Isotope Exchanges at Equilibrium

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In the previous chapter, we saw the utility of isotopes (radioisotopes in particular) in elucidating enzyme mechanisms. They were examples when the net chemical reaction was occurring in one direction. Isotope exchange kinetics is also possible in a system at equilibrium. Isotope exchange experiments at equilibrium are useful for defining kinetic mechanisms. Data from carefully executed isotope exchange studies are powerful supplement to steady-state kinetic analysis (Boyer 1978; Rose 1995). More importantly, they provide excellent evidence in discriminating ordered and random sequential mechanisms. We recall that, in some cases, steady-state kinetic data gives quantitative information, and this is used to answer qualitative questions. For example, the presence of a K_{iA} term with finite value is used to conclude against parallel initial velocity patterns (Chap. 19 Analysis of Initial Velocity Patterns). However when these values are extremely small, a clear-cut decision becomes difficult. In such cases isotope exchange data gives unambiguous yes–no answers (see below).

We can assemble together substrates and products in their equilibrium concentrations (K_{eq} = [products]/[substrates]); but a net flow of matter (either substrates → products or products → substrates) will not occur if the activation energy barrier is very high – the system is said to be at *static equilibrium*. Addition of an enzyme catalyst opens the channel so to speak and makes it dynamic. In a *dynamic equilibrium*, there are both forward and backward fluxes, but no net reaction takes place because the two rates match (Chap. 10 Concepts of Equilibrium and Steady State, Part II). Isotope exchange between reactant–product pairs in the presence of the enzyme is the first evidence of the dynamic nature of equilibrium. Glucose isomerase provides a simple example of this concept (Chap. 10 Concepts of Equilibrium and Steady State, in Part II). Similar label transfer experiments can also be performed for a multiple substrate/product reactions.

Nature of Experiments Conducted Equilibrium isotope exchanges are conducted with the help of a suitably labeled substrate or a product. Most often such experiments are analytical – used to decide reaction types (ping-pong, ordered, or

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random) and not to obtain kinetic constants. Nevertheless, the following points are worth considering in the design of a clean isotope exchange study:

(a) Label should be incorporated into a remote position of the reactant such that isotope effects are negligible. The label should serve only as a marker and should not lead to a different mechanism altogether.
(b) The reaction mixture at equilibrium is so assembled that a very small quantity of the tracer with high specific radioactivity is introduced. The concentration of the labeled component added should be insignificant and not perturb the equilibrium set up.
(c) Apart from the purity of the radiolabeled reactant, it is critical to ensure functional purity of the enzyme. High sensitivity of tracer detection makes the interference by contaminating enzyme activities problematic. ATP ↔ ADP exchange rate for a kinase (such as hexokinase) is wrongly estimated if the enzyme sample contains another ATPase as a contaminant. It must be ensured that additional activities, if any, exhibited by the enzyme sample are due to the same enzyme. For instance, the γ-glutamyl transferase activity is catalyzed by the same glutamine synthetase active site where glutamine synthesis occurs. One can ascertain that two different activities are due to the same enzyme (Chap. 14 Quantification of Catalysis and Measures of Enzyme Purity, in Part II), if they co-purify to a constant ratio of specific activities during various stages of purification.
(d) Since isotope exchanges are set up with all the reactants (substrates and products) present, abortive dead-end complexes (see Chap. 20 and later in Chap. 28, Fig. 28.3) may be formed. Their formation influences and severely impedes the interpretation of exchange data.

26.1 Partial Reactions and Ping-Pong Mechanism

Ping-pong mechanisms involve double displacements, and a substituted form of the enzyme (denoted as *F* form) occurs during the catalytic cycle (Chap. 19, Analysis of Initial Velocity Patterns). This implies that atom/group transfer onto the enzyme from a particular substrate can occur even in the absence of the rest of the substrates. Because of this partial reaction, isotope exchange can be demonstrated between a reactant and product in the absence of other reactants and products. In fact an early and brilliant example of this work was by H.A. Barker and colleagues on bacterial sucrose phosphorylase. Sucrose phosphorylase catalyzes the following reaction.

$$\text{Sucrose} + \text{Phosphate} \rightleftarrows \text{Fructose} + \text{Glucose-1-phosphate}$$

The isotope exchanges arising out of its two partial reactions are:

(a) Glucose-1-phosphate + [^{32}P]Phosphate ⇄ Glucose-1-[^{32}P]phosphate + Phosphate
(b) Sucrose + [^{14}C]Fructose ⇄ [^{14}C]Sucrose + Fructose.

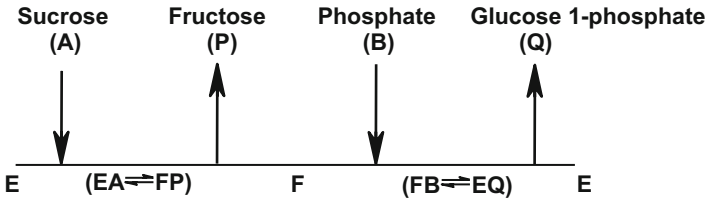

Fig. 26.1 Kinetic scheme for sucrose phosphorylase. The two enzyme forms *E* (free enzyme) and *F* (glucosyl-enzyme) are shown

Notice that sucrose is not required for the first exchange and phosphate is not required for the second exchange. Such isotope exchanges are best evidence of ping-pong mechanism, and glucosyl-enzyme as an essential covalent intermediate is indicated in this case. The following kinetic scheme (Fig. 26.1) adequately accounts for the observed exchanges.

The enzyme form "*F*" is the glucosyl-enzyme intermediate of sucrose phosphorylase. Either sucrose or glucose-1-phosphate can charge the enzyme with glucose to form the glucosyl enzyme. This glucosyl group can be picked up either by [^{32}P] phosphate ($B \leftrightarrow Q$ exchange, "a" in the box above) or by [^{14}C]fructose ($A \leftrightarrow P$ exchange, "b" in the box above) from the medium.

Other examples involving partial reactions include enzymes that transfer amino groups (such as aspartate transaminase), acyl groups (such as 3-oxoacid CoA-transferase, serine transacetylase, and transpeptidase), carboxyl group (like transcarboxylase), a three-carbon unit (a transaldolase), a two-carbon unit (a transketolase), phosphate group transfer (nucleotide diphosphokinase), phosphate group migration (phosphoglyceromutase), two-electron transfer (like in methylenetetrahydrofolate reductase), etc. As expected, in all these cases, isotope exchanges between the first substrate and the product occur in the absence of other substrate and/or product. The different *F*-forms for these reactions may be referred to in a later section (Chap. 31 Nucleophilic Catalysis and Covalent Reaction Intermediates, in Part IV).

26.2 Sequential Mechanisms

The full complement of substrates needs to assemble at the enzyme active site for any chemistry to take place in a sequential mechanism. Therefore as expected, no partial reactions are possible. In such situations isotope exchanges are possible only when all reactants and products are present. Accordingly the design of experiments is somewhat involved so as to maintain equilibrium condition.

Typically, in a two substrate–two product enzyme reaction of the type,

$$A + B \rightleftarrows P + Q$$

the $A \leftrightarrow Q$ isotope exchange rate is monitored as a function of increasing concentrations of $B-P$ pair. The concentrations of B and P are simultaneously raised to maintain the $[B]/[P]$ ratio and hence the position of equilibrium $K_{eq} = ([P][Q]/[A][B])$. For the converse experiment, $B \leftrightarrow P$ isotope exchange is measured as $[A]$ and $[Q]$ are simultaneously raised while maintaining the $[A]/[Q]$ ratio. For other possible combination of exchanges (viz., $A \leftrightarrow P$ and $B \leftrightarrow Q$) to occur, atoms/groups must be transferred between the respective substrate–product partners. With hexokinase reaction, for example, there is nothing common between ADP and glucose, and a study of ADP↔glucose exchange is not possible. Therefore which exchanges are experimentally observable depends on the reaction chemistry under consideration.

Ordered Mechanism With the addition of substrates and release of products being ordered, only the outer pair – A and Q – can interact (respectively, bind or leave) with the E form of the enzyme:

$$A + E \rightleftarrows EA + B \rightleftarrows (EAB \rightleftharpoons EPQ) \rightleftarrows EQ + P \rightleftarrows E + Q$$

Increasing the concentrations of $B-P$ pair (but maintaining the $[B]/[P]$ ratio) will initially increase the rate of $A \leftrightarrow Q$ isotope exchange (hyperbolic). At higher concentrations however, most of the enzyme gets locked up into central complexes, and very little E form will be available for interaction. Therefore, $A \leftrightarrow Q$ isotope exchange rate is inhibited as the concentration of $B-P$ pair is raised. In a fully ordered mechanism, this inhibition can drive the $A \leftrightarrow Q$ exchange rate to zero. This profile for $A \leftrightarrow Q$ exchange rate is shown in Fig. 26.2 (panel I). The exchange profile for the inner pair – B and P – is quite different however. Increasing concentrations of the $A-Q$ pair facilitates $B \leftrightarrow P$ isotope exchange by driving more and more enzyme into central complexes. Ultimately the $B \leftrightarrow P$ exchange rate reaches a plateau and a hyperbolic profile results (Fig. 26.2, panel III).

From the rates of exchange between cognate pairs of reactants, Silverstein and Boyer (Boyer 1978) showed that lactate dehydrogenase follows an ordered mechanism with the coenzymes (NAD^+ and NADH) forming the outer Michaelis complexes. Malate dehydrogenase also shows such exchange patterns and is an example of fully ordered mechanism (Fig. 26.3).

The $NAD^+ \leftrightarrow$ NADH exchange rate increased and eventually fell to zero when [malate-oxaloacetate] was raised; whereas malate ↔ oxaloacetate exchange rate followed hyperbolic pattern and reached a plateau with increasing [NAD^+-NADH]. The substrate inhibition of exchange rate is typical for the outer pair, and thus we conclude that NADH is the first substrate to add, while NAD^+ is the last product to leave the enzyme.

While Theorell-Chance mechanism is an example of ordered mechanism (but without central complexes), it is not possible to pile up central complexes. Therefore no substrate inhibition (by alcohol–aldehyde pair) of $NAD^+ \leftrightarrow$ NADH exchange rate occurs with liver alcohol dehydrogenase.

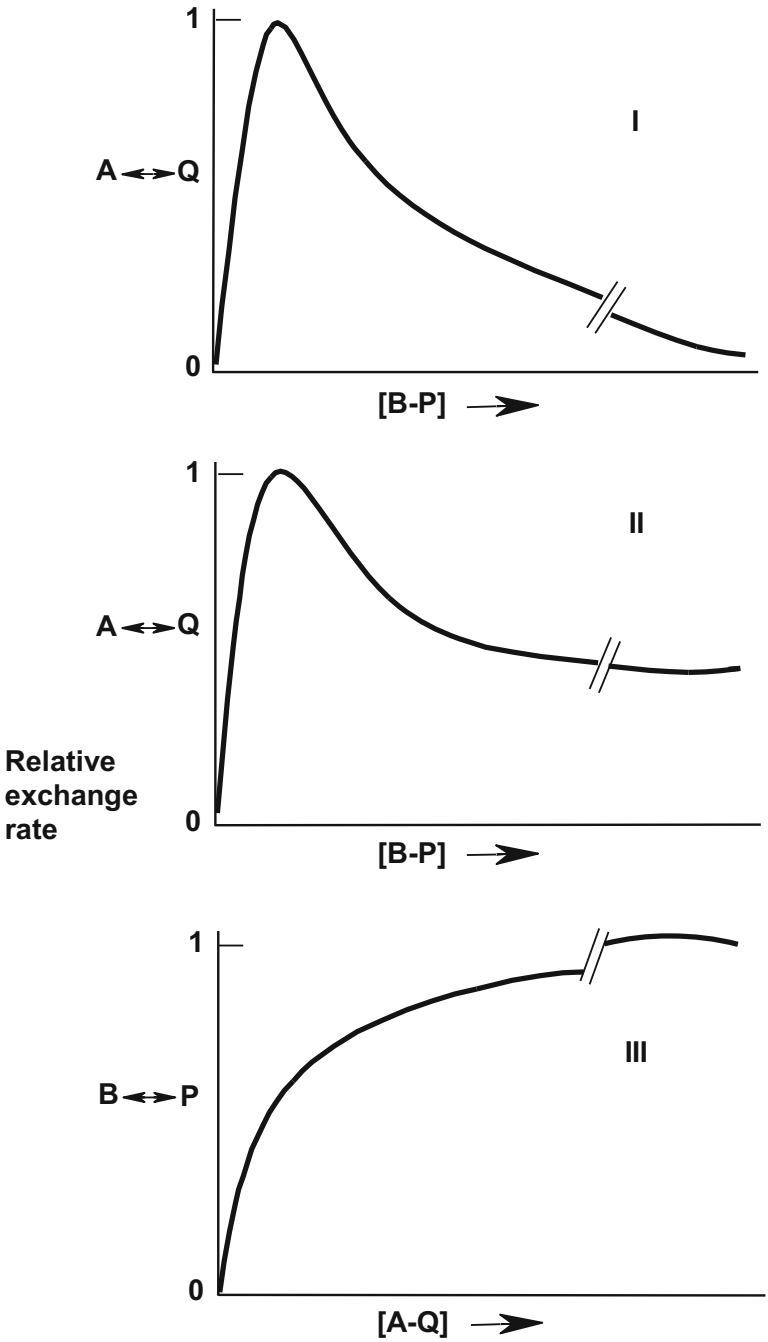

Fig. 26.2 Equilibrium isotope exchange profiles observed with enzymes. Schematic isotope exchange data for complete inhibition (panel I), partial inhibition (panel II), and hyperbolic (panel III) patterns of exchange is shown

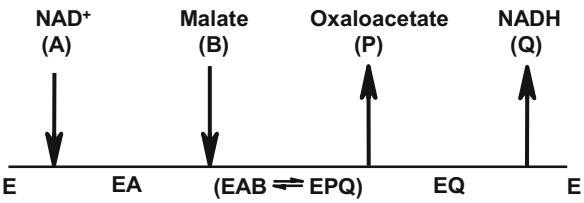

Fig. 26.3 Kinetic scheme for malate dehydrogenase. The coenzymes form the outer substrate–product pair

Random Mechanism As random addition of substrates (and release of products) can occur, no fixed inner and outer pairs can be defined. The *E* form of the enzyme can bind to all the reactants. Because of this the enzyme is not locked up into central complexes, and the $A \leftrightarrow Q$ isotope exchange rate is not inhibited as the concentration of $B-P$ pair is raised. In effect the rates of exchange between both cognate pairs of reactants ($A \leftrightarrow Q$ as well as $B \leftrightarrow P$) follow hyperbolic kinetics (Fig. 26.2, panel III). The three isotope exchanges possible with hexokinase reaction – glucose↔glucose-1-phosphate, ATP ↔ glucose-1-phosphate, and ADP ↔ ATP – are hyperbolic and show no substrate inhibition. Other examples of sequential random mechanisms where such exchange data is available include creatine kinase and yeast alcohol dehydrogenase.

Formation of a ternary complex in random mechanisms can follow both the routes, namely, $E \rightarrow EA \rightarrow EAB$ and $E \rightarrow EB \rightarrow EAB$ (see Chap. 19 Analysis of Initial Velocity Patterns). It is however not necessary that these alternative paths are used equally by an enzyme. If one of the routes is taken most of the time, then such a random mechanism is termed as preferred ordered sequential mechanism. In such cases (say $E \rightarrow EA \rightarrow EAB$ is preferred), one observes an inhibition of $A \leftrightarrow Q$ isotope exchange. However even at very high concentrations of $B-P$ pair, the $A \leftrightarrow Q$ exchange does not go to zero but reaches a limiting value (Fig. 26.2, panel II). This partial inhibition of exchange is characteristic of a *preferred-order* in the random mechanism, and the relative magnitude of inhibition gives the extent to which the two paths are followed. In fact isotope exchange is a very sensitive tool to detect minor reaction pathways.

In summary, (a) isotope exchange in the absence of the second (other) substrate is diagnostic of a ping-pong mechanism, (b) isotope exchanges at equilibrium are possible for sequential mechanisms only when the full complement of reactants (all substrates and all products) are present, and (c) substrate inhibition of $A \leftrightarrow Q$ exchange is observed by raising *B–P* levels only in an ordered mechanism.

References

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