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meeting point

Hijacking nature—new approaches to unravel enzyme mechanisms and engineer improved biocatalysts

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The second EMBO conference on 'Catalytic Mechanisms by Biological Systems' took place in Groningen, the Netherlands, in October 2012. Structural, molecular and computational biologists, as well as chemists, biophysicists and engineers discussed technologies to improve our mechanistic understanding of enzymes, as well as the design of robust biocatalysts.

Introduction

As our ability to analyse biological systems increases, our capacity to synthesize them for practical applications grows. However, before designing biological systems 'à la carte' [1], it is necessary to take a closer look at their mechanisms. Only when our mechanistic understanding of enzymes is enriched, we will improve their properties more efficiently and usefully. The first half of the conference focused on structures, dynamics and new approaches to understand enzyme mechanisms, whilst the second part dealt with the discovery, design and evolution of biocatalysts for practical applications.

Four-dimensional crystallography

Ever since Jan Drenth solved the first crystal structure of a protein (papain) in Groningen, crystallography has been essential to investigate enzyme mechanisms. However, X-ray three-dimensional protein crystallography (3DC) rarely achieves the resolution required to place hydrogen atoms exactly (less than approximately 1.2 Å)—information that is crucial to understand proton shuffling and redox steps. To overcome this limitation, collecting Raman and ultraviolet spectra *in crystallo* allows the real-time monitoring of biocatalysis and complements the static information gained by 3DC. Andrea Mattevi (U. Pavia, Italy) illustrated the application of this new approach, also called microspectroscopy or X-ray four-dimensional crystallography (4DC), to the oxidation mechanism of phenylacetone monooxygenase (PAMO). He used 4DC to highlight the role of the ligand $NADP^+$ in flavin-hydroperoxide formation, which seems to have the dual role of reducing the flavin cofactor and stabilizing a crucial catalytic intermediate. Although traditional 3DC experiments did not reveal any difference between the oxidized and reduced structure of the enzyme, 4DC measurements *in crystallo* revealed transient changes in oxidation states during X-ray data collection. In particular, 4DC data suggests that the flavin peroxide intermediate is not cumulated *in crystallo* due to the experimental conditions and that the intermediate is possibly photo-oxidized rapidly by the X-rays [2]. This highlights that the study of catalytic mechanisms of redox systems explored by 3DC needs to be coupled with microspectroscopy to provide a time-resolved enzyme characterization, and to take into account oxidation and the damaging effects of X-rays on the crystalline sample.

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Enzymes and atoms in motion

In the late 1970s, Arieh Warshel took the first steps in computational enzymology when he applied molecular dynamics to rhodopsin, before its crystal structure was known. Since then, molecular dynamics has been extended to many other biological systems, from electron transfer reactions to ion transport in proteins. Thanks to the development of computational methods and computer power, a great level of accuracy and speed has been achieved in molecular dynamics, which can be coupled with molecular mechanics and quantum mechanics to understand enzyme mechanisms in molecular and atomic detail. Molecular mechanics force fields allow the mechanisms to be modelled from nanoseconds to microseconds, but it cannot be applied to chemical reactions where bond making and breaking is involved. To overcome this, quantum mechanics methods are introduced to model the distribution of electrons in the system explicitly. Adrian Mullholland (Bristol U., UK) applied quantum mechanics and molecular mechanics to elucidate the mechanism behind the drug resistance in mutants of influenza virus H1N1-2009. H1N1 uses haemagglutinin and neuraminidase to interact with sialic acid receptors in cell membranes for host cell invasion and subsequent infectivity. Sialic acid derivatives intended to interfere with the ability of the virus to infect the host were designed

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initially on the basis of the transition state of neuraminidase and through decades of structural studies, leading to a new generation of therapeutics including oseltamivir (Tamiflu®, Roche) and zanamivir (Relenza®, GSK). However, shortly after the 2009 flu outbreak and owing to the widespread use of antivirals, drug resistance against oseltamivir and zanamivir started to be reported. The I223R and H275Y mutations in neuraminidase turned out to be responsible for the drug resistance. Although the crystal structure of apo-neuraminidase is known, the position of a loop suggested a closed protein conformation. Molecular dynamics simulations revealed an opening of the protein, resulting in a conformational change localized at loop 150 in the catalytic site. When the same approach was applied to the inhibitor-bound structure, the transition to the open conformation in the double mutant I223R/H275Y clearly revealed a loss of binding [3]. In consequence, the emergence of drug resistance was due to a loss of hydrogen bonds between the inhibitor oseltamivir and the enzyme.

From molecular physics to archaeology

Molecular mechanics used to involve the study of rather simple molecular systems, but nowadays it is possible to investigate complex systems such as proteins. One aspect of this is palaeoenzymology, which can be used both to uncover general evolutionary principles by resurrecting extinct proteins and to discover ancient enzymes that have potential applications in the present day. Julio Fernandez (U. Columbia, New York, USA) has developed a technique called 'single protein force spectroscopy' (SPFS), in which a clamp applies a constant pulling force to a given protein in the sub-millisecond range. By using a cantilever tip to stretch the probe, single protein length changes can be measured with a resolution from 1 nm up to 0.1 nm achievable through some extra tricks. Fernandez demonstrated the power of this technology through his studies of the length and mechanical stability of Precambrian thioredoxin (Trx) enzymes. Trx are sulphur-based oxidoreductases involved in redox signalling and are ubiquitously conserved across the tree of life. By using SPFS to analyse disulphide bond reduction in seven reconstructed enzymes from different common ancestors and environments, Fernandez could elucidate the chemical mechanism of Trx enzymes. It turns out that both ancient and modern Trx enzymes use basically the same mechanism, but with differences in thermostability and activity depending on pH [4]. Given its usefulness, Fernandez also noted that his device is now in the process of being commercialized.

Teaching old enzymes new tricks

Just as stem cells differentiate into specialized cells in developmental biology for evolutionary biologists, ancestral enzymes were thought to be unspecific or promiscuous, catalysing similar reactions; in contrast to modern enzymes, which are usually specific for only one reaction. Romas Kazlauskas (U. Minnesota, USA) presented an interesting phylogenetic-based approach to obtain biocatalysts useful for the efficient asymmetrical synthesis of nitro alcohols—important building blocks in the chemical industry. Members of this family containing the ancestral α/β-hydrolase fold include dioxygenases, hydrolases, esterases and hydroxynitrile lyases. Previous studies have shown that the reaction of nitroalkanes with aldehydes (Henry reaction) catalysed by hydroxynitrile lyases is possible, albeit with negligible activities. Nevertheless, by looking at the probabilities of amino acid substitutions on specialist esterases and hydroxynitrile lyases inferred from phylogenetic analyses, it was possible to resurrect promiscuous enzymes showing nitroaldolase activity with high efficiency for this unnatural reaction.

Ab initio protein design

The ultimate challenge in protein engineering is the true rational design of practical biocatalysts. Instead of performing expensive and time-consuming *in vitro* experiments, it would be more economical and faster to design enzymes *in silico* first, and then go to the bench. Although the first *ab initio* examples were published recently [5], their enzymatic activity remains minimal, far from any real practical applications. Nevertheless, addressing the causes of these performance issues should help to reveal subtle details important to the design of the enzymes. By using 3DC of a Kemp eliminase, Stephen Mayo (California Institute of Technology, USA) explained how an active site that is both strongly flexible and heavily exposed to water molecules results in a folded yet inactive enzyme. The use of molecular dynamics suggested the need of an enlarged space for the active site for catalysis, which, upon rational amino acid mutagenesis, yielded a several hundredfold more active enzyme compared with the initial one [6]. Although there is no general guide to the design of *de novo* useful catalysts, ongoing research at Mayo's lab is providing promising results. The *in silico* screening of existing databases, together with molecular dynamics simulations, is defining a reduced set of protein scaffolds that can be further selected for experimental testing. In any case, the *status quo* in *de novo* protein design requires further mutagenesis and directed evolution. This was exemplified by Donald Hilvert (ETH Zurich, Switzerland) who, in collaboration with Mayo, evolved the Kemp eliminase, despite strong competition with other groups trying to optimize this important reaction in organic chemistry [7].

Evolving more efficiently

Previously known as 'the search for a needle in a haystack', the field of 'directed evolution' has matured to the point that it is now possible to enhance the activity, thermostability and selectivity of virtually any protein. In the past, libraries with thousands of variants had to be screened to find modest improvements. Now, smaller yet smarter libraries of hundreds of variants contain a higher number of improved biocatalysts. On the basis of the exploration of modern protein databases containing primary amino acid sequences or tertiary structures, Uwe Bornscheuer (Greifswald U., Germany) presented state-of-the-art strategies to discover useful biocatalysts, including the enantioselective synthesis of amines from prochiral ketones, which are useful precursors for a wide range of commodity chemicals. Although any enzyme can be engineered through directed evolution to fit the process specifications [8], the issue of non-additivity—synergistic or cooperative effects that cannot always be predicted by combining different mutations—remains a challenge [9].

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Real-world issues and solutions

As our oil-based resources disappear, our quest for a bioeconomy based on renewable sources becomes more important. Most of the biomass of Earth is found in trees in the form of lignocellulose. Thus, future biorefineries will require technologies to degrade recalcitrant polysaccharides. Glycoside hydrolases are a group of enzymes involved in the collective degradation of polymers. Although the mechanism of a hydrolytic reaction sounds trivial, glycoside hydrolases are not the only factors required for lignocellulose degradation. Vincent Eijsink (Norwegian U. of Life Sciences, Aas, Norway) gave a presentation about how the cooperation between glycoside hydrolases and specialized oxidases can improve the efficiency of enzymatically converting complex polysaccharides. Whereas glycoside hydrolases allow the substrate to access its active site through a tunnel or cleft, the oxidases have a planar substrate-binding surface, adapted to bind to surfaces rather than single polysaccharide chains. Eijsink presented recent results based on nuclear magnetic resonance spectroscopy and experimental metal-binding studies that provide deep insight into the copper-dependent lytic mechanism of these new oxidases [10]. This was a nice example of how a deeper understanding of enzyme mechanisms through basic research can lead to new avenues for addressing biotechnological challenges.

The scientific programme was excellent. However, we felt that allostery, the emerging role of which in protein mechanics and dynamics is gaining momentum [11], was almost underrepresented. Arthur Gora (Masaryk U., Brno, Czech Republic) did his best to promote this growing research area by demonstrating how communication pathways in enzymes can be redesigned to alter activity and selectivity [10]. Hopefully the next meeting will do more to embrace allostery and the possibilities it offers. For now, we thank all the organizers for this unique and engaging EMBO meeting on behalf of all participants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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