conducted with a time resolution of around 1 s. To trap and observe an intermediate whose lifetime is normally 10 ms, its lifetime must be prolonged to around 10 s. That is, the rate coefficient for its breakdown must be reduced by a factor of 1000, corresponding to an increase in the free energy of activation for its breakdown of 2.8 kcal/mol. This is a relatively small energetic perturbation and one which may well be obtained by, for example, site-specific mutagenesis of a single key amino acid residue at the active site that is directly involved in the chemical step through which this intermediate breaks down. However, the rate coefficient for formation of this intermediate must remain largely unaffected, since the goal is both to prolong the lifetime of the intermediate into the experimentally convenient time range and to maximize its peak population. If the peak population is less than 100%, the intermediate is not homogeneous. If, alternatively, the lifetime of the intermediate is much shorter, e.g., 1 ns, then the free energy of activation for its breakdown must be increased by 12.4 kcal/mol, a substantial energetic perturbation. The more extensive chemical manipulations necessary to generate and trap such an intermediate are likely to affect other steps in the overall reaction mechanism. The authenticity of the trapped intermediate becomes more questionable. That is, chemical trapping is less likely to be successfully applied to shorter-lived intermediates whose trapping requires extensive energetic perturbation.

An alternative to "chemical trapping" of short-lived species is "physical trapping", which is achieved by lowering the temperature and literally freezing out the intramolecular motions that are essential for activity. Careful study of the temperature dependence of the activity of several macromolecular systems has revealed that they all undergo a marked change in dynamic properties at a particular temperature sometimes known as the glass transition temperature,[8] denoted T_g. Above T_g, the motions of the macromolecule are spatially extensive, diffusive in nature, and dependent on temperature and activity is retained; below T_g, the motions are spatially restricted, the dynamics can be characterized by a normal-mode analysis, and activity is largely lost. Attempts to trap intermediates by lowering the temperature below T_g take two distinct forms.[7,9] In "trap-freezing", the structural reaction is initiated at temperatures above T_g and allowed to proceed for a

fixed time, previously determined to be that required to attain a high concentration of the desired intermediate. The reaction is then terminated by rapidly lowering the temperature below T_g. The time resolution here is set by the time required to bring the temperature of the sample below T_g and hence depends on its surface area, thermal mass, thermal conductivity, and the efficiency of heat transfer from the sample to the cryogen. Values in the millisecond range are common for crystals of biological macromolecules of 100 μm average dimension. In freeze-trapping, the temperature of the sample is first reduced below T_g, then the reaction is initiated in the frozen crystal, for example by illuminating it by a laser pulse. All subsequent structural reactions either do not occur or are extremely slow. The time resolution is set, in principle, by only the duration of the laser pulse.

Difficulties arise in both classes of physical trapping if there are unsuspected inhomogeneities of the sample, either inherent in the reaction mechanism or introduced in the freezing process. The reaction mechanism and the magnitude of the rate coefficients governing the interconversion of species may be such that the sample is chemically and structurally heterogeneous immediately prior to freezing. That is, it does not consist cleanly of a single intermediate. Even if the sample is indeed chemically homogeneous, it consists of an extensive set of more or less rapidly interconverting configurational substates at all temperatures above T_g, as discussed further below. The distribution of molecules among these substates and the mean structure are temperature-dependent. If the sample were frozen infinitely fast, then no redistribution among these substates could occur as the temperature is lowered. The distribution subsequently observed at temperatures below T_g would be that associated with the initial temperature. If, alternatively, the sample were frozen infinitely slowly, then it would remain in thermodynamic equilibrium at all temperatures. Complete redistribution would occur among all substates, and the distribution at low temperature would be that associated with the final temperature. The difficulty is that experimental freezing rates lie somewhere between these two extremes. Different parts of the macroscopic sample experience different thermal histories during the freezing process. The distribution among configurational substates is not in thermodynamic equilibrium. Both the form of the distribution and its mean — the space-average structure represented in the sample — are likely to vary within a single sample and from sample to sample, depending on the exact thermal history. Variation of the distribution across the sample may well contribute to the substantial increase in crystal disorder (mosaicity) commonly seen on rapid freezing of macromolecular crystals. Further, if energy is subsequently deposited in the crystal by illumination with an intense X-ray beam or a laser pulse, then redistribution among the configurational substates may occur: structural annealing.

Experimental trapping approaches are thus most effective when clean trapping of a particular intermediate can be assured. Interpretation of the structural results of such experiments is hindered if substantial chemical or structural heterogeneity is present. Since longer X-ray exposures are possible, crystallographic studies of trapped structures generally yield higher resolution and better-refined sets of atomic coordinates. They are more precise but not necessarily more accurate; that requires demonstration that the trapped structures are indeed authentic.

In contrast, time-resolved experimental approaches do not attempt chemical or physical trapping of intermediates, accept that chemical and structural heterogeneity is inherent in the sample at all times as the reaction proceeds, and attempt to resolve this time-dependent heterogeneity into the structures of homogeneous intermediates during the subsequent data analysis process. As might be expected, the time-resolved approach also poses substantial challenges which should not be underestimated. The major difficulties with the time-resolved approach are 3-fold. Since structural intermediates can be very short-lived, excellent experimental time resolution is required over an extended time range and the large diffraction data sets must be acquired very rapidly. Second, exposure times are brief and even after accumulating numerous subexposures, the diffraction patterns can be weak and consequently may exhibit limited resolution. Third, the subsequent resolution of structurally heterogeneous data into an overall mechanism and time-independent structures of intermediates is a challenging conceptual, structural and computational problem. Stoddard[7b] points out clearly that the trapping and time-resolved approaches represent two experimental extremes distinguished by the methods used to isolate the intermediate, the time allowed for data collection, and the X-ray method employed. If authentic intermediates are to be isolated experimentally (as in the trapping approaches) or computationally (as in the time-resolved approach), then a "simple", chemical kinetic model must accurately represent the course of the reaction rather than a "complex" model. This point is elaborated on below.

I distinguish here between those approaches in which X-ray diffraction data are collected during the (relatively prolonged) lifetimes of particular intermediates and the data are ultimately interpreted as though they were static and time-independent and those in which the explicit time dependence of the diffraction data is measured and all subsequent analysis and interpretation is based on that fact. I restrict the use of the phrase "time-resolved" to the latter, in which time is an explicit and critical experimental variable. This restriction coincides with well-established uses of the phrase, as, for example, in "time-resolved spectroscopy".

II. Experimental Basis of Time-Resolved Crystallography

A. Reaction Initiation

As noted above, two classes of experiments are aimed at elucidation of the mechanism through the identification and characterization of intermediates.

In both classes, a structural reaction must be initiated, rapidly, uniformly, and nondestructively, in the molecules in the crystal.[10] In principle, any physical or chemical parameter on which structure depends can be used for reaction initiation: a change in reactant concentration (for example, diffusion of a reactant into the crystal through the solvent channels), a temperature or pressure jump, X-ray irradiation (where radiolytic products are reactive; see, for example, ref 11), or illumination by light (in the case of naturally light-sensitive reactions or of reactions on which light sensitivity may be conferred by preparation of a light-activable, reactant precursor). Of these, diffusion and light activation are the simplest and most widely used. Reaction initiation remains the most challenging experimental component in time-resolved crystallography. It has proved difficult to achieve activation of a large fraction of the molecules in a crystal in a nondamaging manner since reaction initiation deposits energy in the crystal that ultimately appears as heat. Crystals of macromolecules are often damaged by temperature jumps or temperature gradients. Irreversible reaction initiation (for example, by photoactivated bond breaking) may be essential but has the large disadvantage that each reaction initiation then requires a fresh crystal and these may be in short supply.

After reaction initiation, the working assumption is that the changes in structure from one molecule to another are uncorrelated in space and time and the molecules behave independently of one another in the crystal, as if they were in dilute solution.[10] That is, the fact that one molecule is in state A_i does not affect the probability that its neighbors in the crystal lattice are also in state A_i or in any other state A_j. The assumption arises from the weakness of the intermolecular forces that stabilize the crystal lattice; a molecule imposes only weak constraints on its neighbors in the lattice. If the assumption is correct and if a chemical kinetic model holds (see below), then the time variation in experimental observables arises from the variation in population of the statistically large number of molecules in each intermediate, time-independent structural state.

B. Experimental Design

Ultrafast time-resolved experiments have been conducted in pump−probe mode: a short laser pulse, the pump, initiates a light-driven structural reaction in the crystal whose progress is then monitored after a time delay t by a synchrotron X-ray pulse, the probe. The duration of the laser pulses used ranges from 200 fs to 7 ns and of the X-ray pulses from around 100 ps to 2 μs (in which the last is derived from a train of much shorter subpulses). The time resolution depends on the duration of the laser and X-ray pulses and on the jitter in the time delay between them and has varied between roughly 300 ps and 2 μs depending on the experiment. When a femtosecond laser is used, the time resolution of roughly 300 ps is set by the X-ray pulse duration and the jitter in the relative timing of the X-ray and laser pulses. A time resolution of 2 μs is set by the duration of the X-ray pulse train transmitted by a fast shutter. Experimental details are provided by Bourgeois et al.[12] and reviewed by Ren et al.[13] It is sufficient to state here that structural crystallography with nanosecond time resolution has been successfully conducted on light-sensitive, strongly scattering crystals of smaller macromolecules such as the heme protein, myoglobin,[14] and the blue light photoreceptor, photoactive yellow protein,[15] using the pulses of polychromatic X-ray radiation emitted by beam line BL3 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, and more recently at the BioCARS sector 14 at the Advanced Photon Source (APS) at Argonne National Laboratory, IL. Experiments aimed at a time resolution of a few hundred picoseconds require a femtosecond laser and are more challenging but still feasible.[16]

Slower, time-resolved experiments have been conducted by illumination of a light-activable or caged precursor, by continuous illumination of a crystal to establish a photostationary state, or by establishing steady-state conditions by continuous infusion of new reactants and allowing multiple turnovers. Time-independent states such as a photostationary state or a chemical steady-state can, of course, be studied at leisure or the time variable can be reintroduced by initiating decay from the time-independent state and following the process in time.

C. Data Acquisition and Reduction

The Laue X-ray diffraction patterns[17] stimulated by the polychromatic probe pulse are acquired on a sensitive, low-noise CCD detector. The raw intensities associated with the Laue spots in these patterns must be reduced via the derivation and application of a set of X-ray wavelength-dependent and wavelength-independent correction factors to yield ultimately the desired four-dimensional X-ray data sets, the time-dependent X-ray structure amplitudes $|\mathbf{F}(hkl,t)|$. Algorithms and software to accomplish this have been developed by several groups.[18] Time-resolved Laue data sets of high quality must possess several attributes. Coverage of reciprocal space spanned by the variables (hkl) must be complete, exceeding 90% in all resolution ranges (except that at the very highest resolution). Coverage must also be highly redundant: each structure amplitude is typically derived from 5 to 13 separate observations, thus yielding high precision in the mean structure amplitude and a good measure of its random error. The data must extend to high resolution. High completeness is essential to avoid series termination errors that may compromise structural interpretation; known precision permits the use of effective weighting schemes for combination of error-prone data and identification of measurement outliers,[19] and high resolution is essential to accurately model the critical yet small structural changes that occur during the overall reaction.

Each crystal will yield data sets corresponding to one or two time delays t before radiation damage compels its replacement. This damage evidently originates in the illumination by the intense, repetitive laser pulses rather than in the relatively weaker X-ray pulses. Complete time-resolved data presently

contains 9−25 values of the time delay t, distributed quasi-uniformly in log(t) to ensure unbiased sampling in time. However, it is clear that more values would be highly desirable and that, depending on the structural complexities and total duration of the reaction to be unraveled, measurements at 100 or more values of the time delay should be sought. A significant source of experimental error lies in the fact that in the present data collection strategy[14,15] each data set at time delay t is acquired separately. These must be pieced together to derive the entire time course, a process that introduces substantial systematic error. This strategy is dictated largely by the fact that no suitable X-ray area detector with excellent time resolution exists, and hence, data cannot be acquired at many time delays after a single reaction initiation. In an alternative strategy, data are acquired at all desired values of the time delay t for a single crystal but cover only a small fraction of reciprocal space (hkl). Many other crystals must then be used to complete the coverage of the unique volume in reciprocal space.

With values of $|\mathbf{F}(hkl,t)|$ distributed through the course of the structural reaction in hand, together with $|\mathbf{F}(hkl,0)|$ from control data sets acquired on the same crystal immediately prior to the laser pulse, then Fourier (electron density) maps with coefficients $\{2|\mathbf{F}(hkl,t)| - |\mathbf{F}(hkl,0)|\}$ or difference Fourier (difference electron density) maps with coefficients $\{|\mathbf{F}(hkl,t)| - |\mathbf{F}(hkl,0)|\}$ may be calculated. In each case, such maps use suitable unbiased "omit" phases derived from the reactant structure. Conventional difference Fourier maps display an approximation of the true difference in electron density between the structure at time delay t and that at time 0, point by point in the crystallographic asymmetric unit. These maps are very sensitive to small structural differences but are subject to a number of errors[19,20] arising from random and systematic errors in the time-dependent X-ray structure amplitudes $|\mathbf{F}(hkl,t)|$ and from the phase approximation inherent in the difference Fourier approach. The former sources of error may be mitigated by applying suitable weighting schemes to the amplitudes of the difference Fourier coefficients.[19] The latter may be attacked by seeking to derive explicitly the phase and magnitude of the vector difference between $\mathbf{F}(hkl,t)$ and $\mathbf{F}(hkl,0)$ via an "isomorphous noise suppression" (INS) approach.[19b] The Fourier transform of an error-free vector difference of this kind would yield the accurate difference in the electron density distributions between time t and time 0. The INS approach is analogous to density modification approaches[21] in which experimental errors from whatever source generate unusual electron density distributions. Comparison of these unusual distributions with those expected for error-free maps can be used to modify the phases used to generate the maps in an iterative manner. In the case of time-dependent difference Fourier maps, general expectations about the distribution of difference electron density in true, error-free maps can be applied to the observed, error-prone maps and exploited to yield phase information.

III. Interpretation of Structural Results: Simple and Complex Reactions

Suppose a time-resolved pump−probe experiment had been conducted on a light-sensitive biological system and perfect error-free X-ray structure amplitudes $|\mathbf{F}(hkl,t)|$ were available to high resolution at 100 values of the time delay t, spanning the time range from 100 ps to 1 s. What would the time-dependent electron density maps (or difference electron density maps) reveal? This is a rather more subtle question than originally realized,[10a] and there is unlikely to be a single answer. First, the X-ray data arise from a time average over the duration of the X-ray pulse. The duration of this pulse is assumed to be short with respect to the lifetime of all desired structures and affords effectively instantaneous sampling. Clearly this assumption begins to break down as the lifetimes diminish. Second, the crystal contains a statistically large number of molecules, typically 10^{12}−10^{13}, and the individual X-ray patterns (Bragg as well as non-Bragg scattering) arise from the interaction of X-rays with all electrons in these molecules. Third and most interestingly for this question, there is extensive evidence from spectroscopic data that macromolecules exist in a very large number of substates with similar but not identical configurations.[8,9] Although the most detailed form of these data derives from studies in solution, the solution-like nature of macromolecular crystals noted above and the existence of substantial crystal disorder embodied in the Debye−Waller factors demonstrates that molecules in crystals also exist in a very large number of distinct substates. These configurational substates may be arranged in a hierarchy in which different tiers of the hierarchy are distinguished by the height of the energy barriers between the substates in a particular tier (for an example, see Figure 1).[8,9,22] Alternatively, these barriers may be more or less uniformly distributed and a continuum of substates exists lacking discrete tiers. The existence of numerous configurational substates separated by energy barriers of differing magnitude is embodied in the idea that in structural reactions the molecules traverse a highly rugged "energy landscape".[23] The energy landscape concept is held to be equally applicable to such functionally distinct processes as enzymatic activity, protein folding, and the response to ligand binding or to absorption of a photon.

If these configurational substates have different functional properties and interconvert at defined rates, then it becomes important to ask how these impact a particular experimental observable. In seeking to probe mechanism in a chemical sense, we are interested in elementary steps such as bond breaking, isomerization, conformational transitions without change in covalent structure, etc. If these steps are probed by optical spectroscopic means, the observable is the time evolution of (for example) absorption or fluorescence features in the spectrum that arise from transitions between electronic or vibrational states extending over several atoms. Structure is not observed directly; it must be inferred from the spectroscopic observations, often by invoking theoretical calculations of spectra. In contrast, if we

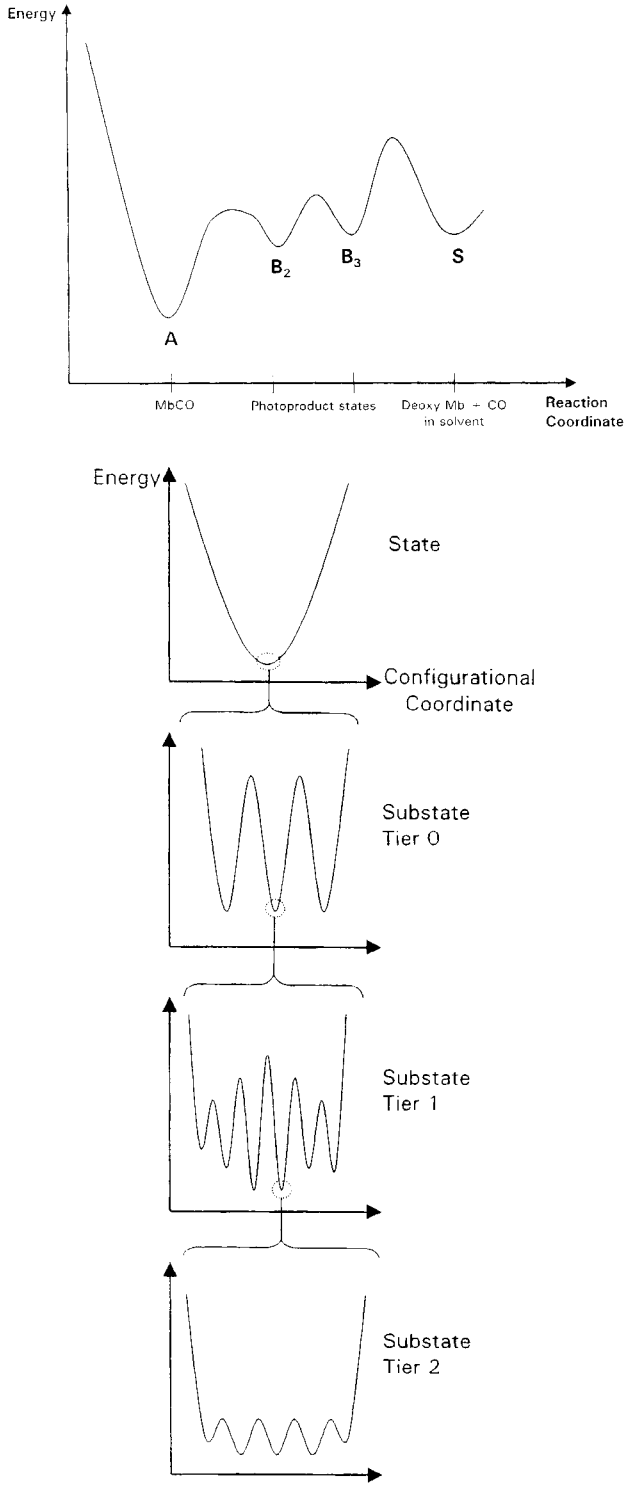

Figure 1. Reaction surface and conformational energy landscape of carbonmonoxy myoglobin, MbCO. (Top) Schematic reaction surface for ligand binding in which A corresponds to the CO-bound state, B_2 and B_3 to photoproduct states in which CO remains in the heme pocket or elsewhere in the globin, and S to the state where CO has dissociated completely from the protein. See also Figure 4 of ref 42g. (Bottom) Simplified scheme of the conformational energy of MbCO in three different tiers of the hierarchical energy landscape as a function of a configurational coordinate. Adapted from ref 9.

probe these steps by X-ray crystallography, the observable is the time evolution of the intensities of the diffracted X-rays from which may eventually be derived the spatial location of all atoms in the structure. X-ray photons provide a much more direct structural probe than UV−VIS−IR photons. Spectral changes may occur that are localized to a few bonding orbitals and are essentially invisible to any X-ray experiment, and conversely, many configurational substates (or even quite distinct structural states) of a macromolecule may possess identical spectroscopic properties. It follows that there need not be an exact parallelism between the time evolution of a spectral feature in a given reaction and the time evolution of the atomic structure.[24]

It is then important to ask the following: do the rates at which the configurational substates interconvert affect the overall course of the chemical reaction? If the answer is no, then the reaction may be classed as "simple", but if it is yes, then the reaction is "complex". Further, are the differences between particular configurational substates that differ substantially in functional properties localized to a particular structural region such as the active site or chromophore or do they involve the macromolecule as a whole? If the former holds, then some parallelism between optical and structural properties is to be expected, but if the latter holds, then optical and X-ray experiments may yield quite different results. For example, one might be simple and the other complex.

I base the discussion of simplicity and complexity and the application of these concepts to time-resolved crystallography on that of Karplus[25] and focus initially on a single step in what is more commonly a complicated multistep process. A reaction is said to be "simple" if a phenomenological expression involving rate constants describes its time dependence and if the temperature dependence of the rate follows the Arrhenius expression. A rate constant can be defined if three requirements are met.[25] A reaction coordinate (or progress variable) must exist for the transition from reactants to products; a well-defined energy barrier of a height several times kT separates the reactant and product states along the reaction coordinate; and critically, the relaxation times for all the very numerous degrees of freedom other than the reaction coordinate are short with respect to motion along the reaction coordinate. That is, there is a separation of time scales. The existence of a substantial energy barrier along the reaction coordinate means that the rate constant can be written in the form

$$K = A(T) \exp[-\Delta G^{\ddagger}/RT]$$

in which the activation energy $\Delta G^{\ddagger}$ is nearly independent of temperature. If the preexponential $A(T)$ is at most weakly dependent on temperature, then Arrhenius behavior will be exhibited: log K varies linearly with $1/T$. If these criteria hold for all steps in a reaction, then a chemical kinetic model holds, described by a set of discrete states A_i whose interconversion exhibits exponential behavior. A chemical kinetic mechanism then consists of a complete characterization of each state A_i, elucidation of the set of rate constants for their interconversion, and an understanding in chemical, structural, and energetic

terms of each state and of the transition states that separate them.

However, it is by no means clear that these criteria will hold when examining structural transitions in macromolecules. Systems such as disordered crystals and glasses contain highly numerous, slowly equilibrating configurational substates and exhibit nonexponential relaxations and non-Arrhenius behavior. For many macromolecular reactions, the free energy barrier $\Delta G^{\ddagger}$ has a large and variable entropic component and the Arrhenius relation does not hold. In the most extensively studied protein, myoglobin, the rebinding of ligands such as O_2, CO, and NO after photodissociation at cryogenic temperatures is highly nonexponential in time, varies from ligand to ligand, and is strongly dependent on temperature in a markedly non-Arrhenius manner[26] that conforms more closely to the Ferry relation in which log K varies linearly with $1/T^2$. The authors infer that the Ferry relation arises from many nonlocal degrees of freedom that give rise to a rough potential along the reaction coordinate superimposed on a larger, smooth barrier and that the two cannot be separated.[26] The energy landscape is significantly and inescapably rough, and the separation of time scales necessary for "simple" behavior is lost.

It must also be recognized that a reaction that is simple under one set of conditions when examined by one experimental probe may be complex under others. For example, the binding of O_2 by myoglobin under physiological solvent conditions and temperature is a straightforward bimolecular reaction with no evident kinetic complexities, very unlike the situation with O_2 and other ligands at lower temperatures or higher solvent viscosities. In a multistep reaction, the fastest steps may proceed sufficiently briskly along the reaction coordinate that protein relaxations along the other degrees of freedom do not occur fast enough to ensure the separation of time scales necessary for "simple" behavior, but later steps may proceed much more slowly along the reaction coordinate. Hence, early steps may be complex, nonexponential, and non-Arrhenius, unlike the simple later steps. A reaction that is simple when probed optically may be complex when probed by X-ray crystallography. This might arise because the optical transition is uncoupled from slower protein relaxations that are nevertheless evident in the X-ray experiment. A reaction that is simple in dilute solution may be complex in the crystal, if the effects of the crystal solvent and the crystal lattice are to greatly slow macromolecular relaxations, as in a highly viscous solution.

I return to the question posed at the beginning of this section: what would time-resolved electron density maps reveal? Much evidently hinges on whether the time dependence of structure is "simple", that is, expressible as a single exponential (or more likely as a sum of exponentials in a multistep reaction) over all observable time scales. If so, then a chemical kinetic model holds and the next objective is to analyze the data to identify the number of time-independent structures A_i and to derive their structures, the pathways by which they interconvert, and the rate constants for their interconversion. However, the temperature dependence is unlikely to have been determined in the crystal (at least in the early stages of the experiment) and conformity to Arrhenius behavior is therefore unknown. But, alternatively, the time dependence of structure may be "complex", expressible perhaps as a stretched exponential that is indicative of slower relaxation over configurational substates. No chemical kinetic model holds; the data reflect the superposition of numerous substates closely related in configuration whose separation is not readily achieved. Information may be derived about the physics of the system but much less about the chemistry — the mechanism.

The above discussion has concentrated on the interpretation of time-resolved crystallographic experiments. However, note again that trapping experiments also depend for their validity on the existence of a chemical kinetic model, populated by discrete intermediates that can indeed be trapped.

With experimental data, errors will tend to mask the real features in the difference electron density maps and distort their time dependence. The initial stages of inspection of these maps thus explore broad-brush questions: do certain features make chemical sense, i.e., are they located on or near groups likely to be involved in the reaction? If so, is the time dependence of their peak value or of their total electron content expressible as a sum of exponentials? Are the exponents identical when several such features are examined in this way?[27] If, for example, N distinct exponents (that is, relaxation times or transients) can be identified, then an underlying chemical kinetic mechanism must contain at least $(N + 1)$ states. If N exponents exist in real space in the difference electron density maps, then by the Fourier transform relationship the identical N exponents must exist in the time dependence of the structure amplitude of every reflection $|\mathbf{F}(hkl,t)|$ in reciprocal space.[10a] This permits the time dependence of each reflection to be fitted and by Fourier transformation permits the generation of a continuous, time-smoothed set of maps in real space. As in conventional kinetic analysis, the smoothed maps may be compared point by point in real space and in time with the experimental maps and the question asked, do the smoothed maps fit the experimental maps to within the known error or are there significant residual features for which an alternative mathematical form must be sought? If the fit is good, then it yields a set of N difference electron density values (or electron density values), one for each exponent, spanning the crystallographic asymmetric unit.

This set of electron density values does not reveal directly the set of structures A_i that populate a mechanism. Each set arises, in general, from a complicated superposition of several of the structures A_i, and some structures may never attain a high enough population to be detectable in the data. Deduction of the true structures A_i requires a final step: examination of each of the finite number of different candidate mechanisms that would display N relaxations and identification of each of the exponents with a particular rate constant (or combination

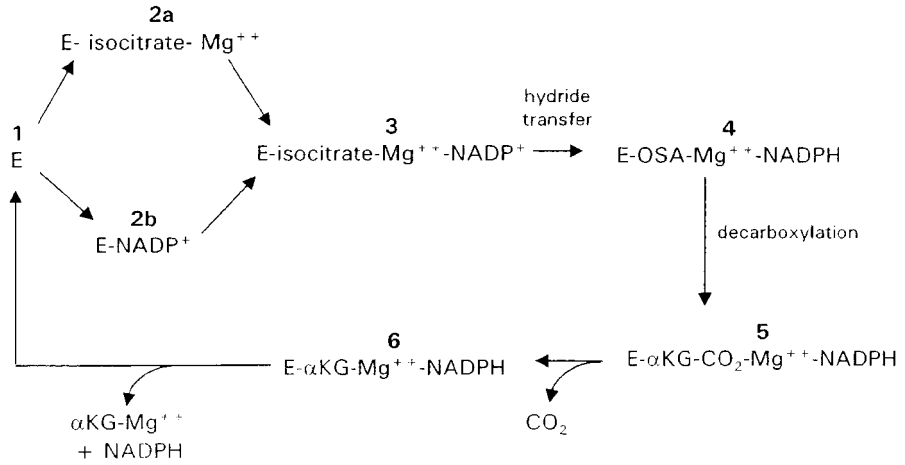

Figure 2. Reaction pathway of isocitrate dehydrogenase. The productive, ordered, ternary complex **3** is formed from the free enzyme **1** via a random binding mechanism. Hydride transfer from isocitrate to the cofactor yields the oxalosuccinate intermediate **4**, which undergoes enzymatic decarboxylation to the α-ketoglutarate intermediate **5**. Free enzyme is liberated by product release, initially of CO_2 to yield **6** and finally of α-ketoglutarate and NADPH. E, isocitrate dehydrogenase; OSA, oxalosuccinate; α-KG, α-ketoglutarate. Adapted from ref 28.

of rate constants). For each candidate mechanism and identification, a candidate set of time-independent structures A_i can be produced.[27] A candidate set may turn out to be a superposition of macromolecular structures and will not be able to be cleanly refined; that mechanism can be rejected. Other candidate sets may be single, refinable structures; those mechanisms are retained. These structural constraints may be sufficiently powerful that only a single mechanism is retained. Note that this type of constraint is not available in the analysis of time-dependent optical data, where no constraints exist on the form of candidate spectra associated with a particular intermediate.

However, the time-resolved X-ray data may not be well-fitted as a sum of exponentials but rather as a single, stretched exponential. No explicit structural data can then be extracted about intermediate structures that lie on the reaction coordinate between reactant and product, other than to state that they are both numerous and likely to interconvert slowly with respect to progress along that coordinate.

IV. Examples of Results

Time-resolved Laue studies have been published on 10 separate biological macromolecules at varying levels of mechanistic detail and with time resolutions ranging between 5 s and 10 ns. A recent review [13] lists nine of these, provides citations to the original literature, and discusses the experiments at some length. Rather than repeat this discussion here, I select studies of three of these macromolecules, isocitrate dehydrogenase, photoactive yellow protein, and myoglobin, and add the most recent one, that of cytochrome P450cam.[11] These studies exemplify the general principles of both trapping and time-resolved experiments outlined above.

A. Isocitrate Dehydrogenase

The enzyme isocitrate dehydrogenase catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and carbon dioxide via formation of an oxalosuccinate intermediate (Figure 2). When substrate is introduced into the crystal by diffusion, the enzyme is fully active in the crystalline state and under typical conditions (pH 7.5, 295 K) exhibits a relatively low turnover rate of 60−70 s^{-1}. Earlier, more rapid steps are associated with the formation and breakdown of intermediates prior to this rate-limiting step, that of product release. The rate constants for product release may be reduced by modest manipulation of the pH and temperature (e.g., pH 6.5, 277 K) or by site-specific mutagenesis aimed at amino acid residues directly involved in the breakdown of particular intermediates. Two such mutants separately allowed the steady-state accumulation of the enzyme−substrate complex prior to hydride transfer[28] and of the oxalosuccinate intermediate prior to decarboxylation[29] (Figure 2). The most prominent structural features evident in these chemically trapped intermediates were the substrate-dependent and charge-dependent ordering of the nicotinamide and ribose moieties of the NADP cofactor. In these steady-state experiments, the enzyme is undergoing multiple turnovers, but no such multiple turnover strategy could be devised that would lead to significant accumulation of the enzyme−product complex. Instead, a single-turnover experiment was performed on the wild-type enzyme. Since the half-life of the enzyme−product complex is around 10 ms, diffusion could no longer be used as a means of reaction initiation and rapid Laue data collection was essential. Reaction initiation was achieved by the photolytic liberation of either isocitrate (from caged isocitrate) or NADP (from caged NADP) using three separate photoactivable compounds, each possessing a different mechanism leading to the formation of the initial enzyme−substrate complex and subsequently to the enzyme−product complex.[30] The structure of this complex suggests that carbon dioxide dissociation is rapid and may help to drive product formation and that small conformational changes may contribute to slow product release.

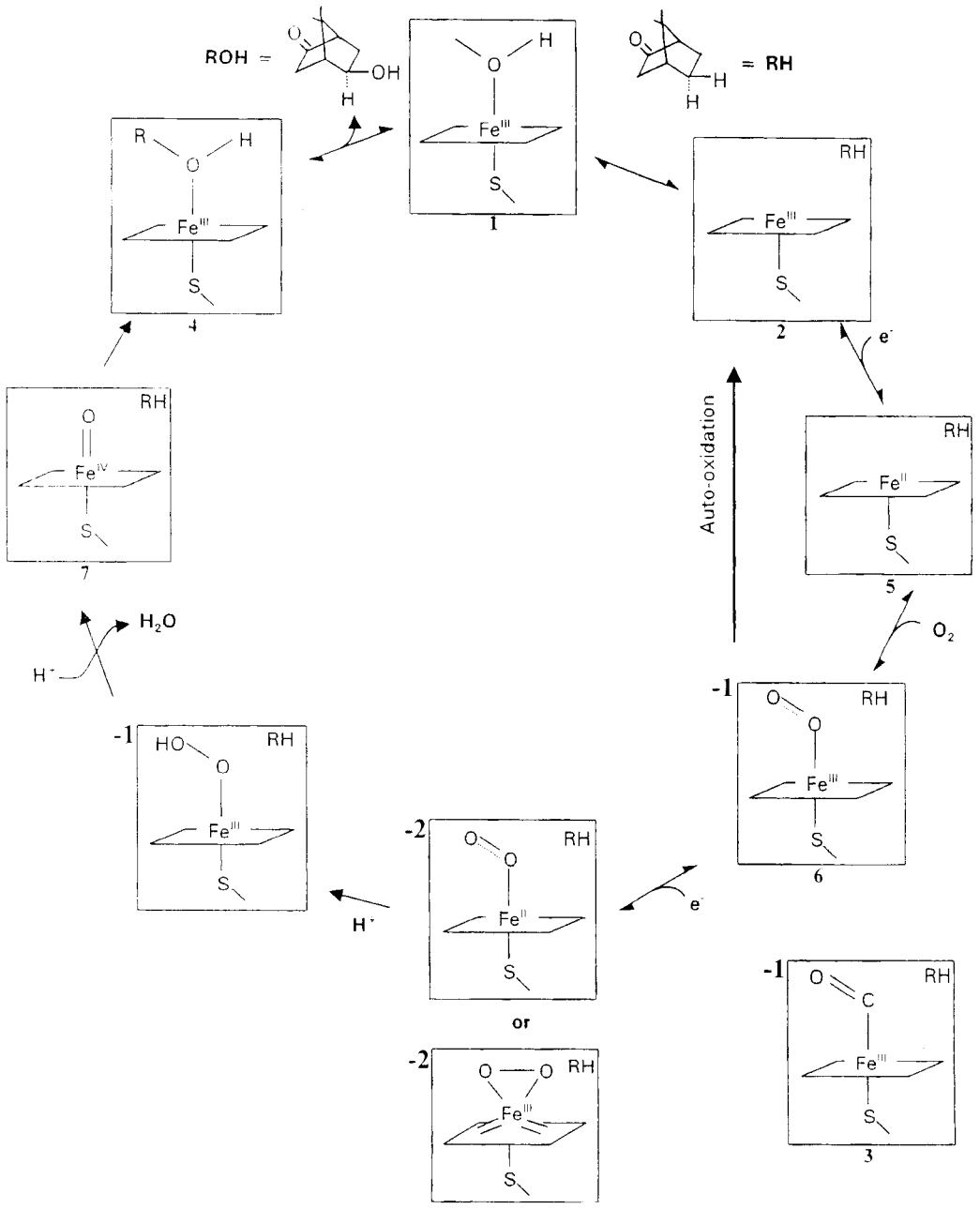

Figure 3. Reaction pathway of cytochrome P450cam. Reversible substrate binding to the six-coordinate, low-spin, ferric form **1** results in the five-coordinate, high-spin ferric camphor complex **2**. Addition of the first electron to **2** reduces the enzyme to the five-coordinate ferrous camphor complex **5**, which then binds O_2 to give the six-coordinate ferrous dioxygen intermediate **6**. Addition of the second electron and two protons produces one molecule of water and the so-called activated oxygen intermediate **7**. Insertion of the iron-bound oxygen into the substrate produces 5-exo-camphor **4** and product release to regenerate the free enzyme **1**. The unnumbered species between **6** and **7** represent other candidate species along the reaction pathway. The CO complex **3** is an analogue of the dioxygen intermediate **6**. Adapted from ref 11.

B. Cytochrome P450cam

Cytochrome P450 enzymes are widely distributed heme monooxygenases that catalyze the stereospecific hydroxylation of nonactivated hydrocarbons at physiological temperatures and pressures. Since, in contrast, the uncatalyzed reaction requires extremely high temperatures to proceed, these enzymes have been described as "the biological equivalent of a blowtorch".[11] The best-characterized member of this family is cytochrome P450cam, which catalyzes the regio- and stereospecific hydroxylation of camphor to 5-exo-hydroxycamphor, probably according to the mechanism shown in Figure 3 that depicts 10 candidate structures, 9 of which lie along the reaction coordinate. In addition to the free enzyme **1** (in which the heme iron is six-coordinate, low-spin, ferric), three other structures (the binary, ferric enzyme−substrate complex **2**, the enzyme−product complex **4,** and the ferrous carbon monoxide complex **3**) are stable and could therefore be determined by standard crystallographic techniques.[31] However, proposed key intermediates such as the binary ferrous enzyme−substrate complex **5**, the dioxygen intermediate **6** (which may differ significantly from the analogous carbon monoxide intermediate **3**), and the unusual activated oxygen species **7** are short-lived and inac-

cessible to standard crystallographic techniques. The structures shown were proposed by analogy with the reactions of other heme proteins[32] and from chemical and spectroscopic results. Direct preparation and structural characterization of intermediates is clearly essential, and this has very recently been achieved by "time lapse" crystallography.[11]

A characteristic of the proposed reaction mechanism is that multiple reactants are involved: camphor itself, electrons in a stepwise two-electron reduction, protons, and oxygen (Figure 3). Certain intermediates will accumulate until the essential reactant for the next step is provided. A novel feature of the experiments[11] is that electrons as reactants for the second of the two reduction steps are supplied by X-ray radiolysis of water in the crystal. That is, the probe for the reaction — X-rays — is simultaneously the pump that initiates this step. Production of the ferrous enzyme−substrate complex **5** was achieved by reduction with dithionite, conversion to the dioxygen intermediate **6** by exposure to high partial pressure of oxygen, and progressive conversion to the activated oxygen species **7** by X-ray irradiation at low temperature. No explicit time-resolved measurements were made; rather, ingenious experimental conditions were established that were designed to trap the normally short-lived species **5**, **6,** and **7** by both chemical and physical means. The structural results themselves were obtained at crystallographic resolutions between 1.6 and 2.3 Å and largely support the mechanism shown in Figure 3. However, there are indications that the intermediates (or more precisely the structures designed to mimic particular intermediates) are not homogeneous. For example, the difference electron density for the activated oxygen intermediate **7** suggests that incomplete O−O bond cleavage has occurred, the transition from the dioxygen intermediate **6** has not gone to completion, and a mixture of species is present. It is also something of a puzzle why **7** has accumulated at all. The authors speculate that an experimental feature such as constraints imposed by the crystal lattice, the unusual source of electrons, or the low temperature may have conspired to alter the overall rate-determining step and hence to trap a species identified as **7**. Nevertheless, this study represents the state-of-the-art in the preparation and chemical and physical trapping of hypothetical intermediates along a complicated reaction pathway, their characterization by careful, quasi-static crystallographic experiments and structural refinement, and their relation to parallel chemical and spectroscopic measurements.

C. Photoactive Yellow Protein

Photoactive yellow protein, PYP, is a small, water-soluble, blue light photoreceptor that contains a simple 4-hydroxycinnamic acid chromophore covalently attached via a thioester linkage to the sole cysteine in the protein.[33] Upon absorbing a photon, PYP undergoes a fully reversible photocycle (Figure 4) that contains several spectrally distinct intermediates whose lifetimes range from a few hundred femtoseconds to 1 s.[34] PYP is of increasing biological interest since it is the structural prototype for the so-called

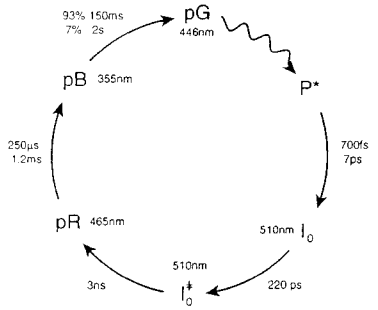

Figure 4. Photocycle of photoactive yellow protein, PYP, at room temperature. The absorption maximum of each species and the relaxation times for interconversion between states are shown. Relaxation from the pR state (also known as I_1) via the pB state (also known as I_2) to the ground-state pG proceeds via biexponential decays. From ref 27. See also ref 34 for the data on which this figure is based.

PAS domain-containing class of signal transduction molecules.[35] The key structural question is how absorption of a photon by PYP leads to the generation of a structural signal, initially in PYP itself and presumably in as yet unidentified molecules downstream in the signal transduction pathway. Ultimately, the swimming behavior of bacteria containing PYP is altered, and it is believed that PYP is the primary blue light photoreceptor for this biological process.[36]

In the dark (or ground) state denoted pG, the chromophore exists as the *trans*, phenolate anion in which the fully buried, negatively charged chromophore is stabilized by numerous specific hydrogen bonds in the chromophore pocket.[37] Time-resolved crystallographic studies with 10 ms time resolution of the decay from a saturated, photostationary state associated with the late, blue-shifted intermediate pB (Figure 3) showed that the chromophore in pB was *cis*, protonated, and exposed to solvent[38] and that several significant tertiary structural changes in the surrounding protein had occurred. In an attempt to identify earlier structural changes associated with normally short-lived intermediates such as pR, Perman et al.[15,27] conducted time-resolved crystallographic studies at room temperature with nanosecond time resolution, and in parallel, Genick et al.[39] undertook experiments to freeze-trap structural intermediate(s) at low temperature by illumination of frozen crystals. The published results differ in the configuration and location of the cis, strained chromophore. These differences may arise from distinct experimental difficulties in the two approaches. Perman et al.[15] concentrated on the interpretation of data acquired at a single time point, 1 ns after initiating the photocycle. They did not explicitly examine the subsequent time evolution of what is likely to be a rather complicated structural process in which several structurally distinct species may be present at 1 ns. Further, the extent of photoactivation in the crystals was low, which reduces the structural signal and only unweighted maps were examined. These difficulties compromise the original structural interpretation.[15] Genick et al.[39] also considered only a single data set (or time point), that of a species freeze-trapped at 90 K. However, the photocycle of

PYP takes a different form at low temperature and at room temperature,[40] with intermediates that differ substantially in their optical absorption maxima and presumably also in the structure of the chromophore and its immediate environment. The correspondence in spectra and structure between low-temperature and room-temperature intermediates has not been established. At temperatures well below the transition temperature T_g, little protein relaxation can occur and structural transitions in the chromophore such as isomerization occur within an essentially rigid protein framework. It appears that in PYP, effective coupling between light-driven structural transitions initiated in the chromophore and the surrounding protein is essential to generate the high quantum yield for entering the photocycle and to suppress that for the competing fluorescence pathway.[15] There is therefore some question as to whether the freeze-trapped structure represents, as intended, an extremely short-lived room-temperature intermediate whose lifetime has been greatly prolonged by freezing. There can be no question that the structure itself is very precise since it was obtained at exceptionally high resolution, 0.85 Å.

These difficulties in interpretation of the time-resolved data made it clear that the explicit time dependence had to be measured of the structural changes in the photocycle of PYP over the presently accessible time range from nanoseconds to seconds. The time dependence of improved difference electron density maps[19,27] could be modeled as a sum of exponentials. That is, the reactions were "simple" in the sense defined above and a chemical kinetic mechanism holds. This mechanism is analogous to, but not necessarily identical to, that based on spectroscopic data shown in Figure 4. Studies are in progress to identify the time-independent structures that populate the mechanism(s).

D. Myoglobin

Myoglobin is arguably the most widely studied of all proteins, yet continual surprises emerge as new experimental techniques are applied. The carbon monoxide complex of myoglobin (MbCO), in common with that of other heme proteins, is light-sensitive. Absorption of a photon leads to prompt rupture of the covalent iron−CO bond, to the relaxation of the heme and the globin toward the stable, deoxymyoglobin (deoxyMb) structure, to the diffusion of the CO through the globin, and ultimately to the rebinding of the CO and re-relaxation of the protein. Rebinding of CO to the heme that it left is expected to be describable by a geminate, first-order reaction exemplified by the transition from substates B to A in Figure 1 and rebinding from the solvent by a bimolecular, second-order reaction exemplified by the transition from substate S via B to A in Figure 1.

Comprehensive studies of this reaction have been carried out in solution over a wide range of time scales and of such variables as temperature, pressure, solvent viscosity, and pH for wild-type proteins of several species and for several variants.[41] Most such studies follow some spectroscopic feature associated with the heme group or the ligand and are sensitive to protein structural changes only to the extent that these are coupled to the heme or ligand. The question naturally arises: how does this solution behavior arise from the underlying structures and the dynamics of their interconversion?

Extensive freeze-trapping crystallographic studies[42] have been performed at low temperature in which frozen crystals of MbCO are illuminated to rupture the iron−CO bond and the progress of the reaction is then followed as a function of time, temperature, or both. Although the results obtained by several groups agree in general terms, they disagree in such details as the location of the photodissociated CO and how it evolves with time and the extent of changes in heme stereochemistry and in the tertiary structure of the globin. Even for a system as apparently well understood as myoglobin, it appears that small (and perhaps unsuspected) experimental differences may lead to quantitatively different structural results. Comparison of the highest resolution, static structures of deoxyMb and MbCO[43] reveals a number of significant differences in the heme stereochemistry and in the tertiary structure of the globin. To explore the time course of appearance of these structural changes, the first nanosecond time-resolved crystallographic studies were applied to myoglobin.[14] More recent studies[44] have substantially extended the number of time points in the nanosecond to millisecond range at which the time-dependent average structure of the photoproduct, denoted Mb*(t), was determined. Two observations suggest that the overall photodissociation, relaxation, rebinding, and re-relaxation reaction may be complex in the sense discussed above. First, when the time dependence of two prominent structural features at the heme is examined, one feature associated with the loss of CO and the other with motion of the iron atom, neither the single-exponential behavior expected for the geminate rebinding process nor the second-order behavior with time-independent rate coefficient expected for rebinding from solvent is observed. Rather, the time dependence is more complicated and may contain a combination of stretched exponential and second-order behavior.[44] Second, although the overall structural differences between Mb*(t) and MbCO resemble at all times t those between deoxyMb and MbCO (see Figure 1 of ref 14), they do not exactly match. That is, prompt and complete relaxation to deoxyMb does not occur and intermediates exist. There may be competition between the chemical step along the reaction coordinate of CO rebinding (and subsequent re-relaxation toward the stable MbCO structure) and protein relaxation toward deoxyMb. If this interpretation indeed holds, then the separation of time scales necessary for the existence of a simple reaction is lost; no simple chemical kinetic mechanism is valid. It is worth emphasizing that if the photodissociation reaction were examined in dilute solution under conditions similar to those in the crystal, a rather different behavior is seen. Stretched exponential behavior is exhibited in dilute solution at lower temperatures or in solutions of higher viscosity, where the rates of protein relaxation are evidently

slowed with respect to that of CO rebinding.[45] The dynamic behavior of myoglobin may be quantitatively affected by embedding the molecules in a crystal lattice. Experimental data on this critical point must be sought in parallel, quantitative, spectroscopic studies on crystals and solution.

V. Summary and Outlook

True time-resolved crystallography is still in its early days, particularly in comparison with the better-established trapping experiments that are also aimed at the structural identification of reaction intermediates. The time-resolved experiments themselves can readily be conducted with nanosecond time resolution on simple, photoreversible systems, but robust procedures for the analysis of time-dependent data in terms of a chemical kinetic mechanism and time-independent intermediate structures are still being developed. In many respects, trapping experiments and time-resolved experiments are complementary: certain systems lend themselves better to one approach than to the other. The strengths of the time-resolved experiment lie in probing faster reactions (to date, both fully reversible and light-driven) where the larger energies and structural perturbations associated with trapping experiments may cloud the interpretation. As emphasized above, both classes of experiment hinge on the applicability of a chemical kinetic model to the system in question.

The structural data presently available raise the very interesting question of why one small protein (here, PYP) apparently exhibits simple behavior derived from the coupling of fast structural relaxation with slow progress along the reaction coordinate but another small protein (here, myoglobin) apparently exhibits complex behavior derived from slower structural relaxation. A possible, biologically based answer is that as a signaling protein, PYP has been under selection pressure to efficiently generate a specific structural signal or signals in the face of competing fluorescence and thermal de-excitation pathways, and this requires rapid structural relaxation. Myoglobin, as an oxygen storage protein, is not under pressure to develop or maintain a specific pathway for exit and re-entry of the ligand nor necessarily to have rapid structural relaxation. Is it PYP that is unusual among proteins or myoglobin? Similar ultrafast, time-resolved studies on other proteins are called for to begin to answer this question and to develop further mechanistic insights.

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