Isotopes in Enzymology

25

Isotopes are atoms of an element that contain different number of neutrons in their atomic nucleus. They have the same atomic number, i.e., same number of protons (of course an equal number of electrons), but differ in their atomic mass due to the neutron numbers. For example, the three isotopes of hydrogen have one proton and one electron each but contain zero (hydrogen), one (deuterium), or two (tritium) neutrons. Chemical properties of any element are determined by its electronic configuration (and atomic number), and therefore all the isotopes of that element react similarly. Isotopes provide an excellent tool for the study of reactions because they are isoelectronic and isosteric. This feature makes isotopes eminently suitable as tracers in enzyme research. Both radioactive and stable isotopes commonly used in enzymology are listed in Table 25.1. While remarkably similar in their chemical reactivity, their mass differences inflict subtle but definite changes in the rate of bond-forming/bond-breaking events involving them. These "isotope effects" – valuable in understanding enzyme mechanisms – are discussed in a subsequent section (Chap. 27 Isotope Effects in Enzymology).

Substrates/products labeled with radioactive isotopes are used in enzyme assays. Stable isotopes are less commonly used to monitor enzyme reactions. Their detection is either inherently less sensitive or requires involved instrumentation like NMR spectroscopy or mass spectrometry. Such nonradioactive methods are not easily amenable for routine assays. NMR active labels (like ^{15}N and ^{13}C) are of value in solution dynamic studies of enzymes. These heavy isotopes (^{13}C in particular) are very valuable also in the study of metabolism (metabolic flux measurements and *metabolomics*). Coupled with accurate mass measurements, substrates appropriately labeled with heavy isotopes provide the necessary data for kinetic isotope effect analysis.

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25.1 Enzyme Assays with a Radiolabeled Substrate

Radioisotope measurements are particularly resorted to when other simpler methods of assay like colorimetry, spectrophotometry, fluorimetry, or polarography are not feasible. A substrate bearing one or more radioisotopes is used in enzyme kinetic measurements so that the product formed is radioactive. Almost always enzyme assays with radiolabeled substrates are fixed-time, end-point assays – the reaction is stopped, and using an appropriate technique, labeled product is separated from the remaining substrate. A chromatographic step is often employed for this purpose. For example, galactokinase reaction is monitored by using ^{14}C-galactose as substrate; the labeled galactose-1-phosphate formed is resolved from unreacted ^{14}C-galactose by a suitable ion exchange resin and its radioactivity counted. The amount of radioactivity in the product, as a function of time, gives the measure of reaction progress. While using radiolabels to monitor reaction rates, following considerations are important:

1. The position of the radiolabel in the substrate should be carefully chosen. Atomic positions away from and not involved in bond-breaking/bond-forming steps must be used. Such remote labeling ensures that no "isotope effects" are introduced.
2. Sensitivity of radiotracer detection requires that a good post-reaction separation of labeled substrate from the product formed be achieved. This is critical in obtaining satisfactory blanks and controls.
3. In enzyme kinetic studies, the labeled substrate is usually mixed with "cold" (unlabeled) substrate to achieve required substrate concentration without having to use high quantities of radioactivity. The specific radioactivity is so adjusted that minimal amount of the radiolabel will provide good signal-over-background readings.

Commonly used radioisotopes are β-emitters, and the radioactive decay process follows first-order kinetics (Chap. 9, Chemical Kinetics: Fundamentals, in Part II). Each radioisotope is associated with a characteristic half-life (Table 25.1). The standard unit for radioactivity is the Curie (Ci) – the quantity of any substance that decays at a rate of 2.22×10^{12} disintegrations per minute (dpm). The proportion of

Table 25.1 Isotopes commonly used in enzyme studies

Element (most abundant form)	Radioactive isotope			NMR active isotopes	Heavy isotopes
	Isotope	β-Emission (MeV)	Half-life		
Hydrogen (^{1}H)	^{3}H	0.018	12.3 years	^{1}H, ^{2}H	^{2}H
Carbon (^{12}C)	^{14}C	0.154	5700 years	^{13}C	^{13}C
Nitrogen (^{14}N)	–	–	–	^{15}N	^{15}N
Oxygen (^{16}O)	–	–	–	^{17}O	^{17}O, ^{18}O
Phosphorus (^{31}P)	^{32}P	1.718	14.3 days	^{31}P	–
Sulfur (^{32}S)	^{35}S	0.167	87.1 days	–	–

radiolabeled molecules in the given substrate sample is expressed as specific radioactivity. Convenient units for specific radioactivity are dpm/μmol and μCi/mmol.

In practice, one cannot directly measure radioactivity in a given sample because the efficiency of counting the number of disintegrations (in a scintillation counter) is never 100%. Therefore the counts per minute (cpm) are measured, and using a factor for counting efficiency, the sample radioactivity can be calculated in dpm units. Going from cpm to dpm to specific radioactivity, one can easily convert the data into conventional enzyme velocity units.

25.2 Isotope Partitioning

Apart from their use as tools in enzyme assays (as mentioned above), radiolabels are employed to probe the reaction mechanism (Boyer 1978; Rose 1995). Exchange of label from the product to its cognate substrate can be followed even while the reaction is actually proceeding in the forward direction. For example, in a reaction catalyzed by glucose-6-phosphatase, the reverse flow of label from ^{14}C-glucose back to glucose-6-phosphate was monitored even while the net forward reaction was occurring. Such experiments provide insights into order of product release.

Radioisotopes are also used to probe the fate of a substrate molecule sitting on the enzyme as *EA* complex. Recall the earlier Michaelis-Menten formalism (Chap. 15, in Part II) employed to derive the rate equation. We can now imagine the same enzyme-substrate complex but with a radiolabeled substrate (A^*). Once formed, this EA^* has two possible fates – A^* gets converted to product (catalysis and product release with a forward rate constant k_{cat}) or A^* is released from the complex (with a rate constant k_{-1}) even before catalysis can occur (Fig. 25.1).

With suitable experimental design, it should be possible to measure the ratio of two rate constants – k_{cat}/k_{-1}. This ratio – without units – is a measure of *commitment-to-catalysis*; it is also variously referred to as *partition coefficient* (also see Chap. 16, More Complex Rate Expressions, in Part II) or *stickiness ratio*. This ratio is negligible when k_{cat} is very small compared to k_{-1} – the commitment-to-catalysis is very low – the *EA* complex has greater tendency to dissociate rather than to convert A to P. If k_{cat} is much greater than k_{-1}, then commitment-to-catalysis is very high – the substrate is very sticky, and all the substrate that binds *E* goes on to form

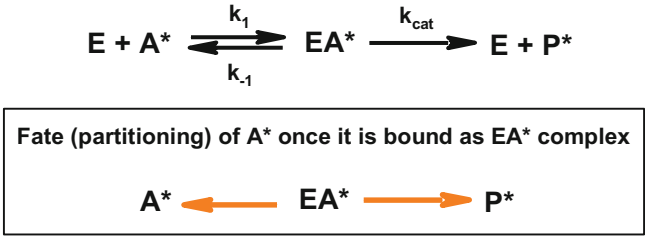

Fig. 25.1 Equilibria showing the fate of enzyme-bound isotopically labeled substrate

product. It should however be clear that stickiness (given by k_{cat}/k_{-1}) and affinity (the association constant, k_1/k_{-1}) are not the same.

The commitment-to-catalysis can be experimentally measured by the substrate-trapping procedure (called Rose experiment). A schematic of this elegant but powerful protocol is shown below.

1. Generate EA^* complex in a small volume (say 20 μl) by mixing known amount of enzyme and labeled substrate.
2. Dilute this with rapid mixing into a cocktail containing other reaction components also containing >1000 fold excess of unlabeled A.
3. Incubate briefly (10–15 s) for the enzyme to go through several catalytic turnovers and permit enzyme bound A^* to react.
4. Quench the reaction (usually with acid) and measure radiolabel in A^* and P^*.
5. The steps 1 through 4 are repeated at several concentrations of the other substrate (other than A, say B). The label trapped as P^*, at several concentrations of B, is recorded. Suitable controls are taken each time.

From a double reciprocal analysis of the isotope trapping data ($1/P^* \rightarrow 1/[B]$), we obtain (a) maximal labeled P^* formed in the first turnover and (b) the apparent K_M for B, for the trapping process. The commitment-to-catalysis is determined at saturating [B]. Such an isotope trapping strategy was first described for yeast hexokinase by Irving Rose, to show that glucose was very sticky. Isotope partitioning analysis of inosine phosphorylase (reaction shown below) provides an excellent example of this procedure.

$$\text{Inosine} + \text{Phosphate} \rightleftarrows \text{Hypoxanthine} + \text{Ribose-1-phosphate}$$

Of the 30 μM of enzyme-[8-^{14}C]inosine complex, almost 20 μM was trapped and recovered as [8-^{14}C]hypoxanthine at saturating phosphate concentration. The remaining 10 μM of the EA^* complex dissociated back to release the bound substrate, [8-^{14}C]inosine. A commitment-to-catalysis of about 2 was calculated from this data ($k_{cat}/k_{-1} = 20\ \mu M/10\ \mu M = 2$).

There are practical limits as to when an isotope partition study is feasible. For single-substrate enzymes, for example, the reaction begins as soon as enzyme and substrate are mixed together. Measuring partition coefficient (stickiness) then requires rapid mix, chemical quench approaches – this is equipment and technique intensive (Chap. 11 *ES* Complex and Pre-steady-state Kinetics, in Part II). A simpler alternative in such cases is to measure stickiness by monitoring micro-viscosity effects on the V_{max}/K_M of the enzyme (refer to Chap. 15 in Part II and Chap. 38 in Part V, relating to significance of k_{cat}/K_M).

One other variation of isotope partitioning experiment is positional isotope exchange (PIX). This method measures the rate of internal isotope exchange within a substrate molecule. PIX rate is usually expressed as a ratio to the overall reaction rate. The PIX analysis of argininosuccinate lyase reaction is a good example of this approach.

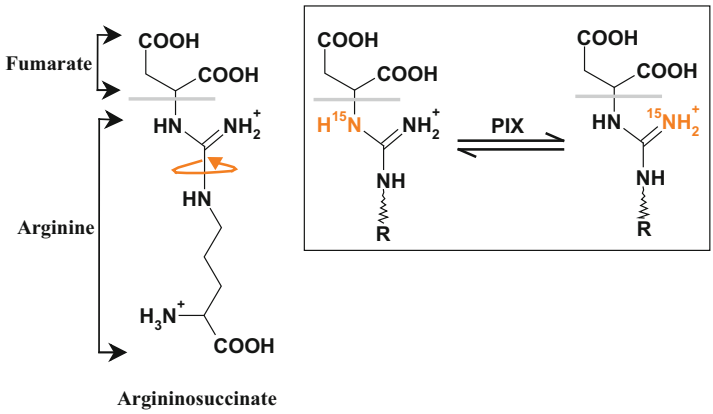

Fig. 25.2 Positional isotope exchange (PIX) in argininosuccinate lyase reaction. Gray line shows the C-N bond cleaved by the enzyme, and the cyclic arrow indicates a free rotation around that bond at the enzyme active site

$$\text{Argininosuccinate} \rightleftarrows \text{Fumarate} + \text{Arginine}$$

The PIX rate was measured by ^{15}N NMR, while its overall reaction was progressing toward arginine formation. The enzyme mobilizes the bond between the bridge nitrogen and the second carbon of the dicarboxylic acid (Fig. 25.2).

The bridge to non-bridge ^{15}N exchange rate, as a function of fumarate concentration, was hyperbolic. It could thus be concluded that (a) the guanidinium group of arginine can freely rotate in the active site and (b) fumarate leaves much faster than arginine from the enzyme surface. A similar PIX study (of the β-γ bridge oxygen of Mg-ATP) provided crucial mechanistic evidence for γ-glutamyl phosphate as an intermediate in glutamine synthetase reaction (Chap. 36 Integrating Kinetic and Chemical Mechanisms: A Synthesis, in Part IV).

References

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Rose IA (1995) Isotopic strategies for the study of enzymes. Protein Sci 4:1430–1433