Biochimica et Biophysica Acta 1864 (2016) 65–69

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Physiological enzymology: The next frontier in understanding protein structure and function at the cellular level☆

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ARTICLE INFO

Article history:
Received 4 June 2015
Received in revised form 21 July 2015
Accepted 23 July 2015
Available online 13 August 2015

Keywords:
Enzymology
Kinetics
Spectroscopy
Protein structure
Single molecule

ABSTRACT

Historically, the study of proteins has relied heavily on characterizing the activity of a single purified protein isolated from other cellular components. This classic approach allowed scientists to unambiguously define the intrinsic kinetic and chemical properties of that protein. The ultimate hope was to extrapolate this information toward understanding how the enzyme or receptor behaves within its native cellular context. These types of detailed *in vitro* analyses were necessary to reduce the innate complexities of measuring the singular activity and biochemical properties of a specific enzyme without interference from other enzymes and potential competing substrates. However, recent developments in fields encompassing cell biology, molecular imaging, and chemical biology now provide the unique chemical tools and instrumentation to study protein structure, function, and regulation in their native cellular environment. These advancements provide the foundation for a new field, coined physiological enzymology, which quantifies the function and regulation of enzymes and proteins at the cellular level. In this Special Edition, we explore the area of Physiological Enzymology and Protein Function through a series of review articles that focus on the tools and techniques used to measure the cellular activity of proteins inside living cells. This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

In the late 1600s, Antoine van Leeuwenhoek was the first scientist to use simple microscopy techniques to observe and report on organisms such as protists and bacteria. While his initial reports were met with amusement and skepticism, science has come to appreciate his pioneering work and recognize him as the father of cellular biology. Since then, scientists have been fascinated by the inner workings of prokaryotic and eukaryotic cells. One of the more exciting challenges has been to completely decipher the complexities surrounding the structural diversity of proteins and how this diversity impacts their function within the confines of the cell. From a historical perspective, one could argue that organic chemists had the earliest and most significant influence on examining protein structure and its relationship to function. In this respect, organic chemists introduced a reductionist philosophy to study the individual contributions of defined molecular forces such as hydrogen bonding interactions, electrostatic potential, and solvation effects on the rates, stoichiometries, and stereochemistry of chemical reactions. In this case, quantifying how variations in an individual component affect a chemical reaction allowed one to deduce the role of a defined physical force on chemical reactivity. These efforts lead to the development of structure–activity relationships such as Hammett plots which provide quantitative descriptions of free energy relationships as they relate to reaction rates and equilibrium constants.

Biochemists quickly adopted this reductionist approach and began applying this philosophy to study the behavior of enzymes that perform similar reactions to those reported by organic chemists. The major difference, of course, is the remarkable ability of enzymes to catalyze reactions with faster rates and increased regioselectivity, especially in aqueous media which contrasts most organic reactions that are typically constrained to non-aqueous solutions. Despite these advantages, however, it was nearly impossible to study the activity of an individual protein within the confines of the cell due to overwhelming complexities. Complications caused by competition with thousands of other proteins coupled with numerous other biological macromolecules such as lipids, carbohydrates, and metals required biochemists to apply a reductionist approach to define the activity of a specific protein or enzyme. As a result, enzymology relied heavily on characterizing the biochemical properties of a single, highly purified protein that was removed from all other cellular components. Using this reductionist approach, enzymologist could define intrinsic thermodynamic and chemical properties of that particular enzyme. The ultimate hope was to take the mechanistic information derived from an isolated enzyme or protein and extrapolate

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http://dx.doi.org/10.1016/j.bbapap.2015.07.011
1570-9639/

it to understand its behavior within a cell. The ability to perform structure–activity relationships on enzyme-catalyzed reactions was made possible by applying the groundbreaking work of Leonor Michealis and Maud Menten that generated a mathematical model to define the rate of product formed by an enzymatic reaction by relating the initial velocity of the reaction with variations in substrate concentration [1]. This mathematical model allowed three important parameters to be quantitatively defined. These include V_{max} (the maximal speed of the reaction), K_m (the concentration of substrate needed to obtain one-half of the maximal velocity), and V_{max}/K_m (the catalytic efficiency of the enzyme catalyzed reaction). While initially published in 1913, the mathematical model developed by Michealis and Menten is still widely applied today to understand enzyme function.

In the 1960s, the field of enzymology was revitalized by the pioneering work of several research groups that help redefine our understanding of enzyme catalysis and function. One group led by W.W. Cleland and colleagues continued to develop mathematical models to quantify enzyme behavior. Realizing that the overwhelming majority of enzymes perform reactions using two or more substrates, these authors published a series of seminal publications that specifically address this complication [2–4]. In these papers, the simplistic model (and associated limitations) initially derived by Michaelis and Menten was expanded to account for more complex reactions involving multiple substrates. In addition, a series of rules were developed using initial velocity patterns based on product and dead-end inhibitors. Information derived from these patterns could be logically applied to determine the order of substrate binding and product release as well as to define the location of the rate-limiting step along the reaction pathway.

Meanwhile, other scientists were applying principles of organic chemistry to uncover many of the mysteries surrounding enzyme catalysis. The field of bioorganic chemistry was spearheaded by scientific luminaries including William Jencks [5–7], Robert Abeles [8–10], Thomas Bruice [11–13], Stephen Benkovic [14–16], Frank Westheimer [17], Jeremy Knowles [18], Myron Bender [19], Chris Walsh [20], Alan Fersht [21], Ronald Breslow [22] and many others. These scientists and their colleagues helped develop concepts such as transition state stabilization, ground state destabilization (the Circe Effect), mechanism-based inhibitors, catalysis involving acyl and phosphate transfer reactions, and later on, dynamic protein motions associated with enzyme catalysis, that form the basis for much of our current understanding of enzyme catalysis.

Other approaches such as rapid chemical quenching techniques, originally developed by Tonomura [23], Gutfreund [24], and Hess [25] and then popularized by other scientists including Paul Boyer [26], Ken Johnson [27], and Gordon Hammes [28], also expanded the field of enzymology. Applying rapid-mixing techniques allows scientists to study enzyme behavior on extremely short time scales (microsecond to seconds). Information obtained from these pre-steady-state measurements is vital to define kinetic steps encompassing substrate binding, chemistry, product release, and intervening conformational change steps during catalysis. In general, these types of traditional enzyme kinetic studies investigate the behavior of an ensemble of molecules. However, to gain a more in-depth understanding of an enzymatic reaction or the function of a protein, single molecule experiments are performed to study the behavior of an individual molecule within an ensemble using techniques such as fluorescence energy transfer. Performing a combination of bulk (ensemble) and single-molecule experiments provides a powerful platform to study complex protein function and dynamics. Indeed, applying these approaches to enzymes such as dihydrofolate reductase [29] has generated unique insight into the behavior of enzymes as catalysts.

Concomitant with increases in understanding enzyme function came major advances in structural biology that provided unprecedented insight into how proteins perform chemical reactions. From a historical perspective, inorganic chemists initially made the largest impact in the field of X-ray crystallography as they used this technique to solve the structures of numerous metal-complexes. Based on their success, biological chemists quickly adopted this scientific platform, and in the 1960s, two Nobel prizes were awarded for remarkable achievements in applying X-ray crystallography to better understand the complexities of biological compounds. One award was given to Dorothy Crowfoot Hodgkin for her efforts in solving the structures of complex biomolecules such as the enzyme cofactor, vitamin B-12, and the antibiotic, penicillin [30,31]. The second was awarded to Perutz and Kendrew for their structural studies on globular proteins such as hemoglobin [32–34]. The contributions of Perutz and Kendrew are particularly noteworthy as their research validated how events associated with the binding of small molecules such as molecular oxygen can produce significant conformational changes in protein structure. Visualizing these structural changes in hemoglobin advanced many new concepts in biochemistry, most notably allostery in ligand/substrate binding. To this day, X-ray crystallography plays a pre-eminent role in providing information on the secondary, tertiary, and quaternary structure of many proteins and enzymes.

Spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy, infrared spectroscopy, mass spectroscopy and fluorescence spectroscopy are often employed to further characterize how enzymes and proteins function as well as to detect protein complexes that exist under physiologically relevant conditions. Initially used as analytical tools to identify and characterize the structures of small molecules, important advancements in these techniques over the past 20 years have propelled their utilization by biological chemists to study significantly larger biomolecules. For example, bull seminal protease inhibitor was the first protein structure solved using 2-dimensional NMR [35]. Through the pioneering work of Nobel Prize laureates Richard R. Renst and Kurt Wüthrich [36] and others, advances in NMR spectroscopy allowed for the determination of the solution structures for much larger protein complexes such as the 900 kDa GroEL/GroES [37]. Finally, structures of membrane proteins are routinely determined using solution or solid state NMR specroscopy.

Infrared spectroscopy also plays an important role in defining protein structure and function. Using Fourier transform infrared (FTIR) spectroscopy, the mechanisms of protein misfolding and aggregate formation have been investigated [38]. Extension of this technique further allows for the monitoring of chemical changes in cells, thereby providing insight into global changes in the content and localization of proteins in normal *versus* diseased cells. With recent advances in detection, sensitivity, and data analyses, innovative experiments can now be performed to monitor the behavior of substrates or ligands on cellular proteins as well as the transient interactions amongst cellular proteins in biochemical pathways. Many of these approaches are highlighted and described within this Special Issue to illustrate their power in deciphering how proteins and enzymes function under physiologically relevant conditions.

Another recent development in cell biology is the use of fluorescently labeled proteins such as green fluorescent protein (GFP) to study protein function. Seminal work on the discovery and development of GFP originally isolated from the jellyfish, *Aequorea victoria*, lead to a Nobel Prize in Chemistry for Martin Chalfie, Osamu Shinomura, and Roger Tsien in 2008 [39–41]. Fusing the gene for GFP to the N- or C-terminus of a gene of interest allows for the expression of a fusion protein that can be monitored by fluorescence microscopy. With this technique, scientists can now easily visualize the cellular localization of any polypeptide that is stably or transiently expressed. Applying the mantra for real estate — "location, location, location" — to enzymology allows scientists to accurately define how protein localization influences its function, activity, and regulation. Today, GFP-fusion proteins are routinely introduced and expressed in numerous organisms ranging from simple bacteria and yeast to more complex organisms including plants and animals.

While GFP and other fluorescently-labeled proteins have expanded our understanding of protein localization, they suffer from several drawbacks. The most notable deficiency of this technology is that it is