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New Concepts in Bioorganic Chemistry

Beyond Enzyme Kinetics: Direct Determination of Mechanisms by Stopped-Flow Mass Spectrometry

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Abstract—The development of soft ionization techniques has made mass spectrometry an efficient and essential tool for the determinations of the primary structures of peptides and proteins. Recently the technique has been extended at an explosive rate to noncovalent structures as well as dynamics of protein–protein interactions. We propose here that interfacing mass spectrometry with a stopped-flow mixing device and applying these new techniques of soft ionization to enzymes undergoing catalysis will provide direct access to enzyme mechanisms, both kinetic mechanisms (which describe the comings and goings of substrates, products, and inhibitors) and chemical mechanisms (which describe the order of breaking and making chemical bonds). Transient-state measurements will provide the order of reaction events; steady-state measurements will provide the distribution and therefore the relative energy level of enzyme forms participating in those events; combining transient-state and steady-state measurements is therefore expected to provide sufficient information to construct a free energy diagram of the enzyme-catalyzed reaction. © 1997 Elsevier Science Ltd.

Fantastic developments in mass spectrometry such as fast atom bombardment (FAB), matrix-assisted laser ionization techniques (MALDI), and electrospray ionization (ESI) have burst upon the scene and are poised to revolutionize enzymology. The ability to measure the mass of large proteins, including covalent and noncovalent enzyme–ligand complexes, to within a fraction of a dalton is absolutely breathtaking.[1–4] It occurs to us that if such a detection system were interfaced with a rapid-mixing device such as a stopped-flow apparatus, both old and new questions of enzyme kinetics could be answered by direct observation, rather than by the inferential analytical geometry of classical enzyme kinetics. Listed below are possibilities that, based on selective examples of published literature, are immediately attainable, some without the aid of a rapid-mixing device. Short of these exciting possibilities, however, is the simple idea that if enzymologists replaced their Gilford absorption spectrophotometers with mass spectrometers and focused on the traditional low-mass region of the spectra, *virtually all products of all enzyme reactions*† could be monitored directly, obviating the need for chromophoric substrates and coupled assays, and at the same time provide much greater accuracy and precision as well as access to lower concentrations of substrates. These advances alone could revolutionize enzymology.

†Racemates are the only exception.

Determination of inhibitor dissociation constants

This has already been done. For example, Grieg et al.[5] have measured dissociation constants of oligonucleotide-serum albumin complexes (MW 72,870 and 79,273 corresponding to the oligomer and its disodium adduct, respectively) by electrospray mass spectrometry and found dissociation constants of 3.1 ± 0.3 and 11.9 ± 0.6 μM, respectively. These values compare to 2.8 ± 0.3 and 10.0 ± 0.2 μM obtained from a Scatchard plot of solution data. Hence, fears of underestimating complexes due to fragmentation are unfounded when soft ionizations are employed.

Dissociation constants of tight-binding inhibitors are difficult to measure because of the very low concentrations of inhibitor and enzyme required, which induce slow onsets of inhibition.[6] But Gao et al.[7] have used mass spectrometry to measure dissociation constants as low as 1.1 nM for inhibitors of carbonic anhydrase. Moreover, they did so using a combinatorial library of 289 peptide inhibitors, identifying the few tight-binding inhibitors within the library from peak locations, and determining their dissociation constants from relative peak heights—in, effectively, a single experiment! A tremendously powerful screening device for inhibitors and antagonists has been invented.

Determination of kinetic mechanisms of inhibition

Cheng et al.[8] have demonstrated competitive binding within a pool of 16 inhibitors of carbonic anhydrase by the absence of $E(I)_2$ complexes, where E represents enzyme and I an inhibitor. It follows that if an inhibitor and enzyme were mixed together with the substrate, S, it should be possible to determine whether or not any complexes of EIS are formed, and to what degree in comparison to EI. The absence of EIS signifies competitive inhibition. Such an experiment has been done by Li and Henion[9] using ion-spray mass spectrometry to examine the hydrolysis of a hexasaccharide of *N*-acetylglucosamine, NAG_6, by hen egg white lysozyme in the presence of an inhibitor, tetra-*N*-acetylchitotetraose δ-lactone. While no EIS complexes were observed, $E(I)_2$ was clearly present. Probing mechanism-based inhibitors, Knight et al.[10] observed stable complexes with inhibitors of human leucocyte elastase, thus verifying their covalent nature, and were able to probe the chemical mechanism of the suicide substrates by the magnitudes of masses of the complexes.

Determination of substrate and product dissociation constants

Dissociation constants of substrates are often not determinable from steady-state kinetics.[11] It follows from studies with inhibitors described above that if an enzymatic reaction were examined during initial velocity conditions in the steady-state, then dissociation constants could be determined by relative peak heights of E and ES as a reaction approaches equilibrium. Thus the perennial uncertainty of whether the Michaelis–Menten constant, K_m, is equal to the dissociation constant, K_d, can be resolved. Because enzyme-complexed products accumulate as equilibrium is approached, then dissociation constants of products could be determined as well. Li and Henion[9] were able to observe the progress of lysozyme catalysis over a period of an hour without the aid of a rapid-mixing device because NAG_6 is a poor substrate. Free enzyme was observed at m/z 1789 and 2045, the enzyme–NAG_6 complex was observed at m/z 1943, and the latter peak decreased with time. The product complex enzyme–NAG_4 (K_d = 8.5 μM) was also observed, at m/z 1892, and this peak increased with time. The complex enzyme–NAG_2 was not observed, but NAG_2 binds approximately 100 times weaker than NAG_4,[12] which will account for the absence of a corresponding mass spectrometry peak, and gives us a lower limit of binding tightness necessary for detection by mass spectrometry.

Determination of diffusion-controlled rate constants

A severe limitation of contemporary kinetics is the absence of a reliable method for determining the rate constants for the combination of substrates and enzymes. Most kinetic studies rely on calculations such as that of Alberty and Hammes,[13] which in turn must rely on assumptions concerning shape and angles of attack. On the other hand, rate constants for the combination of tight-binding inhibitors and enzymes can be determined directly if concentrations of both enzyme and inhibitor are very low.[14] Slow, tight-binding inhibition arises from second-order reaction kinetics where $k_{app}=k_{on}[E][I]$. Because of the remarkable sensitivity of modern mass spectrometry—concentrations in the *femtomole* range can be measured accurately—it follows that slow onsets of catalysis at very low $[E]$ and $[S]$ can be achieved as well, allowing measurement of k_{app} and calculation of k_{on}.

These measurements will have considerable value to enzyme kinetics. For example, the concept of a 'perfect enzyme' in which V_{max}/K_m is diffusion-controlled, is a popular one (e.g., ref 15) but it also depends on calculated values of diffusion-controlled rate constants. It is possible that some enzymes currently thought to be perfect because of high V_{max}/K_m values will turn out not to be so, while others with low V_{max}/K_m values will be found to approach diffusion-controlled kinetics despite the low values because of hindered access to the active site or of inactive conformers of the substrate. In the latter case, knowing the conformation which undergoes binding provides a guide to inhibitor design.

Determination of enzyme kinetic mechanisms

The possibilities of mechanistic determinations are many—and sufficient to render classical steady-state kinetics obsolete. We will examine three: ping-pong, sequential, and abortive-ordered sequential mechanisms. If a ping-pong mechanism is operative, then incubation with either the first substrate or second product will generate a new form of enzyme with a different mass, such as an acyl-enzyme. The latter has been observed by mass spectrometry[16] using very slow substrates of chymotrypsin and subtilisin. Moreover, the competency of the alternate form of enzyme to account for rates of turnover should be testable by mass spectrometry as well, by rapidly combining a mixture of the first substrate and enzyme with the second substrate (water, in the case of serine proteases), and ascertaining to which form of enzyme the second substrate combines—a determination that has not been possible within traditional kinetics.

Distinguishing between random sequential and ordered sequential mechanisms would appear to be straightforward; an EB complex would form in the former, but not in the latter (taking care to explore a range of concentration of both substrates). However, a special case of sequential mechanism makes the mass spectrometry approach especially attractive. In the abortive ordered sequential mechanism an unproductive EB complex is formed, and if binding is in rapid equilibrium this mechanism cannot be distinguished from a random one.[7] But as described above, rapid-equilibrium binding can be rendered slow-onset binding at the low concentrations available to detection by mass spectrometry. Hence, stopped-flow mass spectrometry will distinguish between these mechanisms.

Determination of iso-mechanisms

The substrate combines with one form of enzyme, E, and the product dissociates from another form, F, in an iso-mechanism:

$$E + S \rightleftharpoons ES \rightleftharpoons FP \rightarrow P + F \rightarrow E$$

Scheme 1.

In order to complete the turnover, free enzyme must undergo an isomerization, represented by F—E. Despite a 40-year history, few examples have been described because of the lack of a good detection method.[18] Analysis of progress curves, however, either in the form of oversaturation plots or Foster–Neiman plots has increased the sensitivity of detection[19] and led to the identification of several new iso-mechanisms, including proline racemase, triosephosphate isomerase, fumarase, pepsin, and alanine racemase. Indeed, most chemical mechanisms proposed for enzymatic catalysis, such as general acid or general base mechanisms, presuppose an iso-mechanism of some sort, albeit kinetically insignificant, perhaps. Nevertheless, difficulties remain in the new detection methods, notably a necessity for a direct assay method, as coupled assays cause problems.

Mass spectrometry should make the detection of iso-mechanisms relatively easy, and with a level of sensitivity as to detect even kinetically insignificant iso steps, which while unimportant to kinetic mechanisms, can be useful in establishing chemical mechanisms.[20] If an isomerization is rate-limiting and kinetically very significant, as is the case with carbonic anhydrase[21] then "when the enzyme is saturated there's nothing on the enzyme" (Irwin Rose, personal communication). Free F will be observed under steady-state conditions, not ES or EP of Scheme 2, and in the case of carbonic anhydrase in which the iso step is a proton transfer, the difference in mass from free E will be one mass unit. Can we see that? Incredibly, the answer to that question is, yes! Gao et al.[7] obtained a value of MW_{obs} = 28,996.6 ± 0.1 Da for bovine carbonic anhydrase II.

Determination of chemical mechanisms

By extension, given the remarkable precision evidenced with carbonic anhydrase, it may be possible to document step-wise catalysis and identify intermediates. Putative intermediates have been suggested for many enzymatic reactions on chemical grounds, but proving their kinetic competence can be a difficult matter.[24] For example, oxaloacetate has long been considered an intermediate in the oxidative decarboxylation of malate to pyruvate catalyzed by malic enzyme, and deuterium isotope effects on heavy-atom isotope effects are consistent with hydride transfer preceding decarboxylation.[25] However, oxaloacetate does not dissociate during reaction turnovers to allow direct observation of its formation, although free oxaloacetate will act as a weak substrate for the formation of both malate and pyruvate.[26] Stopped-flow mass spectrometry can provide direct evidence for the intermediate because the noncovalent complex *E-NADPH-oxaloacetate* should differ from *E-NADP-malate* by one mass unit and one charge, and thus may be separable. If not, then the 'softness' of the ionization techniques can be modulated to fragment intermediate complexes with or without fragmenting the intermediates themselves, and thereby separate intermediates from substrates and products. Moreover, using tandem mass spectrometry, a soft ionization in the first stage would separate enzyme complexes; an intermediate enzyme complex would be collected and subjected to moderate ionization in the second stage, causing only a noncovalent complex to dissociate to intermediate and free enzyme, thus providing a means to identify covalent complexes; and in the third stage, the intermediate from a noncovalent complex would be subjected to hard ionization causing fragmentation of the intermediate to produce a spectra from which the identity of the intermediate could be determined by conventional means.

Determination of free energy diagrams

Identifying the so-called rate-limiting or rate-controlling step of an enzymatic reaction has been the goal of many investigations, but as Laidler[22] has pointed out, unless the free energy diagram is known for a particular reaction, a rate-controlling step lacks definition, and if the free-energy diagram is known, identifying a rate-controlling step is unnecessary. Constructing a free-energy diagram from kinetic data, however, is a difficult process, requiring extensive kinetic analyses[23] and usually is limited to mechanisms of only three steps. Mass spectrometry offers the possibility of immediate and direct access to one form of diagram, albeit limited to enzyme forms of different mass. Their distribution during steady-state conditions would define the ground-states of the diagram, and the kinetics of their formation during transient-state conditions would define both the order of their appearance along the reaction coordinate and energy levels of the transition-states.

This idea is illustrated in Figure 1, in which a hydrolytic reaction is simulated using the following reaction mechanism:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EPQ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EQ \overset{k_1}{\rightarrow} E + Q$$

Scheme 2.

The mechanism is assumed to have a rate-limiting release of the final product, Q, signified by the highest barrier. The final step might include the hydrolysis of a covalent acyl enzyme intermediate, EQ. Covalent acyl intermediates and noncovalent but tightly-bound complexes can be difficult to distinguish kinetically, but if ES and EPQ differ in size by the mass of a water molecule, 18 Da, an acyl mechanism would be ruled out.

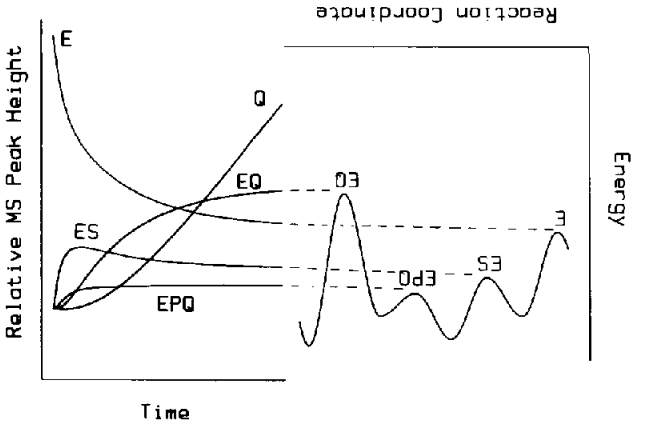

Figure 1. Transient-state and steady-state kinetics of an enzymatic reaction as they would appear in stopped-flow mass spectrometry. On the left are simulated progress curves for the different forms of enzyme participating in the mechanism of Scheme 2. The relative values for the rate constants employed in the simulation were 2, 4, 3, 5, 6 and 1 for k_1-k_7, respectively. Relative concentrations of free Q were multiplied by 100. On the right is a simulated free-energy diagram associated with Scheme 2 in which the data were plotted on an antilog scale to exaggerate differences, and plotted upside down to illustrate the inverse relationship between energy levels and concentrations. Rotate the figure one-half turn to view the energy diagram in the normal way.

Conclusion

We are sure by now that the reader has imagined other possibilities of application of stopped-flow mass spectrometry to enzyme mechanisms. In sum, most of the questions addressed by traditional enzyme kinetics can be answered more simply experimentally and with less ambiguity by stopped-flow mass spectrometry, putting to rest the old saw "you can't prove a kinetic mechanism, you can only disprove." More importantly, many questions out of the reach of traditional kinetics can now be answered as well. Nearly 20 years ago, Cleland[27] prepared a detailed review of how to "ferret out the secrets of how enzymes really catalyze their reactions" thus making the discipline available to "a new generation of enzymologists, armed with a brain and a Gilford." Stopped-flow mass spectrometry promises to replace both.

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