Caught in the act

Femtochemistry opens the lid on biology's third vital criterion

Philip Hunter

Along with replication and mutability, living beings are set apart from the mineral background they inhabit by their metabolism—their ability to catalyse chemical reactions. Since Linus Pauling first proposed that these reactions are made possible by enzymes that recognize and bind tightly to their substrates at a crucial transition point [1], it has become increasingly clear that understanding these reactions requires details of the precise molecular alignments that take place at the level of femtoseconds (10^{-15} s).

This transition state is the 'point of no return' for colliding molecules in a chemical reaction. Beyond it, the reactants inevitably go on to form new products; before it, the reaction does not take place. It lasts for tens to hundreds of femtoseconds, when the molecules are at a state of maximum energy from which they will fall either towards completing the reaction, or with equal likelihood, away from it. The role of the enzyme is to enable the molecules to negotiate this energy summit and to reach the point of completing the reaction.

Many processes, including protein folding and the splitting of water during photosynthesis, pass through more than one transition state. Unravelling them all is a challenging task, but the potential prizes are great and might include the ability to harness reactions to produce carbon-neutral energy, for example, by mimicking or exploiting photosynthesis. There are also great therapeutic possibilities, as cell replication in cancer or metabolic processes in pathogens could be halted by intervening at transition states to block key reactions.

This therapeutic avenue was first explored in 1986 by Richard Wolfenden, now at the University of North Carolina at Chapel Hill, USA, who calculated that conformational changes in the active site of an

enzyme at the transition state should enable it to bind to the reactants with huge strength to overcome the energy barrier [2]. This, in turn, suggested that suitably designed analogues, mimicking the reactants at the transition state, could intervene by binding to the enzyme during that brief window, thus rendering the enzyme ineffective.

However, the technologies needed to gather information about transition states have only become available during the past decade. The principle technology in use is X-ray absorption spectroscopy (XAS), which is combined with an ultra-fast laser in an arrangement known as a 'pump probe'. This setup determines the geometrical shape of the approaching molecular orbitals and the distribution of electrostatic charge around them. The XAS provides information about charge distribution, whilst the pump probe yields details of the geometrical structure during the crucial femtoseconds of the transition state.

The pump probe splits a short laser pulse into two separate pulses by a time-scale corresponding to the period of the relevant molecular vibrations. The first pulse—the pump—excites the sample, whereas the second pulse—the probe—measures the changes caused by the first. This information can be used to determine the structural details of the transition state, thus enabling the hunt for suitable analogues. Vern Schramm's laboratory at the Albert Einstein College of Medicine of Yeshiva University, in New York, USA, is doing exactly this. "Our approach gives geometry and electrostatic information for the transition state," Schramm explained. "We can use computational approaches to compare these to large numbers of related molecules to see which best mimic the transition state." Schramm's team has

already applied this to develop a drug that targets *Plasmodium falciparum*—the protozoan parasite that causes malaria. The drug blocks the crucial purine pathway with a transition-state analogue [3]. *Plasmodium* is a purine auxotroph, meaning that it cannot manufacture the molecule directly. Instead, the parasite makes purines indirectly, through an enzyme called purine nucleoside phosphorylase that synthesizes a purine precursor called hypoxanthine. Schramm's transition analogue, BCX4945, binds to the active site of the enzyme at the transition state and so blocks its action, starving the parasite of purine.

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In trials, BCX4945 cleared *P. falciparum* infection in night monkeys of the *Aotus* genus—a model close to that of human malarial infection. But there was some re-emergence of the parasite at reduced levels after a few days, similar to the pattern observed with conventional anti-malarial drugs. The drug has been licensed to BioCryst Pharmaceuticals, which is providing it to third parties, under license, for clinical trials. "One such party is now evaluating the drug for a go/no-go decision to go forward into a small-controlled human trial," commented Schramm. We expect that party to make that decision by mid-2013."

Meanwhile, Schramm has planned laboratory studies to determine the exact mechanism of drug action, off-target effects and the

efficiency of different drug combinations in night monkeys, as well as the rate of resistance formation in the parasite to BCX4945. However, he is having trouble finding funding for the research, as the eventual treatment would require more than three doses per day, making it difficult to deploy in regions that suffer from malaria and have poor health infrastructure. Nevertheless, Schramm is convinced that the drug has great potential because of its low toxicity and different mode of action, which starves the parasite. It has certainly demonstrated that transition-state analogues can work.

In the meantime, Schramm's group is targeting human immunodeficiency virus (HIV), which has also resisted attempts to develop satisfactory therapies that are both effective and have acceptably low side effects. The aim is to inhibit the HIV-1 protease that cleaves newly synthesized polypeptides to enable the virus particles to become infectious and invade new cells. HIV protease inhibitors have been used for years, but resistant strains of HIV have emerged.

Schramm believes that a transition-state analogue might overcome this problem of resistance. "We recently solved the transition-state structures of HIV protease native and drug-resistant enzymes," he said. "Surprisingly, the transition states are identical. Thus, the resistance does not come from altered transition-state structure. The result shows that if a transition-state analogue can be found for the reaction, it should efficiently inhibit both the native and resistant enzymes."

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Although such an approach holds great promise, there are significant challenges for developing drugs that mimic transition states. One is that solving the structure of the transition state itself is not sufficient, as the analogues might still not be suitable for use in humans. Kinases, for example, perform

a wide variety of signalling and other functions by transferring phosphate groups. "In kinases, we understand the transition states, but biologically compatible mimics of the transition state have not been achieved," Schramm said.

Even when biologically compatible, effective mimics are available, they might still prove inappropriate owing to unanticipated effects on other pathways. Schramm also pointed out that an inhibitor can be too powerful, irrespective of its mode of action. "Some human targets are essential and it will be harmful to cause complete inhibition for long periods. An example is the target of statins, HMGCoA reductase, which is the pacemaker enzyme for cholesterol, but also for all other steroid hormones," he explained. This biochemical knowledge of the target is crucial for using transition-state analogues, Schramm noted. "When the target is unique to a pathogen, for example, their use is ideal. But when the target is a host enzyme, for example in cancer, animal experiments are essential to show that the analogue has the desired effect with limited toxicity."

Femtobiology is not only focused on identifying transition-state analogues for drug development; researchers are also digging into photosynthesis, given its potential for yielding carbon-neutral fuels and electric power. Photosynthesis involves two photoreactions that harvest light to energize electrons through a plethora of associated enzymes and co-factors. The crucial first step is carried out by photosystem 2 (PS2), which uses light energy to split two water molecules into oxygen and four electrons. The electrons are transferred to the Calvin cycle in which they convert carbon dioxide into carbohydrates.

The water-splitting part of PS2 is the crucial component for solar energy conversion because it is the engine of the whole system and the key to its high efficiency [4]. "Understanding the water-splitting reaction and identifying the various reaction steps and intermediates is of key importance and will be very important for the development of new and efficient artificial systems," explained Villy Sundstrom, whose team at Lund University in Sweden works on solar energy conversion research.

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The water splitting occurs in a cluster of four manganese ions and one calcium ion in a five-state cycle. To analyse the process accurately requires elucidating the precise structure of each stage, each of which lasts for only a short period. An important step forward was made in 2011, with the production of a model of the complex in the ground S1-state by X-ray crystallography at a resolution of 1.9 Å [5]. This still left the great challenge of determining the structure of the transient S2-, S3- and S4-states, but provided essential information that stimulated further work on the structure of the S2-state [6]. The study of the S2-state, by Khandavalli Lakshmi and colleagues at The Baruch '60 Center for Biochemical Solar Energy Research in Troy, New York, USA, involved the use of PS2 isolated from three species—two cyanobacteria and spinach. The researchers trapped the oxygen-evolving complex (OEC) in the S2-intermediate-state by low temperature illumination.

Lakshmi's team used a technique called two-dimensional hyperfine sublevel correlation spectroscopy to detect weak magnetic

interactions between the manganese cluster of the S2-state and the surrounding protons. "The major breakthrough of the 1.9 Å X-ray crystal structure [of the S1-state] is that it identifies all of the amino acid ligands of the Mn₄Ca-oxo cluster and four water molecules that are directly coordinated to the metal ions," Lakshmi said. This helped the team with their detective work in locating all the structural units within 5 Å of the Mn₄Ca-oxo cluster that might be involved. "This leads to several likely candidates that include amino acid ligands that are directly co-ordinated to the cluster, amino acid side chains that are not co-ordinated to the cluster, two water molecules that are co-ordinated to the manganese ion, two water molecules that are co-ordinated to the Ca²⁺ ion and nine water molecules that form a hydrogen bond network in the vicinity of the Mn₄Ca-oxo cluster in the crystal structure," Lakshmi explained.

One of the interesting findings was that the S2-states of the three organisms studied were almost indistinguishable. "In an unexpected but welcome surprise, we observe that the hyperfine spectra of the S2-state of the OEC of PSII from *Thermosynechococcus vulcanus*, the PsbB variant of *Synechocystis* PCC 6803 and spinach are identical," Lakshmi said. "This suggests that the OEC of PSII is highly conserved in the three species".

There is still some way to go to unravel all S-states of PS2, Lakshmi conceded. "There are several open questions on the fate of the water molecules in the S-states that warrant immediate attention," she said. These include the precise location and binding of the substrate water molecules, the oxidation state of the manganese ions that ligate the substrate water molecules and precise geometry of the Mn₄Ca-oxo cluster.

In parallel with the structural and functional analysis of the S-states of PS2, research has been ongoing into artificial systems that use catalysts other than the Mn₄Ca-oxo for water splitting. Such systems had only achieved levels of efficiency usually two orders of magnitude lower than PS2 itself, in terms of the rate of oxygen production. But a major advance uses a ruthenium catalyst to achieve water oxidation rates similar to PS2 [7]. There is just one important caveat: the catalyst does not use light to drive the oxidation, it uses an acidic solution of ammonium cerium

nitrate, a compound of the rare earth metal cerium. However, the team believes that the high rates of oxidation achieved with the ruthenium catalyst could lead to water oxidation technology based on more abundant elements, such as the first-row metals rather than rare earth ones. In the future, they hope that the knowledge gained about these artificial catalysts and how they work will pave the way to the light-driven generation of molecular hydrogen by water splitting.

Whilst the ultimate aims of directly harnessing photosynthesis for human benefit, and the creation of an artificial system that rivals the water-splitting efficiency of PS2 would be huge steps forward with profound implications for energy production, the end is a long way off. In the meantime, the growing interest in split-second moments at the catalytic centres of many enzymes continues to enhance our knowledge of the metabolism and lays the groundwork for progress in drug development, energy production and other areas.

CONFLICT OF INTEREST

The author declares that he has no conflict of interest.

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