Closure – Whither Enzymology

40

नासतो विद्यते भावो नाभावो विद्यते सतः।
उभयोरपि दृष्टोऽन्तस्त्वनयोस्तत्त्वदर्शिभि।।2.16।।
The unreal has no existence, and the real never ceases to be,
the reality of both has been perceived by the seers of truth.

(Bhagavad Gita; 2.16)

Enzymes are the only biomolecules that combine three hallmark features, namely, *catalysis*, *specificity*, and *regulation*. No wonder that study of enzyme catalysis continues to occupy prime position in modern biology (and chemistry!). Up until the fashion of *omics* took over studying one enzyme at a time formed the thrust of enzymology. Importance of enzyme function in vivo will form one of the key frontiers with the present emphasis on systems biology as a pointer in this direction. Enzymes in sequence and in combination with other enzymes and other cellular components often manifest interesting features like coupled reactions, regulatory networks, and distributed control of metabolism. While single enzyme studies have taken the back seat, the knowledge gleaned from such research is important for a systems biology approach. After all *any biologist who follows their research interest to the finest level of detail will become an enzymologist* (quote by Perry Frey).

Enzymes are catalysts *par excellence*. A three-pronged strategy to comprehend them has involved mechanism, structure, and function. The following topics will continue to attract much attention of enzymologists in the foreseeable future.

Transition State Analysis and Computational Enzymology Transition state theory of reaction rates was meaningfully extended to enzyme catalysis. The highest point along the imaginary reaction coordinate is called the *transition state*; this is of highest free energy and is an ephemeral species. Kinetic isotope effects provide direct information on the enzymatic transition state. Besides kinetic measurements, isotope effects, time-resolved spectroscopy, NMR, X-ray crystallography, and

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_40

computational enzymology are making significant contributions to understand the enzyme transition state. This frontier in computational enzymology and complex enzyme models was recognized with chemistry Nobel Prize in 2013 to Martin Karplus, Michael Levitt, and Arieh Warshel. The emergence of the quantum mechanical/molecular mechanics approach allows one to ask – what are the origins of the catalytic power of an enzyme. Computer simulations will increasingly contribute to modeling molecular enzyme catalysis and capture the contributions of subtle enzyme molecular dynamics to rate accelerations. Such insights to the nature of transition state are valuable in terms of enzyme mechanism as well as design of powerful inhibitors.

Structure–Function Dissection of Enzyme Catalysis A structure-function approach to understand enzyme action uses a combination of kinetic (including rapid transient kinetics) and structural techniques. The protein chemical modifications, spectroscopic tools (like fluorescence, circular dichroism, etc.), and more recently molecular biology tools (like generation of site-directed mutants, truncated or chimeric enzyme proteins) are used. Whereas solution NMR is capable of reporting on enzyme molecular dynamics (albeit at much slower timescales than most events in enzyme catalysis), as of now this structural tool is limited to small proteins. This will improve with more powerful NMR machines. Mass spectrometry of larger polypeptides and oligomeric proteins is also coming of age. Much of direct structural information on enzymes has come from X-ray crystallography, but there is a limit to what a snapshot protein structure can reveal. As late Jeremy Knowles (biochemist at Harvard University) noted, *studying the photograph of a racehorse cannot tell you how fast it can run*. Although snapshots of enzyme bound to substrate, product, or transition state analogs are valuable, they do not capture structural dynamics of catalytic action. This is where progress is expected. Nonetheless, presence of molecular tunnels in enzymes like tryptophan synthase would not be apparent without detailed structural inputs. A fine balance between structural rigidity and conformational plasticity results in the unique catalytic power of enzymes. And structural enzymology of the future will aim to address such catalytic motions in detail.

Site-directed mutagenesis offers a powerful approach for the rational enzyme modifications. It enables enzymologists to selectively replace active site residue (s) and ask some very interesting mechanistic questions. Excellent insights continue to accrue on residues critical for catalysis, binding, and structural stability/flexibility for many enzymes. Nature presents us with 20 naturally occurring amino acids in proteins; we can replace a given residue by any one of the other 19. Nonnatural side chains often provide better mechanistic insights. Sophisticated tools and technology are in place to directly incorporate nonprotein amino acids into proteins in a position-specific manner. This includes expanding the genetic code through in vivo strategies. Without doubt site-specific mutagenesis will continue to extend our knowledge of enzyme mechanism and function.

Single Molecule Enzymology The Michaelis–Menten equation is a highly satisfactory description of kinetic data from a very large number of enzyme molecules in the assay. However, all enzyme molecules are not synchronized with each other in an ensemble-averaged kinetic measurement. A single enzyme molecule gives kinetic signals that reflect the dynamic states of individual catalyst molecules, information that is lost in the average signals from ensembles. Reactions involving single enzyme molecules can now be examined by the advances in fluorescence and related time-resolved spectroscopic techniques. Combined with computational approaches, these techniques allow for dynamic behaviors of individual molecules to be recorded in real time. Single-molecule behavior is powerful in uncovering (a) mechanistic pathways and intermediates, (b) heterogeneities hidden in the ensemble average, and (c) how enzyme conformational fluctuations affect catalysis. Conformational heterogeneity has been experimentally observed in them. Single enzyme molecules display inevitable, stochastic fluctuations in their catalytic activity. Such fluctuations would be less significant for a system comprising many enzyme molecules. However, many critical cellular processes, such as DNA replication, transcription, translation, and protein transport along the cytoskeleton, rely on one or few enzyme molecules. Stochastic fluctuations due to low copy number of enzymes have important physiological implications for cells/organelles. These are now being probed on a single-molecule basis in living cells.

Changing Landscape of Enzyme Allostery Observations on the control of enzyme activity by feedback mechanisms led to the simultaneous discovery of enzyme cooperativity and allostery. While mechanisms of allosteric regulation were developed more than 50 years ago, they continue to be revisited regularly. Present-day structural biology offers unequivocal evidence of multiple conformations in preexisting equilibrium for monomeric enzymes like glucokinase. The new, recent vision of allostery incorporates this more dynamic view of the enzyme protein. Accordingly, allosteric control manifests by a *population shift* in the statistical ensembles of many states, with regions of low local stability and others of high stability. Binding of ligands affects the relative free energies of these states, which differ in their affinities for other ligands and/or their activity. This is different from the earlier concept of a few well-defined (such as R and T states) static conformational states.

Allostery, by definition, involves the propagation of signals between different sites in a protein structure. There is much interest to probe the existence of an entire channel or network of residues through which allosteric signals are communicated. An important issue for further study is to elucidate mechanisms by which an allosteric effect is transmitted via the network of amino acid residues in the protein. The emerging radical view of enzyme function is that each catalytic step corresponds to an ensemble of thermodynamic and structural states. And allosteric regulation and enzymatic catalysis no longer appear as distinct phenomena but as the manifestations of the same intrinsic protein dynamics.

Predicting Enzyme Structure and Function Computational tools have revolutionized the whole of biology. Incorporating new technologies to understand enzymatic catalysis is a recurring theme in enzymology. Computational enzymology has made significant inroads into transition state analysis and molecular dynamics of enzyme action. Besides these, there is a rapid move toward enzyme structure and function prediction through computational approaches. This is because we (a) accumulate a large number of sequenced genomes, (b) come across orphan open reading frames, with no clues of their function, and (c) express sequences into proteins and even crystallize them without knowing what their function(s) are.

Assigning valid functions to unknown proteins/enzymes identified in genome projects is a challenge. While experimental testing remains essential, computational approaches can help guide this experimental design. Bioinformatics approaches are being perfected to (a) identify informative sequence relationships using structure and genome context, (b) homology modeling to allow accurate high-throughput structure prediction, and (c) in silico metabolite docking to provide accurate and testable list of potential enzymes. In view of the exponential growth in genome sequence data sets (with significant proportion of sequences with unknown enzyme functions), an integrated strategy for functional assignment was recently proposed (Gerlt et al. 2011). This enzyme function initiative (EFI) looks to predict the substrate specificities of unknown members of mechanistically diverse enzyme superfamilies, thereby defining their functions.

Enzymes Made to Order Curiosity and the desire to imitate general principles of biological catalysis have led to many developments in the design and construction of artificial enzymes. A major objective continues to be the elucidation of molecular basis of enzyme function. Second, using the knowledge base (which is still far from complete!), one could attempt to design and construct novel catalysts – the tailor-made enzymes. Present approaches to the creation of novel catalysts fall into two general categories: (a) the de novo design and synthesis of catalysts from polypeptides and nonprotein building blocks and (b) the modification/evolution of existing catalysts, such as protein enzymes or ribozymes, by genetic or chemical methods. Enzyme models of increasing complexity are being designed and discovered.

Linus Pauling recognized in the 1940s that the ability of an enzyme to speed up a chemical reaction arises from the "complementarity of its active site structure to the transition state." This has given rise to the productive field of catalytic antibodies (or *abzymes*). There is yet the promise of delivering tailored catalysts for difficult reactions for which natural enzymes do not exist. Such systems may be useful even if they do not attain enzyme-like efficiency. More recently, RNAs have assumed importance as components of (a) gene silencing through double-stranded small interfering RNAs (*siRNA*) generated by Dicer, an RNase III like enzyme, and (b) genome editing through the CRISPR/Cas system where crRNA-guided interference is exploited. The catalytic role of RNA molecules (the so-called ribozymes) has

expanded the realm of biological catalysis beyond proteins. While protein enzymes are superior catalysts, ribozymes might be easier to produce than regular enzymes.

One measure of how well we understand enzymes is to try and build such catalysts from first principles. In this sense, de novo enzyme design is an intellectual endeavor that serves two important objectives. It allows experimental validation of the principles of catalysis that we have gathered so far. Second, tailor-made catalysts can be built for industrial applications, particularly for those reactions for which natural enzymes do not exist. In enzyme engineering (and de novo design), serendipity continues to outstrip design – a clear sign that our basic understanding of enzyme catalysis is far from complete. A major benefit of recombinant DNA technology is the ability to do protein engineering. This powerful method is expected to deliver many enzymes with tailored properties. The strategies to generate novel catalysts include enzyme redesign starting from an existing protein scaffold. Directed evolution of enzymes is making a rapid transition from being a new tool for studying the relationship between sequence and function to being an extremely useful and efficient method for optimizing biocatalysts for industry. The greatest advantage of directed evolution is its independence from the knowledge of enzyme structure and we simply get what we screen for! Rational de novo design of an enzyme continues to be a grand challenge. This is because our understanding of protein dynamics is still very limited and this makes predictions difficult. As of now, the marriage of rational and combinatorial redesign seems to be the way to go.

In the era of systems/synthetic biology, enzymology may not be fashionable, but it will continue to excite and motivate (Cleland 1979; Khosla 2015). As Pasteur famously stated, *there are no applied sciences but only applications of science*. This is so true with the study of enzymes. We rarely find an unemployed enzymologist. Surely, the future will be no different.

References

Cleland WW (1979) Enzymology-dead? Trends Biochem Sci 4:47–48

Khosla C (2015) Quo vadis, enzymology? Nat Chem Biol 11:438–441

Gerlt JA, Allen KN, Almo SC, Armstrong RN, Babbitt PC, Cronan JE, Dunaway-Mariano D, Imker HJ, Jacobson MP, Minor W, Poulter CD, Raushel FM, Sali A, Shoichet BK, Sweedler JV (2011) The enzyme function initiative. Biochemistry 50(46):9950–9962