tion stability and conformational switching rates affect the amount of open junction available at a given time, and that the energetic barrier to branch migration occurs along the path whereby open junctions shift to new positions along the migrating branch[21].

Singling out molecules by FRET removes many of the complications posed by equilibrium distributions of products and revolutionizes the mechanistic study of protein and RNA folding, structural changes in enzymes and proteins, and kinetic processes in macromolecular machines. McKinney *et al.*[16] demonstrate how powerful this approach is for extracting and separating kinetic and thermodynamic information pertaining to junction flipping and branch migration with immobile naked DNA junctions. However, a number of proteins participate in junction formation, resolution and regulation during meiosis, DNA repair and replication. The role of these proteins remains one of the mysteries surrounding homologous and site-specific recombination. It should not be too long before single-molecule analyses of genetic recombination with mobile junctions and a natural complement of essential proteins and enzymes prove how much — or how little — Holliday junction intermediates really do control the outcome of junction resolution and the occurrence of recombination hotspots.

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Snapshots of transition states?

James E. Penner-Hahn

Time-resolved X-ray absorption has been used to investigate the structure of the Zn active site in alcohol dehydrogenase during catalysis. The results support a new mechanistic model for catalysis.

Although alcohol dehydrogenase (ADH) has been studied extensively, the details of its mechanism have remained contentious. On page 98 of this issue, Sagi and coworkers[1] describe time-resolved X-ray absorption spectroscopy (XAS) of the alcohol dehydrogenase from *Thermoanaerobacter brockii*. This is the first time that the complete time-course of a biological reaction has been examined using millisecond time-resolved XAS. These data, together with traditional kinetic studies and quantum chemical computations, support a new model for ADH catalysis.

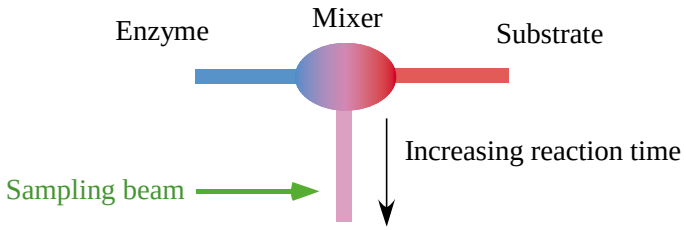

Fig. 1 Schematic illustration of rapid-mixing device used for time-resolved spectroscopic measurements. The distance from the mixer to the sampling beam determines the aging time of the reaction.

Alcohol dehydrogenases

Alcohol dehydrogenases (ADHs) use NAD(H) or NADP(H) to convert between aldehydes or ketones and the corresponding primary or secondary alcohol and have, in most cases, a Zn at their active site. ADHs are ubiquitous and have been studied extensively, particularly the enzyme from horse liver (HLADH). For HLADH, two basic mechanisms have been proposed. In the 'textbook' mechanism, the zinc-bound water molecule is displaced when substrate binds. The role of the Zn is to orient the substrate appropriately for hydride transfer and, perhaps, to polarize the substrate to facilitate the subsequent reaction. This mechanism, in contrast to those for most Zn enzymes, does not propose a catalytic role for Zn-bound water. In the alternative mechanism, the water molecule remains bound to Zn during the catalytic cycle, serving as a site for transient proton transfer during catalysis.

One of the key features distinguishing these mechanisms is the coordination number of the Zn during turnover. If the water is displaced when substrate binds, the Zn will be four-coordinate, with ligation by two cysteines, one histidine, and the substrate (alcohol, alcoholate, and/or ketone/aldehyde). If water is not displaced but rather plays an intimate role in

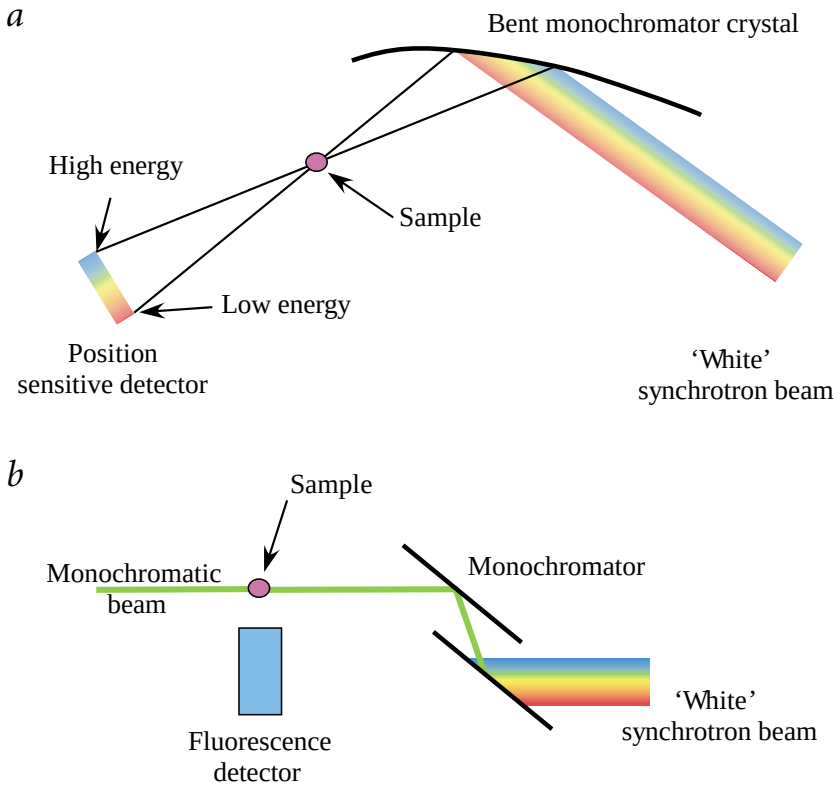

Fig. 2 Geometry for ***a***, dispersive and ***b***, single-wavelength XAS measaurements. In (*b*), the double crystal monochromator (represented by parallel thick black lines) must be rotated to scan through the energy range of interest.

catalysis, then the Zn must pass through one or more five-coordinate intermediates during turnover.

The fact that these mechanisms, which have been debated for over twenty years (see chapters 28–36 of ref. 2), remain controversial is due in part to the difficulty of characterizing the Zn sites in proteins. The gold standard for structural characterization is, of course, X-ray crystallography, and several ADH crystal structures are available. These have generally been interpreted as favoring a mechanism in which the Zn remains four-coordinate, since addition of a fifth ligand would seem to cause collisions with surrounding residues[3,4].

One of the limitations of protein crystallography is that it is generally restricted to stable species (for example, inhibited forms of ADH or ADH that is lacking either substrate or the NAD(H) cofactor). Moreover, protein crystal structures often lack the resolution needed to define the details of the metal site ligation. Spectroscopic probes, while providing less information than crystallography, can be used to follow the structural evolution of an enzyme in solution during catalysis. Unfortunately, zinc, which has a full complement of *d* electrons, is not amenable to most conventional spectroscopies. Consequently, spectroscopic studies of Zn enzymes have generally relied on metal-substitution to provide a spectroscopically accessible probe. For HLADH, EPR and NMR data for the cobalt-substituted enzyme and perturbed angular correlation data for the cadmium-substituted enzyme have indicated the existence of five-coordinate intermediates, in seeming contradiction to the crystallographic results. The inability to make a direct link between spectroscopic results for metal-substituted enzymes and crystallographic data for the inhibited native enzyme presents a challenge to mechanistic interpretations, particularly in view of the fact that Zn, Co, and Cd sites need not be isostructural[5].

Other evidence for five-coordinate intermediates in ADH

The first atomic resolution (1.1 Å) crystal structure for HLADH was reported recently[6] and favors the existence of a five-coordinate Zn. This structure, for HLADH complexed with NADH, shows two alternative water binding sites, with occupancies of 0.4 and 0.6. Both are within bonding distance of the Zn (2.12 and 2.24 Å, respectively), with the former occupying the same location as the oxygen in DMSO-inhibited HLADH. The latter water, located at a longer distance from the Zn, is close (~1.73 Å) to C6 of NADH. Although the data were modeled with only a single water bound in two different sites (that is, with a four-coordinate Zn), the existence of two water sites suggests that a five-coordinate Zn may be possible. This view was strengthened by recognition that the 'collisions' noted above, which seemed to rule out a five-coordinate site, could actually represent chemically relevant modifications of the NADH cofactor (that is, hydroxide binding to C6, thus activating NADH for hydride transfer). These observations propose a new mechanism, in which the active-site Zn is five-coordinate, with the Zn-bound solvent serving both to activate NADH and as a proton source, while the Zn serves both to orient and to polarize the substrate aldehyde[6].

X-ray absorption spectroscopy

The one spectroscopic technique that can be used to obtain direct structural information for Zn in solution is X-ray absorption spectroscopy (XAS)[7]. XAS refers to the structured absorption that occurs on the high-energy side of an absorption 'edge' — the abrupt increase in absorbance that occurs when the incident X-ray energy matches the binding energy of a core electron, typically a 1*s* electron for Zn. With careful analysis[8], X-ray absorption spectra can give bond lengths and coordination numbers for atoms near the Zn, and such spectra are sensitive to both the effective charge and the geometry of the Zn. Static XAS measurements on inhibited bacterial ADH showed that, at least in the inhibited forms, the Zn could adopt a five-coordinate geometry[9]. Bacterial ADH has different Zn ligation than HLADH. Bacterial rather than horse liver ADH was used for XAS since the latter has a second, structural, Zn site that interferes with XAS measurements.

One of the key advantages of a spectroscopic method is that it can be used in conjunction with a rapid mixing apparatus to study reacting systems in real-time. The reaction is initiated by rapidly mixing the reactants (mixing time ~2 ms), and the time resolution is determined by the distance between the mixing point and the sampling point (Fig. 1). The importance of real-time structural information for mechanistic studies is apparent, and this has led to a blossoming of interest in time-resolved XAS over the last decade. Although hundreds of papers have been published on time-resolved

XAS, these have until now focused mostly on questions of materials science and catalysis. This is because the best method for measuring time-resolved XAS is to use a 'dispersive' geometry, in which a polychromatic X-ray beam is focused onto the sample of interest (Fig. 2*a*). Since different energy X-rays traverse different paths, a position sensitive detector can be used for simultaneous measurement of the complete XAS spectrum. Unfortunately, the dispersive geometry is limited to relatively high metal concentrations (>10 mM for modest time resolution; higher for ms time resolution) and is thus not practical for most biological samples.

In order to study dilute samples such as metalloproteins, it is necessary to measure the data as fluorescence excitation spectra (Fig. 2*b*). Unfortunately, fluorescence measurements require that the monochromator be scanned through the energy range of interest, thus destroying the time resolution. If the reaction is slow enough, conventional fluorescence XAS can be used for time-resolved measurements. This was used to study the reaction of carboxypeptidase with a substrate that reacted over several hours[10]. However, even with recent advances in rapid scanning monochromators, it has not proven possible to measure XAS spectra with scan times better than ~50 ms[11] and typical scan times are several seconds long[12]. For an enzyme such as alcohol dehydrogenase that reaches steady state in <100 ms, these are too slow to provide useful time resolution.

There are two solutions to the problem of measuring time-resolved XAS spectra, both of which are used to good effect by Sagi and co-workers[1]. The first is to use rapid freeze-quench (RFQ) to trap samples at particular stages along the reaction pathway. This corresponds to replacing the sampling beam in Fig. 1 with a bath of liquid isopentane in order to trap the sample in a temporally well-defined state. RFQ has been used before to determine the structure of an unstable intermediate (see refs. 13,14), but has not previously been used to follow the complete time-course of a reaction. Sagi and co-workers used RFQ to trap 20 different samples with reaction times varying from ~2 ms (the mixing time of the apparatus) to 110 ms. Because the system evolves continuously during the reaction, with many different species present, it is not possible to take a literal 'snapshot' of intermediates that form. However, by using sophisticated mathematical approaches such as principle component analysis[15], it is possible to come close to this ideal. Sagi and co-workers conclude that two different five-coordinate intermediates form sequentially in the first 70 ms after propanol is added to premixed ADH + $NADP^+$. Further study will be required to test this proposal, since the changes in coordination number and bond-length between the different species are close to the uncertainty in the data. Moreover, it is possible that the present mechanistic results for bacterial ADH are not relevant to HLADH. Nevertheless, the present data provide a clear qualitative demonstration that the structural changes in the Zn site during reaction are more complex than were previously anticipated.

As powerful as RFQ methods can be, they still permit sampling only at a limited number of time points. An alternative is to use single-wavelength measurements (the monochromator in Fig. 2*b* is not scanned) while monitoring the X-ray fluorescence as a function of time. In order to have adequate fluorescence intensities, time-resolved single-wavelength measurements require very intense X-ray excitation, such as those available at 'third-generation' synchrotron sources. Earlier, Dau and co-workers measured the rate of photoreduction (0.57 min^{-1}) of the Mn in the photosynthetic oxygen evolving complex[12]. Sagi and co-workers have now extended this to the millisecond regime, using single wavelength data to confirm the existence of two distinct phases during the first 100 ms of the reaction. Conventional stopped-flow measurements under comparable conditions but using UV-visible absorbance to monitor $NADP^+$ reduction, show only a single phase in this time regime.

The work by Sagi and co-workers demonstrates the potential of time-resolved XAS to reveal important new insights regarding enzyme mechanisms. It is not yet the case that time-resolved XAS is able to provide a routine 'snap-shot' of reactive intermediates. However, as the availability of ultrabright synchrotron sources continues to expand, time-resolved XAS is poised to become a standard tool in the toolkit that biophysicists use to investigate enzyme mechanisms.

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