

[15] Positional Isotope Exchange as Probe of Enzyme Action

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Introduction

The application and development of new mechanistic probes for enzyme-catalyzed reactions have significantly expanded our knowledge of the molecular details occurring at the active sites of many enzymes. Enzyme kinetic techniques have progressed from a simple determination of K_m and V_{max} values through a discrimination between sequential and ping-pong kinetic mechanisms to detailed evaluations of transition state structures by measurement of the very small differences in rate on isotopic substitution at reaction centers. The positional isotope exchange (PIX) technique, originally described by Midelfort and Rose,¹ is one of the newer techniques that has made a significant contribution to the elucidation of enzyme reaction mechanisms over the last decade.

The PIX technique can be used as a mechanistic probe in any enzymatic reaction where the individual atoms of a functional group within a substrate, intermediate, or product become torsionally equivalent during the course of the reaction. This criterion is best illustrated with the example first presented by Midelfort and Rose.¹ Glutamine synthetase (glutamate-ammonia ligase) catalyzes the formation of glutamine via the overall reaction presented in Eq. (1).

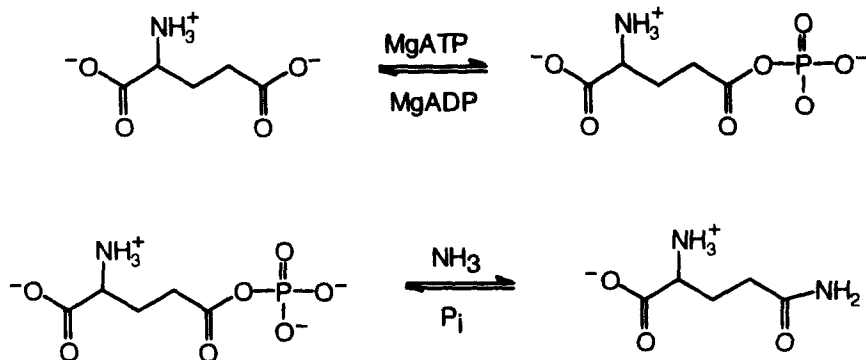


The questions addressed by Midelfort and Rose concerned whether γ -glutamyl phosphate was an obligatory intermediate in the reaction mechanism and if this intermediate was synthesized at a kinetically significant rate on mixing of ATP, enzyme, and glutamate in the absence of ammonia. The putative reaction mechanism is illustrated in Scheme I.

In the first step (Scheme I) glutamate is phosphorylated by MgATP at the terminal carboxylate group to form enzyme-bound MgADP and γ -glutamyl phosphate. In the second step ammonia displaces the activating phosphate group to form the amide functional group of the product glutamine. Previous experiments with this enzyme had failed to detect an equilibrium isotope exchange reaction between ATP and ADP in the presence of enzyme and glutamate.² Thus, if the γ -glutamyl phosphate interme-

¹ C. G. Midelfort and I. A. Rose, *J. Biol. Chem.* **251**, 5881 (1976).

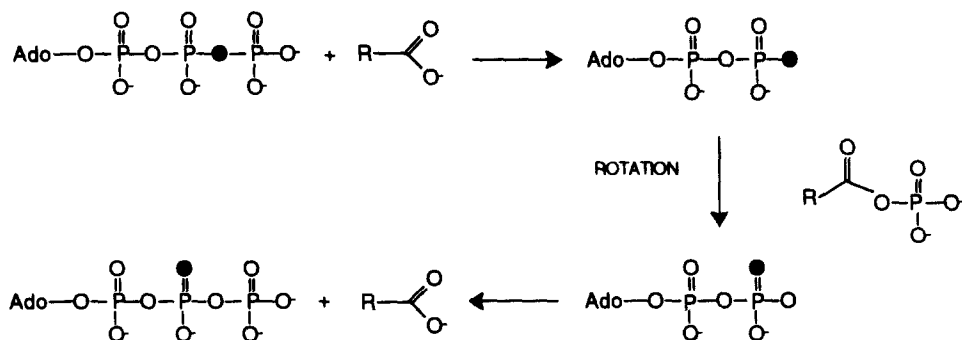
² F. C. Wedler and P. D. Boyer, *J. Biol. Chem.* **247**, 984 (1972).



SCHEME I

diolate was formed at the active site then the release of MgADP from the $\text{E} \cdot \text{MgADP} \cdot$ intermediate complex must be very slow. The formation of the intermediate can be confirmed, however, when the oxygen atom between the β - and γ -phosphoryl groups in the substrate ATP is labeled with oxygen-18.

If the γ -glutamyl phosphate intermediate is formed and the β -phosphoryl group of the bound ADP is free to rotate then an isotopic label that was originally in the β , γ -bridge position will eventually be found 67% of the time in one of the two equivalent β -nonbridge positions of ATP as illustrated in Scheme II. This migration of the isotopic label can only occur if the γ -phosphoryl group of ATP is transferred to some acceptor and if the β -phosphoryl group of the enzyme-bound ADP is free to rotate torsionally. Midelfort and Rose found a PIX reaction with glutamine synthetase, and thus γ -glutamyl phosphate is an obligatory intermediate in the enzymatic synthesis of glutamine.



SCHEME II

Functional Groups for Positional Isotope Exchange

A variety of functional groups common to many substrates and products found in enzyme-catalyzed reactions are suitable for positional isotope exchange analysis. Some of the generic examples are illustrated in Diagram 1. The two most common functional groups that have been uti-

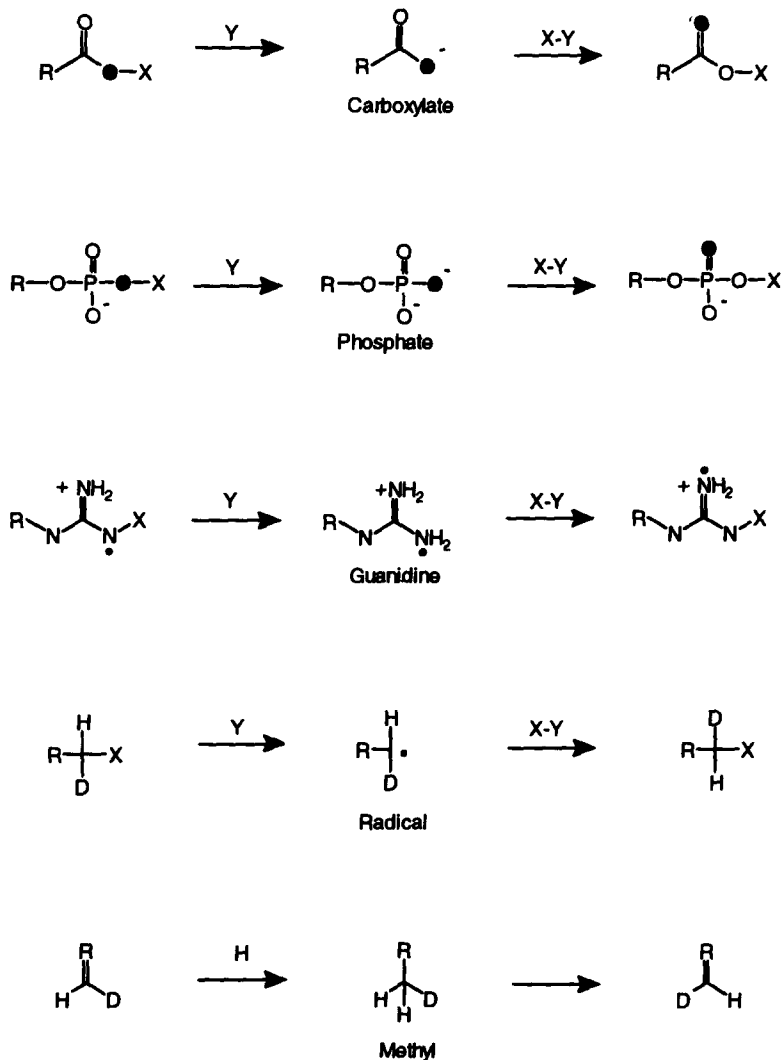


DIAGRAM 1. Functional groups for positional isotope exchange analysis.

lized for PIX studies are esters of substituted carboxylic and phosphoric acids. Thus, all reactions involving nucleophilic attack at either the β - or γ -phosphoryl groups of nucleotide triphosphates are amenable to PIX analysis. Moreover, all reactions utilizing UDP-sugars for complex sugar biosynthesis can use these PIX techniques for mechanistic evaluation. Less common are examples that involve reactions at a substituted guanidino functional group. Specific examples include creatine kinase³ and argininosuccinate lyase.⁴ With these enzymes positional isotope exchange of the two amino groups within arginine or creatine can be monitored by labeling with ¹⁴N and ¹⁵N and following the reaction with ¹⁵N nuclear magnetic resonance (NMR) spectroscopy. Other cases include formation of radical or cationic centers at methylene carbons. Production of achiral acetaldehyde from chiral ethanolamine has been explained by intermediate formation of a C-2 radical during the course of the reaction.⁵ Loss of stereochemistry in *sp*² centers can be exploited as a mechanistic probe when a methyl group is formed as an intermediate or product as for the case in the reaction catalyzed by pyruvate kinase.

Qualitative and Quantitative Approaches

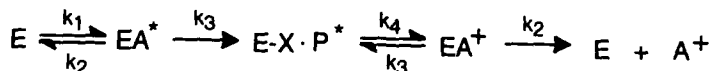
The technique of positional isotope exchange can be used for two interrelated probes of enzyme-catalyzed reactions. In the first, as exemplified by the example with glutamine synthetase, the experimenter is primarily interested in determining whether a particular intermediate is formed during the course of the reaction. In the second approach the primary interest is in a quantitative determination of the partition ratio of an enzyme-ligand complex. The partition ratio is defined here as the fraction of the enzyme-ligand complex that proceeds forward to form unbound products versus the fraction of the enzyme complex in question that returns to unbound substrate and free enzyme.

When one is interested in whether a specific intermediate or complex is formed the experiment is generally conducted with one of the substrates absent from the reaction mixture. Take, for example, the simplified case of an enzyme reaction where two substrates are converted to two products via the covalent transfer of a portion of one substrate to the second substrate. This type of reaction is commonly found in many kinase reactions where ATP is used to phosphorylate an acceptor substrate. The generalized scheme is presented in Eq. (2).

³ R. E. Reddick and G. L. Kenyon, *J. Am. Chem. Soc.* **109**, 4380 (1987).

⁴ F. M. Raushel and L. J. Garrard, *Biochemistry* **23**, 1791 (1984).

⁵ J. Retey, C. J. Suckling, D. Arigoni, and B. Babior, *J. Biol. Chem.* **249**, 6359 (1974).



SCHEME III



If the chemical mechanism involves the phosphorylation of the enzyme by ATP (substrate A) then a positional isotope exchange reaction may proceed in the absence of the acceptor substrate (B) if the acceptor B is not required to be bound to the enzyme in order for the phosphorylation to occur. Because substrate B is not required to be bound to the protein in order for the torsional equilibration of the isotopic label to occur, then all that is required for the PIX reaction to be observed is the incubation of enzyme and the isotopically labeled substrate (A^*). In such cases the velocity of the PIX reaction can be derived from the model presented in Scheme III where A^* represents the substrate as originally synthesized with the isotopic label and A^+ represents that fraction of A where the isotopic label is positionally equilibrated. It can be demonstrated that in such mechanisms the maximum value for the PIX reaction (V_{ex}) will be given by Eq. (3).

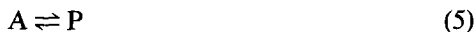
$$V_{ex} = (k_2 k_3 k_4) / [(k_2 + k_3)(k_3 + k_4)] \quad (3)$$

Rose has also demonstrated that in such mechanisms the minimum value for the PIX reaction is given by Eq. (4)⁶:

$$V_{ex} \geq (V_1 V_2) / (V_1 + V_2) \quad (4)$$

where V_1 and V_2 are the maximal velocities of the steady-state reaction in the forward and reverse directions, respectively. This approach can be utilized with great utility to identify activated intermediates in many synthetase-type reactions where ATP is used to activate a second substrate molecule prior to condensation with a third substrate. This can be done either by phosphorylation or adenylation of the second substrate. Equation (4) can be utilized to demonstrate that the presumed intermediate is formed rapidly enough to be kinetically competent.

When one is more interested in a quantitative analysis of the partition ratio of an enzyme-product complex the PIX methodology can easily provide that information. Take, for example, a case where a substrate, A, is converted to a single product, P:



⁶ I. A. Rose, *Adv. Enzymol. Relat. Areas Mol. Biol.* **50**, 361 (1979).

In this example we will assume that the isotopic label originally in A becomes torsionally equivalent in the product, P.

The simplified kinetic scheme can be diagrammed as shown in Scheme IV. In this case the bonds are broken and the isotopes positionally equilibrated in the EP complex. This complex can partition in one of two ways. Either the product P can dissociate irreversibly from the EP complex with a rate constant k_5 , or it can reverse the reaction and form free enzyme and substrate A with a net rate constant of k_4' . The fraction of EP that partitions forward can be quantitated by the amount of P that is formed per unit time, whereas the fraction of EP that partitions backward toward free enzyme and unbound A can be quantitated by the rate of positional equilibration of the isotope labels within the pool of substrate A.

In the steady state the partition forward is given by

$$[EP]k_5 = V_{\text{chem}} \quad (6)$$

whereas the partition backward is given by

$$[EP]k_4' = [EP](k_2k_4)/(k_2 + k_3) = V_{\text{ex}} \quad (7)$$

and thus the ratio of $V_{\text{ex}}/V_{\text{chem}}$ is

$$V_{\text{ex}}/V_{\text{chem}} = (k_2k_4)/[k_5(k_2 + k_3)] \quad (8)$$

Because the maximal velocity in the reverse direction, V_2/E_t , is given by

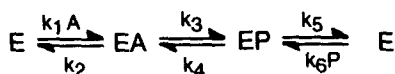
$$V_2 = (k_2k_4)/(k_2 + k_3 + k_4) \quad (9)$$

then

$$k_2/k_5 \geq V_{\text{ex}}/V_{\text{chem}} \geq (V_2/E_t)k_5 \quad (10)$$

Therefore, the lower limit for the experimentally determined partition ratio is given by the maximal velocity in the reverse direction divided by k_5 (the dissociation rate constant for the product P). The upper limit for the partition ratio is given by the relative magnitude for the off-rate constants for A and P from the enzyme. These values would be difficult to obtain directly by any other method.

Experimentally, the positional isotope exchange rate, V_{ex} , is determined by measuring the fraction of positional isotope scrambling equilibrium (F) as a function of time (t) where $V_{\text{ex}} = ([A]/t)\ln(1 - F)^{-1}$. If



SCHEME IV

the isotopically labeled substrate is being chemically depleted during the course of the PIX analysis then the corrected PIX rate is calculated from $V_{\text{ex}} = [(X)/\ln(1 - X)] (A_0/t) \ln(1 - F)^{-1}$, where X is the fraction of substrate lost at time t and A_0 is the initial concentration of the labeled substrate. In the next section we examine the quantitative effects induced by variation of the concentration of the unlabeled substrates and products on the positional isotope exchange rates.

Variation of Nonlabeled Substrates and Products

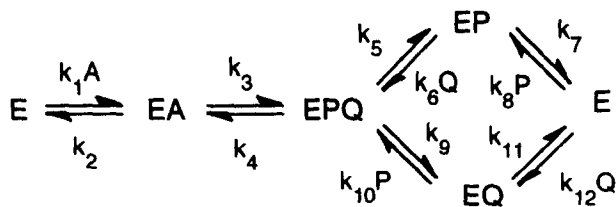
The PIX technique can be used to obtain information about the partitioning of enzyme complexes and the order of substrate addition and product release. This information is obtained by measuring the PIX rate in the enzyme-catalyzed reaction relative to the overall chemical transformation rate in the presence of variable amounts of added substrates or products. In many cases, it is possible with these methods to determine the microscopic rate constants for the release of substrates and products from the enzyme–ligand complexes. The following provides a presentation for how the PIX technique can be applied to the analysis of sequential and ping-pong kinetic reaction mechanisms.

Sequential Mechanisms

In sequential mechanisms, substrate addition and/or product release can be either ordered or random or a combination of various pathways. The PIX reaction can be utilized to distinguish between the possible kinetic mechanisms and to determine the net reaction flux through the various kinetic pathways. The effect of product addition can lead to an *enhancement* of the PIX reaction relative to overall rate (PIXE), whereas the variation of unlabeled substrates may reduce or *inhibit* the PIX reaction relative to overall chemical rate (PIXI).

Positional Isotope Exchange Enhancement

The simplest kinetic mechanism that can be written for an enzyme reaction with multiple products is a Uni Bi mechanism. In this mechanism the release of the products can be either random (Uni Bi Random) or ordered (Uni Bi Ordered). A general model of a Uni Bi Random kinetic mechanism is shown in Scheme V, where A is designated as the substrate with the positionally labeled isotopic atoms and Q is the product in which the positional exchange occurs.



SCHEME V

In Scheme V the ratio of the PIX rate relative to the net chemical rate ($V_{\text{ex}}/V_{\text{chem}}$) is determined by the partitioning of the EPQ complex. Using the method of net rate constants, the partitioning of EPQ can be written as⁷

$$\frac{V_{\text{ex}}}{V_{\text{chem}}} = \frac{(k_2 k_4)/(k_2 + k_3)}{k_5 + (k_9 k_{11})/(k_{11} + k_{10}[P])} \quad (11)$$

As can be seen in Eq. (11), the ratio $V_{\text{ex}}/V_{\text{chem}}$ is dependent on the amount of P added to the reaction mixture. In a random mechanism, the ratio $V_{\text{ex}}/V_{\text{chem}}$ increases as a function of [P] because the addition of P inhibits the flux through the lower pathway and then the ratio plateaus at a level that is determined by the net flux through the upper pathway. The net flux through each pathway can be determined by the ratio $V_{\text{ex}}/V_{\text{chem}}$ as a function of [P]. At [P] = 0, Eq. (11) becomes

$$V_{\text{ex}}/V_{\text{chem}} = [k_2 k_4/(k_2 + k_3)]/(k_5 + k_9) \quad (12)$$

and at [P] = ∞

$$V_{\text{ex}}/V_{\text{chem}} = [k_2 k_4/(k_2 + k_3)]/(k_5) \quad (13)$$

Thus the ratio of k_5 and k_9 can be determined by measurement of the PIX ratio at zero and saturating P.

Figure 1 also illustrates the change in the ratio $V_{\text{ex}}/V_{\text{chem}}$ as a function of [P] as a sequential mechanism changes from random to ordered release of products. The ordered release of the products simplifies Eq. (11). If P is released first ($k_5 = 0$), then the equation becomes

$$\frac{V_{\text{ex}}}{V_{\text{chem}}} = \frac{k_2 k_4/(k_2 + k_3)}{k_9 k_{11}/(k_{11} + k_{10}[P])} \quad (14)$$

The ratio $V_{\text{ex}}/V_{\text{chem}}$ is now linearly dependent on the concentration of added P. The intercept at [P] = 0 becomes

$$V_{\text{ex}}/V_{\text{chem}} = [k_2 k_4/(k_2 + k_3)]/k_9 \quad (15)$$

⁷ W. W. Cleland, *Biochemistry* **14**, 3220 (1975).

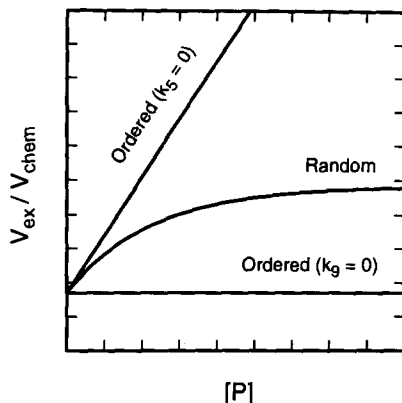


FIG. 1. Enhancement of the ratio of the positional isotope exchange rate and the net rate of chemical turnover as a function of added product inhibitor.

It is possible to determine the lower limits for the off-rate constant from the ternary complex EPQ (k_9) and the binary complex EQ (k_{11}) relative to the turnover number in the reverse direction (V_2/E_t) as shown in Eqs. (16) and (17) since $V_2/E_t = (k_2k_4)/(k_2 + k_3 + k_4)$:

$$k_9/(V_2/E_t) = (V_{\text{chem}}/V_{\text{ex}}) + (k_9/k_2) \quad (16)$$

$$k_{11}/(V_2/E_t) = (1/K_p)[\text{intercept} + 1]/\text{slope} \quad (17)$$

where K_p is the Michaelis constant for the product P.

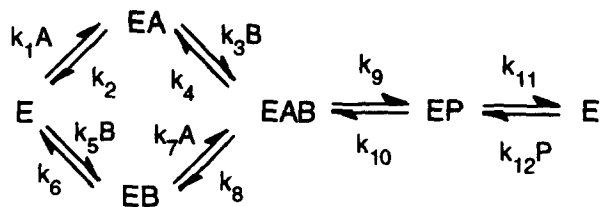
If Q is the first product to be released ($k_9 = 0$) in an ordered mechanism, then there would be no dependence on $V_{\text{ex}}/V_{\text{chem}}$ as [P] is varied, as apparent in Eq. (18):

$$V_{\text{ex}}/V_{\text{chem}} = k_2k_4/(k_2 + k_4) \quad (18)$$

Again, the lower limit for the release of the product from the ternary complex can be determined relative to the turnover number in the reverse reaction:

$$k_5/(V_2/E_t) = (V_{\text{chem}}/V_{\text{ex}}) + (k_5/k_2) \quad (19)$$

Positional isotope exchange enhancement experiments can provide valuable information about enzyme kinetic mechanisms. Utilizing the PIXE technique, a simple inspection of a plot of $V_{\text{ex}}/V_{\text{chem}}$ as a function of [P] readily identifies the kinetic mechanism (see Fig. 1). The PIXE experiments also allow the determination of the microscopic rate constants for product release from both the ternary and binary complexes. An often overlooked advantage of the PIXE experiment is that it can identify a



SCHEME VI

“leaky” product in what otherwise would appear as an ordered product release.

Positional Isotope Exchange Inhibition

The concentration of unlabeled substrate also affects the ratio of the PIX rate relative to the net chemical rate.⁸ An enzyme kinetic mechanism with random addition of substrates is shown in Scheme VI, where A is the substrate with the positionally labeled atoms and P is the product in which the positional exchange occurs.

If A is the first substrate to bind in an ordered kinetic mechanism ($k_8 = 0$), then increasing concentrations of B will inhibit the PIX rate relative to the net chemical rate ($V_{\text{ex}}/V_{\text{chem}}$) because saturation with B prevents A from dissociating from the enzyme. In contrast, if B must bind first ($k_4 = 0$), then its concentration will have no effect on $V_{\text{ex}}/V_{\text{chem}}$. This is because saturation with B would not affect the rate of dissociation of A from the EAB complex. If addition of the substrates to the enzyme is random, then saturation with B will reduce the value of $V_{\text{ex}}/V_{\text{chem}}$ but not eliminate it entirely. A plot of $V_{\text{ex}}/V_{\text{chem}}$ as a function of [B] will plateau at a value of $V_{\text{ex}}/V_{\text{chem}}$ that equals the flux through the lower pathway and thus enables the determination of the ratio of k_4 and k_8 . Figure 2 illustrates the plot of $V_{\text{ex}}/V_{\text{chem}}$ as a function of added substrate. Substrate inhibition by the nonlabeled substrate can be quite diagnostic of the particular reaction mechanism.

In special cases, the investigation of substrate inhibition of PIX reactions can be used to determine the microscopic rate constants for the release of substrates and products from the enzyme complexes. A Bi Bi Ordered mechanism can be used to illustrate this application. The simplest mechanism that can be written for a Bi Bi Ordered reaction is shown in Scheme VII, where A is the substrate with the positional label and P is the product undergoing rotational exchange. For the forward reaction,

⁸ F. M. Raushel and J. J. Villafranca, *Crit. Rev. Biochem.* **23**, 1 (1988).

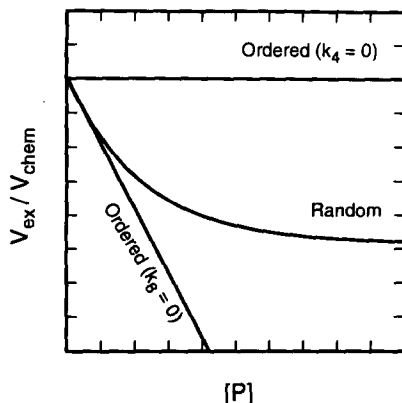


FIG. 2. Inhibition of the ratio of the positional isotope exchange rate and the net rate of chemical turnover as a function of added substrate.

the partitioning of the EPQ complex determines the PIX rate relative to the rate of net product formation. The partitioning of the EPQ complex can be written as

$$\frac{V_{\text{chem}}}{V_{\text{ex}}} = \frac{k_7(k_2k_4 + k_2k_5 + k_3k_5[B])}{k_2k_4k_6} \quad (20)$$

The plot of $V_{\text{chem}}/V_{\text{ex}}$ as a function of the concentration of B yields a straight line. An analysis of the plot gives the following information:

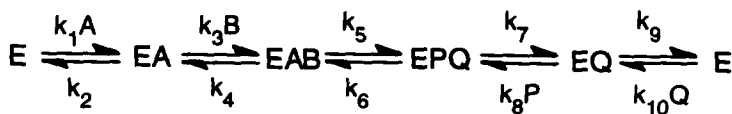
$$\text{Intercept}_{[\text{EPQ}]} = k_7(k_4 + k_5)/(k_4k_6) \quad (21)$$

$$\text{Slope}_{[\text{EPQ}]} = (k_3k_5k_7)/(k_2k_4k_6) \quad (22)$$

If a PIX reaction can be followed for the reverse reaction, then analogous equations can be derived for the partitioning of the EAB complex:

$$\frac{V_{\text{chem}}}{V_{\text{ex}}} = \frac{k_4(k_6k_9 + k_7k_9 + k_6k_8[P])}{k_5k_7k_9} \quad (23)$$

where the slope_[EAB] is equal to $(k_4k_6k_8)/(k_5k_7k_9)$ and the intercept_[EAB] is $k_4(k_6 + k_7)/(k_5k_7)$.



SCHEME VII

It can be shown that the microscopic rate constants for the release of the A and Q from the binary enzyme complexes can be determined from a combination of the respective slopes, intercepts, and the Michaelis constants as shown in Eqs. (24) and (25)⁸:

$$k_2/(V_1/E_t) = [(1 + \text{intercept}_{[\text{EPQ}]})/(\text{slope}_{[\text{EPQ}]})](1/K_b) \quad (24)$$

$$k_9/(V_2/E_t) = [(1 + \text{intercept}_{[\text{EAB}]})/(\text{slope}_{[\text{EAB}]})](1/K_q) \quad (25)$$

The lower limits for the release of B and P from the two ternary complexes can also be obtained:

$$k_7/(V_2/E_t) = (k_7/k_2) + (k_7/k_4) + \text{intercept}_{[\text{EPQ}]} \quad (26)$$

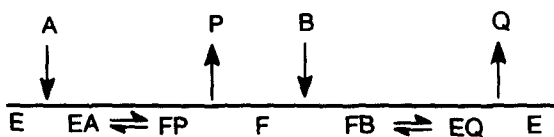
$$k_4/(V_1/E_t) = (k_4/k_9) + (k_4/k_7) + \text{intercept}_{[\text{EAB}]} \quad (27)$$

Therefore, the rate constants k_2 and k_9 relative to the net substrate turnover in the forward and reverse reactions are known from Eqs. (24) and (25), leaving two unknown rate constants, k_4 and k_7 . Because there are two independent equations [Eqs. (26) and (27)] that contain both k_4 and k_7 , both of the rate constants can be determined. Using this analysis of the PIX reaction, the rate constants for the release of all substrates and products from the enzyme complexes can be determined. If these results are combined with the steady-state kinetics parameters, V_1/E_t , V_2/E_t , K_a , K_b , K_p , and K_q , and the thermodynamic parameter, K_{eq} , then it is possible to estimate all 10 microscopic rate constants for the minimal Bi Bi Ordered kinetic mechanism shown in Scheme VII.

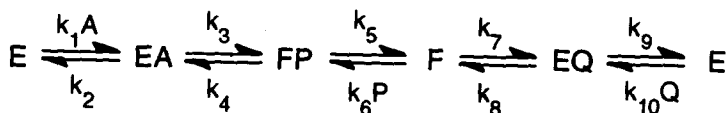
The analysis of PIX reactions as a function of added substrates and products in ordered kinetic mechanisms provides information that would otherwise be difficult to determine. It can also be used to corroborate classic steady-state experiments as well as to give information on the relative "stickiness" of a substrate or product.

Ping-Pong Mechanisms

In ping-pong reaction mechanisms, the first substrate binds and reacts covalently with the enzyme in the total absence of the second substrate to form a stable but modified enzyme-product complex. The second substrate binds and reacts with the modified enzyme to form the second product and regenerate the starting enzyme form. A simple Bi Bi Ping Pong reaction is shown in Scheme VIII.



SCHEME VIII



SCHEME IX

Because the first substrate can bind and react with the free enzyme in the absence of the second substrate, it is often assumed that the ping-pong reaction mechanism would be ideal for analysis by the PIX kinetic technique. The product bound to the modified enzyme could undergo torsional scrambling, and then reformation of the first substrate would yield a positional isotope exchange reaction. However, this is a misconception because in reality no significant PIX reaction is expected to be observed because of the rapid dissociation of the first product from the modified enzyme.⁹ Essentially 1 enzyme equivalent of product is produced, leaving the enzyme in the modified form unable to process more substrate.

The minimal kinetic mechanism for a ping-pong reaction is shown in Scheme IX, where E is enzyme; A, substrate with the positional isotope label; P, product undergoing torsional scrambling; and F, stable modified enzyme. An analysis of the PIX reaction would give the partitioning of the FP complex shown in Eq. (28):

$$V_{\text{chem}}/V_{\text{ex}} = [k_5(k_2 + k_3)]/k_2k_4 \quad (28)$$

However, for a PIX reaction to be observed, a method for reconvert the modified enzyme form F back to free enzyme E is required. This can be achieved with the addition of a large excess of unlabeled product P to the reaction mixture. The large excess of P returns the modified enzyme form F back to E, producing unlabeled A. This process provides a measure of the partitioning of the FP and F complexes. The excess level of P also dilutes the concentration of labeled P. This is important since the purpose of the PIX reaction is to determine the partitioning of the FP complex between F and E. To obtain an accurate determination for the partitioning of the FP complex, the formation of positionally exchanged substrate from the true PIX reaction must be distinguished from a pseudo-PIX reaction (caused by product dissociation and reassociation with the modified enzyme).

The ping-pong PIX experiment is initiated by addition of enzyme to labeled substrate A and unlabeled product P. The first-order equilibration of the positional label between substrate and product gives the partitioning

⁹ L. S. Hester and F. M. Raushel, *J. Biol. Chem.* **262**, 12092 (1987).

between FP and F. Statistical considerations permit the determination of how much of the labeled product that is formed and released into solution will partition back to either the original labeled substrate or the positionally exchanged substrate. The rate of the pseudo-PIX mechanism can be calculated from the exchange rate of the label (positionally exchanged and nonexchanged) between product P and total substrate. The rate of the pseudo-PIX reaction can be used to correct the overall rate of formation of the positionally exchanged substrate to yield the true PIX rate for the interconversion of positionally labeled substrate. The correction factor will decrease as the ratio of unlabeled product relative to labeled substrate increases.

An analysis of the ping-pong PIX experiment is shown in Scheme X, where M, N, and O represent the positionally labeled, unlabeled, and positionally exchanged substrates, respectively. The equilibration of labeled and unlabeled substrates is represented by the interconversion of $M \rightarrow N \rightarrow O$. The formation of the positionally exchanged substrate O by this pathway would be a pseudo-PIX reaction because of the disassociation and reassociation of the product. The interconversion of $M \rightarrow O$ without the formation of unbound labeled product provides the true PIX rate.

The rate constants in Scheme X can be defined as follows:

$$k_a = xy(1 + w) \quad (29)$$

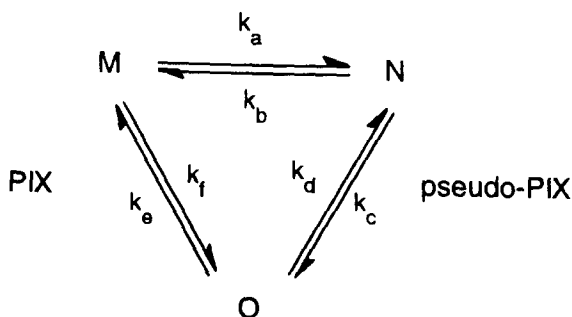
$$k_b = x \quad (30)$$

$$k_c = xw \quad (31)$$

$$k_d = xy(1 + w) \quad (32)$$

$$k_e = xz \quad (33)$$

$$k_f = xzw \quad (34)$$



SCHEME X

where x is proportional to the amount of enzyme used and therefore affects each step equally, y is the ratio of the initial concentration of unlabeled product P and labeled substrate A, w is the equilibrium ratio of positionally exchanged substrate and labeled substrate, and z is proportional to the true PIX reaction.

The factor x can be determined from an analysis of the plot of $([M] + [O])/([M] + [N] + [O])$ versus time since the values of y and w are known. Once x is determined then z can be obtained by an analysis of the plot of $([O])/([M] + [O])$ versus time. A numerical solution for the determination of the rate constants k_a through k_f is thus possible.

The time course for the equilibration of the labeled substrate and the positionally exchanged substrate, represented as a plot of $[O]/([M] + [O])$ versus time, is dependent on the ratio of the PIX and the pseudo-PIX pathways. The flux through the pseudo-PIX pathway can be diminished by increasing the ratio of unlabeled product to labeled substrate. This is represented in Fig. 3, where the curves are simulated with increasing values of z from 0 to 100. In the absence of a pathway for the direct interconversion of $M \rightarrow O$, there is a noticeable lag in the appearance of the positionally exchanged substrate.

To determine the partitioning of FP, the ratio $V_{\text{chem}}/V_{\text{ex}}$ [Eq. (28)] must be expressed in terms of the rate constants k_a through k_f . The chemical rate for the conversion of substrate to product, V_{chem} , is $[X_0]k_a$, and the rate of positional isotope exchange, V_{ex} , is $[X_0](k_e + k_f)$. The ratio $V_{\text{chem}}/V_{\text{ex}}$ can now be expressed as

$$V_{\text{chem}}/V_{\text{ex}} = y/z \quad (35)$$

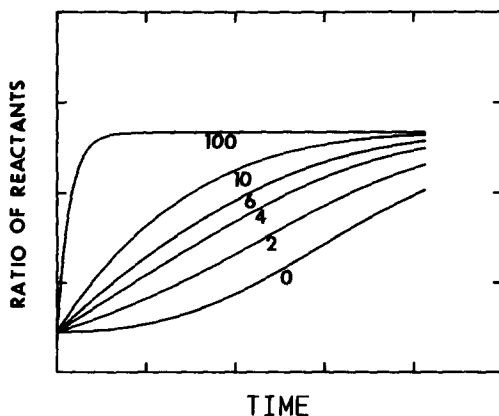


FIG. 3. Simulations for the interconversion of the labeled substrate (M) and the positionally exchanged substrate (O) as a function of time.

The lower limit for the conversion of FP to F, k_5 , relative to the maximal velocity in the reverse direction, V_2/E_t , can be determined since V_2/E_t is less than or equal to $(k_2k_4)/(k_2 + k_5)$:

$$k_5/(V_2/E_t) = V_{\text{chem}}/V_{\text{ex}} \quad (36)$$

Specific Examples

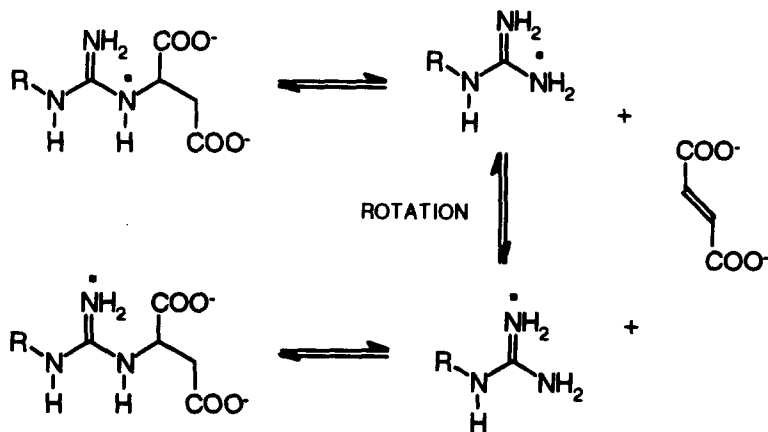
Presented below are examples of five enzyme-catalyzed reactions that have been successfully studied by the positional isotope exchange technique. Three of these enzymes, argininosuccinate lyase, UDPG pyrophosphorylase (UTP-glucose-1-phosphate uridylyltransferase), and galactose-1-phosphate uridylyltransferase (UTP-hexose-1-phosphate uridylyltransferase) give representative examples of the type of information available from a quantitative analysis of enzyme-ligand dissociation rates using positional isotope exchange. The last two examples, D-alanine-D-alanine ligase and carbamoyl-phosphate synthase, are presented to illustrate how the PIX technique can be best utilized to identify reaction intermediates.

Argininosuccinate Lyase

The effect of added products on the observed PIX rate was first investigated by Raushel and Garrard on the reaction catalyzed by argininosuccinate lyase.¹⁰ Argininosuccinate lyase catalyzes the cleavage of argininosuccinate to arginine and fumarate. The effect of fumarate addition on the $^{15}\text{N}/^{14}\text{N}$ positional exchange reaction within argininosuccinate was measured by ^{15}N NMR spectroscopy. Scheme XI shows the positional isotope exchange reaction that was followed with the enzyme. The two external nitrogens in the guanidino moiety of the product arginine are torsionally equivalent. This structural feature enables the scrambling of the ^{15}N and ^{14}N labels within argininosuccinate when the guanidino group is free to rotate about the C-N bond in the enzyme-arginine-fumarate complex.

Raushel and Garrard demonstrated that at zero fumarate there was no observable PIX reaction relative to the net formation of product ($V_{\text{ex}}/V_{\text{chem}} < 0.15$).¹⁰ However, at higher levels of added fumarate the ratio $V_{\text{ex}}/V_{\text{chem}}$ increased until a plateau of 1.8 was reached. These results indicate quite clearly that the release of products from the enzyme-arginine-fumarate complex in the argininosuccinate lyase-catalyzed reaction is kinetically random. Because no exchange was observed at low fumarate,

¹⁰ F. M. Raushel and L. J. Garrard, *Biochemistry* **23**, 1791 (1984).



SCHEME XI

it can also be concluded that the release of at least one of the two products must be very fast. Moreover, the release of arginine from the ternary complex, relative to V_2/E_t , must be greater than or equal to 0.5 (based on the limiting value of 1.8 at saturating fumarate). Therefore, the release of fumarate from the ternary complex (see Scheme V) is very fast relative to the maximal velocity in the reverse direction [$k_9/(V_2/E_t) > 6$], and thus the release of fumarate from the enzyme-arginine-fumarate complex is at least 10 times faster than arginine release.

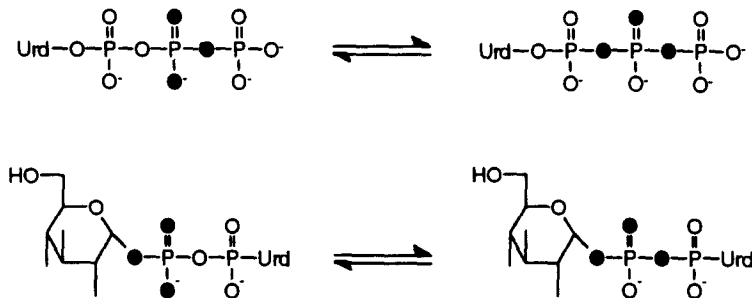
UDPGlucose Pyrophosphorylase

Hester and Raushel investigated the effects of substrate addition on the PIX reaction catalyzed by UDPG pyrophosphorylase.¹¹ UDPG pyrophosphorylase catalyzes the conversion of UTP and glucose 1-phosphate to UDP-glucose and pyrophosphate. The reaction proceeds by the nucleophilic attack of glucose 1-phosphate on the α -phosphate of UTP. The kinetic mechanism has been previously shown to be ordered with UTP as the first substrate to bind and pyrophosphate the first product to be released (see Scheme VII).¹²

Hester and Raushel showed that it was possible to follow a PIX reaction in both the forward and reverse reactions of the enzyme by utilizing the

¹¹ L. S. Hester and F. M. Raushel, *Biochemistry* **26**, 6465 (1987).

¹² K. K. Tsuboi, K. Fukunaga, and J. C. Petricciani, *J. Biol. Chem.* **244**, 1008 (1969).



SCHEME XII

positionally labeled substrates shown in Scheme XII.¹¹ The PIX reactions in the forward and reverse directions were able to be suppressed by increasing the concentration of the second substrate, thus confirming the strictly ordered nature of the reaction mechanism (see Fig. 4).

By combining the quantitative information from the PIX reactions with the steady-state kinetic and thermodynamic parameters, Hester and Raushel were able to obtain estimates for all of the microscopic rate constants in the UDPG pyrophosphorylase reaction. The values calculated for the rate constants (shown in Scheme XIII) reveal that the release of UDPglucose is 3 times slower than the release of pyrophosphate and that the release of glucose 1-phosphate is 5 times slower than the release to UTP. The back-calculated kinetic constants are in excellent agreement

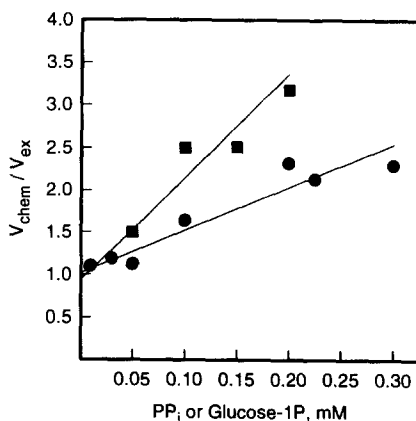
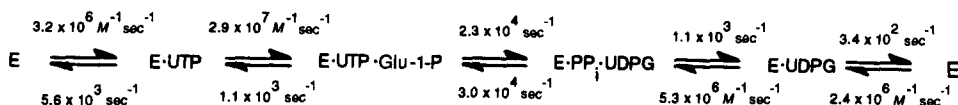


FIG. 4. Plot of the ratio of the net chemical turnover rate and the positional isotope exchange rate as a function of the concentration of added glucose 1-phosphate (●) or pyrophosphate (PP_i) (■).



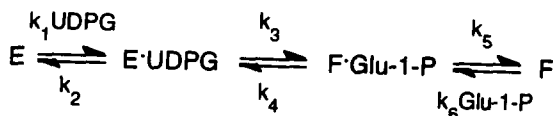
SCHEME XIII

with the experimental values.¹¹ This example demonstrates quite clearly the potential for obtaining quantitative information about the binding and release of substrates and products from a thorough analysis of PIX reactions.

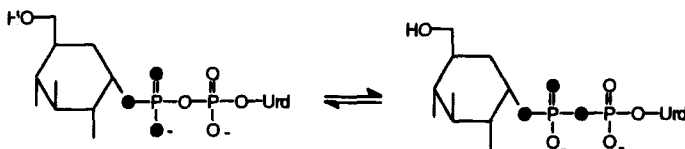
Galactose-1-Phosphate Uridylyltransferase

The PIX technique has rarely been applied to a ping-pong reaction mechanism.¹³ However, Hester and Raushel have investigated the reaction catalyzed by galactose-1-phosphate uridylyltransferase. Galactose-1-phosphate uridylyltransferase catalyzes the transfer of the uridylyl group from UDPglucose to galactose-1-phosphate. A covalent enzyme-uridylyl adduct is an intermediate in the reaction as determined by stereochemical and steady-state kinetic analysis.¹⁴ The PIX experiment monitored only the first half-reaction of galactose-1-phosphate uridylyltransferase as shown in Scheme XIV.

Unlabeled glucose-1-phosphate was included in the reaction mixture to recycle the enzyme so that the partitioning of the uridylyl enzyme-



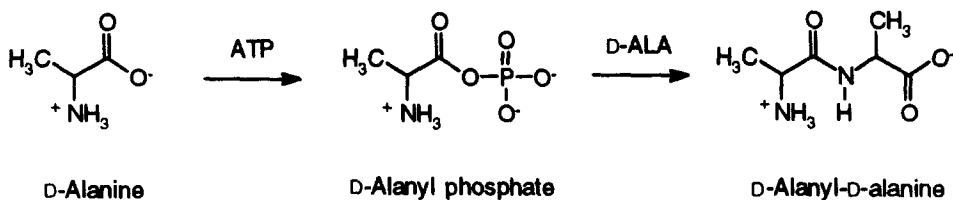
SCHEME XIV



SCHEME XV

¹³ L. S. Hester and F. M. Raushel, *J. Biol. Chem.* **262**, 12092 (1987).

¹⁴ K. R. Sheu and P. A. Frey, *J. Biol. Chem.* **253**, 3378 (1978).

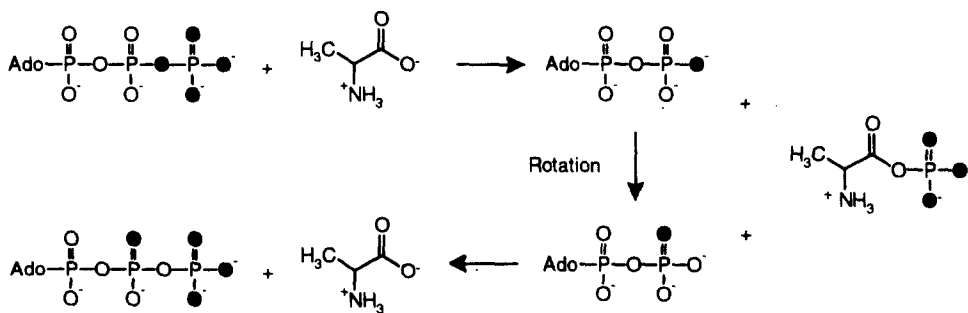


SCHEME XVI

glucose-1-phosphate complex could be determined. The PIX reaction followed the torsional scrambling of ¹⁸O₃ within [β-¹⁸O₃]UDPglucose (Scheme XV) in the presence of variable amounts of unlabeled glucose-1-phosphate as a function of time. No PIX reaction was observed in the absence of glucose 1-phosphate as a function of time. No PIX reaction was observed in the absence of glucose-1-phosphate as expected for a ping-pong reaction. The partitioning of the uridylyl-enzyme–glucose-1-phosphate (V_{chem}/V_{ex}) was determined to be 3.4. Therefore, the release of glucose-1-phosphate from the uridylyl enzyme complex is 3.4 times faster than the maximal velocity in the reverse direction.

D-Alanine–D-Alanine Ligase

Mullins *et al.* utilized the PIX technique in order to obtain kinetic evidence for the intermediate formation of D-alanyl phosphate in the reaction catalyzed by D-alanine–D-alanine ligase.¹⁵ The D-alanine–D-alanine ligase reaction is a difficult reaction to study mechanistically because it utilizes the same substrate twice. D-Alanine–D-alanine ligase catalyzes the formation of D-alanyl-D-alanine from ATP and two molecules of D-alanine. The reaction has been proposed to proceed through an acylphos-



SCHEME XVII

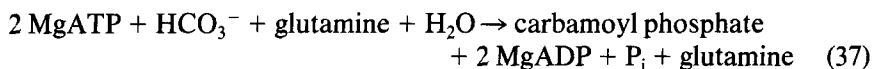
¹⁵ L. S. Mullins, L. E. Zawaszke, C. T. Walsh, and F. M. Raushel, *J. Biol. Chem.* **265**, 8993 (1990).

phate intermediate formed via the phosphorylation of the carboxyl group of the first D-alanine by the γ -phosphate of ATP (Scheme XVI).¹⁶

Mullins *et al.* followed the PIX reaction shown in Scheme XVII. In D-alanine-D-alanine ligase, the difficulty of investigating the kinetic mechanism is increased because the reaction can not be artificially stopped after the addition of the first two substrates to probe for the existence of the intermediate. A PIX reaction is observed only in the presence of D-alanine, and this is consistent with the direct attack of the carboxyl group of one D-alanine on the γ -phosphate of ATP to give an acyl phosphate intermediate. Cleavage of the γ -phosphate of ATP could occur either after the first D-alanine binds or, alternatively, only after the binding of all three substrates. However, the observed positional isotope exchange rate is diminished relative to the net substrate turnover as the concentration of D-alanine is increased. This is consistent with an ordered kinetic mechanism. In addition, the ratio of the PIX rate relative to the net chemical turnover of substrate ($V_{\text{ex}}/V_{\text{chem}}$) approaches a value of 1.4 as the concentration of D-alanine becomes very small. This ratio is 100 times larger than the ratio of the maximal reverse and forward chemical reaction velocities (V_2/V_1). This situation is only possible when the reaction mechanism in the forward direction proceeds in two distinct steps and the first step is much faster than the second step. Thus it appears that formation of the acyl-phosphate intermediate is faster than amide bond formation.

Carbamoyl-Phosphate Synthetase

The PIX technique has been used to identify two reactive intermediates in the reaction catalyzed by carbamoyl-phosphate synthetase (CPS). CPS catalyzes the following reaction:

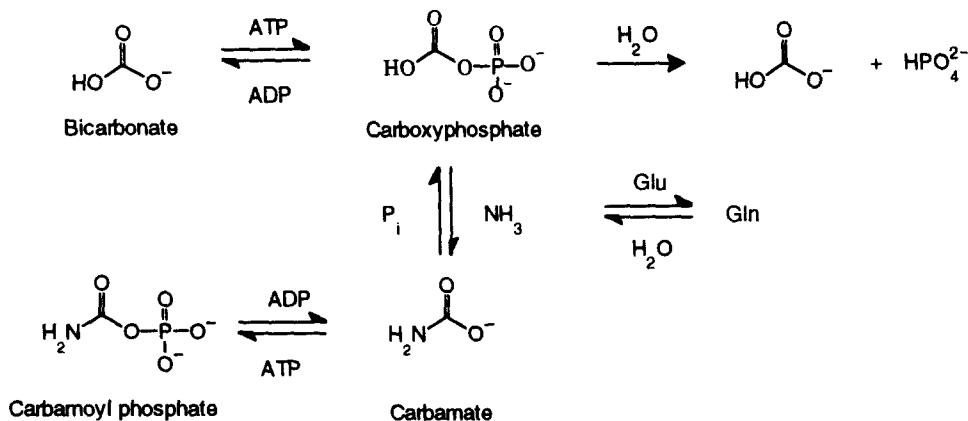


The protein is heterodimeric consisting of a large (120 kDa) and a small (42 kDa) subunit.¹⁷ The proposed mechanism for the synthesis of carbamoyl phosphate (shown in Scheme XVIII) is composed of at least four individual steps.¹⁸ Carboxyphosphate and carbamate have been postulated as the key intermediates in the overall reaction mechanism. The hydrolysis of glutamine to glutamate occurs on the small subunit while the remaining

¹⁶ K. Duncan and C. T. Walsh, *Biochemistry* **27**, 3709 (1988).

¹⁷ P. P. Trotta, M. E. Burt, R. H. Haschemeyer, and A. Meister, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2599 (1971).

¹⁸ P. M. Anderson and A. Meister, *Biochemistry* **4**, 2803 (1965).



SCHEME XVIII

reactions occur on the large subunit. Therefore, the ammonia nitrogen must be transferred from the small subunit to the active site of the large subunit. Free ammonia can be utilized as the nitrogen source by the large subunit in the absence of an active glutaminase reaction from the small subunit. A partial reaction catalyzed by the large subunit is the bicarbonate-dependent hydrolysis of ATP. This reaction is thought to result from the slow hydrolysis of the carboxyphosphate intermediate in the absence of a nitrogen source. In the reverse direction the enzyme can utilize carbamoyl phosphate to phosphorylate MgADP. The other two products of the partial back reaction are CO_2 and NH_3 , which probably arise from the decomposition of carbamate.

Wimmer *et al.* utilized ^{18}O -labeled ATP to probe for a positional isotope exchange reaction that would support the formation of carboxyphosphate as a reactive intermediate (see Scheme XIX).¹⁹ These workers found that in the presence of enzyme and bicarbonate CPS catalyzed a positional isotope exchange within ATP which was 1.7 times as fast as the net hydrolysis of ATP. Therefore the $\text{E} \cdot \text{ADP} \cdot \text{carboxyphosphate}$ complex releases a product into solution about as fast as ATP is resynthesized and released into the bulk solution. These results have been confirmed by three other research groups.²⁰⁻²²

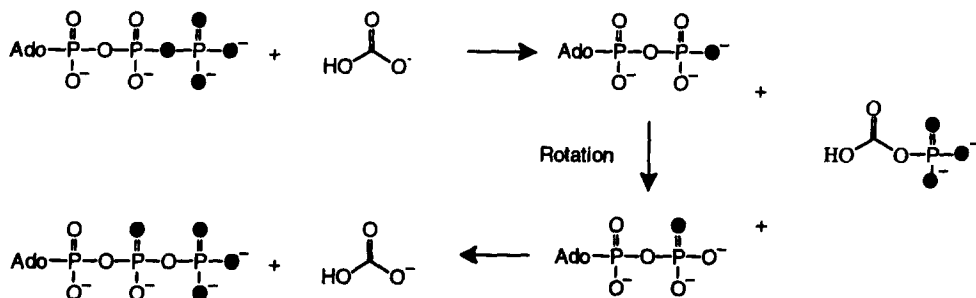
The second intermediate, carbamate, was probed by following the

¹⁹ M. J. Wimmer, I. A. Rose, S. G. Powers, and A. Meister, *J. Biol. Chem.* **254**, 1854 (1979).

²⁰ F. M. Raushel and J. J. Villafranca, *Biochemistry* **19**, 3174 (1980).

²¹ T. D. Meek, W. E. Karsten, and C. W. DeBrosse, *Biochemistry* **26**, 2584 (1987).

²² M. A. Reynolds, N. J. Oppenheimer, and G. L. Kenyon, *J. Am. Chem. Soc.* **105**, 6663 (1983).



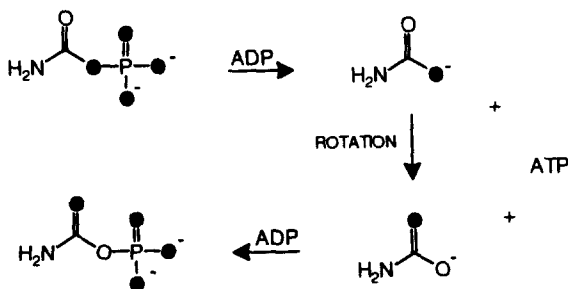
SCHEME XIX

positional exchange of an oxygen-18 label within carbamoyl phosphate.²⁰ Scheme XX shows that if carbamate is formed and stabilized at the active site on mixing of ADP and carbamoyl phosphate, then a positional isotope exchange reaction is possible via the torsional rotation of the carboxyl group of carbamate. The positional isotope exchange rate was found to be 4 times faster than the net synthesis of ATP, and thus carbamate is a kinetically significant reactive intermediate in the carbamoyl-phosphate synthase reaction.

Work from the Meister laboratory has provided evidence that the glutaminase reaction catalyzed by the small subunit requires an essential cysteine residue.²³ It was noted that when the glutaminase reaction was destroyed by incubation with a chloroketone analog of glutamine, the bicarbonate-dependent ATPase reaction was stimulated. Rubino *et al.* later identified the critical cysteine residue by site-directed mutagenesis to be Cys-269.²⁴ The two mutants (C269G and C269S) made by Rubino *et al.* not only lost all glutaminase activity, but more interestingly showed a significant enhancement of the bicarbonate-dependent ATPase reaction relative to wild-type enzyme. The bicarbonate-dependent hydrolysis of ATP normally occurs at approximately 5–10% of the rate of carbamoyl phosphate synthesis. The slow hydrolysis rate represents the protection of the unstable carboxyphosphate intermediate from water. Therefore, the stabilization of carboxyphosphate by the enzyme results from either exclusion of water from the active site or the very slow release of the reactive intermediate from the active site. Apparently, the two mutant enzymes (C269G and C269S) cannot adequately protect carboxyphosphate from water, thus permitting the partial hydrolysis reaction to compete with the overall synthesis of carbamoyl phosphate.

²³ L. M. Pinkus and A. Meister, *J. Biol. Chem.* **247**, 6119 (1972).

²⁴ S. D. Rubino, H. Nyunoya, and C. J. Lusty, *J. Biol. Chem.* **262**, 4382 (1987).



SCHEME XX

The energetics of the bicarbonate-dependent ATPase reaction catalyzed by the wild-type enzyme have been examined by Raushel and Villafranca using rapid quench and PIX experiments.²⁵ Scheme XXI shows the kinetic mechanism for the bicarbonate-dependent ATPase reaction along with the kinetic barrier diagram for that mechanism, where ES represents the enzyme- HCO_3^- -ATP complex and EI represents the enzyme-carboxyphosphate-ADP complex. At saturating levels of substrates the rate of the reaction shown in Scheme XXI is governed by Eq. (38), and the ratio of the PIX rate relative to the rate of net turnover of ATP ($V_{\text{ex}}/V_{\text{chem}}$) is given by the ratio of rate constants presented in Eq. (39):

$$V_{\text{max}} = (k_3 k_5) / (k_3 + k_4 + k_5) \quad (38)$$

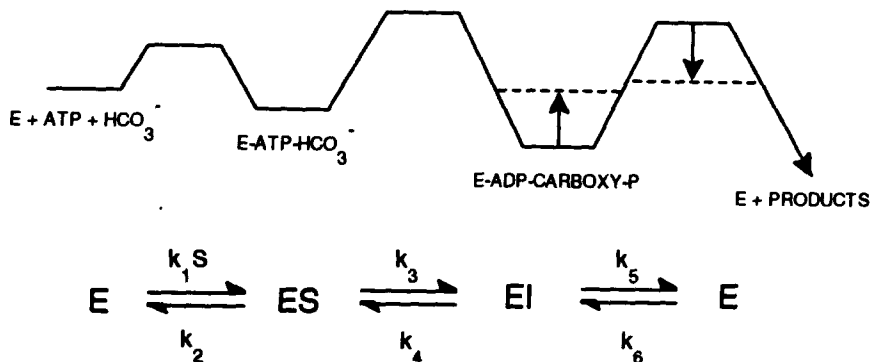
$$V_{\text{ex}}/V_{\text{chem}} = (k_2 k_4) / [k_5 (k_2 + k_3)] \quad (39)$$

The pre-steady-state time course exhibited "burst" kinetics with a rapid formation of acid-labile phosphate followed by a slower steady-state rate. Raushel and Villafranca measured the values of k_3 , k_4 , and k_5 as 4.2, 0.10, and 0.21 sec^{-1} , respectively, from the rapid quench and previous PIX data. They also showed that k_2 is much greater than 3.1 sec^{-1} . Therefore, the formation of carboxyphosphate is very fast, with the rate-limiting steps for net ATP hydrolysis involving either product release or hydrolysis of the intermediate in the active site.

Mullins *et al.* used the PIX technique to examine the changes in the reaction energetics of the ATPase reaction in the C269G and C269S mutants and the isolated large subunit.²⁶ The PIX reaction they followed has been previously shown in Scheme XIX. They found the bicarbonate-

²⁵ F. M. Raushel and J. J. Villafranca, *Biochemistry* **18**, 3424 (1979).

²⁶ L. S. Mullins, C. J. Lusty, and F. M. Raushel, *J. Biol. Chem.* **266**, 8236 (1991).



SCHEME XXI

dependent ATPase reaction for all three enzymes to be 2 to 3-fold faster than the wild-type enzyme, but 4- to 10-fold faster if glutamine is present. As no increase is observed in the NH_3 -dependent carbamoyl phosphate synthesis rate, k_3 is not changed in the mutants. The significant increase in the bicarbonate-dependent ATPase reaction of the mutants can be the consequence of two possible alterations in the kinetic barrier diagram. It could reflect the stabilization of the transition state for the reaction of carboxyphosphate with water. However, this stabilization would only affect k_5 and k_6 while leaving k_4 unchanged. Alternatively, the ground state for the enzyme-bound carboxyphosphate may be destabilized, resulting in an increase in k_4 and k_5 by the same factor. Mullins *et al.* showed that the two cases could be distinguished by measuring the partitioning of the E-ADP-carboxyphosphate complex. The ratio $V_{\text{ex}}/V_{\text{chem}}$ simplifies to k_4/k_5 since $k_2 \gg k_3$ [Eq. (39)]. Therefore, if the ground state of the enzyme-carboxyphosphate complex is destabilized, then the ratio $V_{\text{ex}}/V_{\text{chem}}$ will be identical for the mutants and the wild-type enzyme. If the transition state for the hydrolysis of carboxyphosphate is stabilized, however, the ratio $V_{\text{ex}}/V_{\text{chem}}$ will be reduced in the mutants relative to the wild-type enzyme.

Mullins *et al.* observed that the ratio of the PIX rate relative to ATP turnover was identical for the wild type, C269G, C269S, and the isolated large subunit.²⁶ Therefore, the alteration in the energetics is due to the destabilization of the ground state for the enzyme-bound carboxyphosphate-ADP complex. In the presence of NH_3 , however, the value of $V_{\text{ex}}/V_{\text{chem}}$ was reduced over 20-fold. Thus the increased turnover in the presence of NH_3 results from the substantial stabilization of the transition

state for the reaction of ammonia with carboxyphosphate relative to the reaction with water.

Other Examples

A number of other enzymes have been successfully analyzed using positional isotope exchange methodology. Bass *et al.* have probed the reaction catalyzed by adenylosuccinate synthetase,²⁷ which involves the synthesis of adenylosuccinate from GTP, IMP, and aspartate. Incubation of [β,γ -¹⁸O]GTP, IMP, and enzyme resulted in scrambling of the label from the $\beta\gamma$ -bridge position to the β -nonbridge positions. No scrambling was observed in the absence of IMP, and the addition of aspartate was not required for positional isotope exchange to occur. This result has been interpreted to support a two-step mechanism for the synthesis of adenylosuccinate where the GTP phosphorylates the carbonyl oxygen of IMP to form a phosphorylated intermediate. The phosphorylated intermediate subsequently reacts with the α -amino group of aspartate to generate the final product, adenylosuccinate.

The Lowe laboratory has examined the reactions catalyzed by the aminoacyl-tRNA synthetases using the PIX technique.²⁸⁻³⁰ The enzymes are responsible for the condensation of amino acids to the cognate tRNA. The reaction mechanism has been proposed to involve the formation of an aminoacyl adenyate intermediate from an amino acid and ATP. In support of this mechanism Lowe *et al.* have shown that the isoleucyl-, tryrosyl-, and methionyl-tRNA synthetases all catalyze a positional isotope exchange reaction from the β -nonbridge position of ATP to the $\alpha\beta$ -bridge position in the presence of the required amino acid. No exchange was observed in the absence of the amino acid, nor was any PIX reaction observed in the presence of the dead-end alcohol analogs of the amino acids.

The enzyme CTP synthase catalyzes the formation of CTP from glutamine, ATP, and UTP. The two most reasonable reaction mechanisms involve either the attack of ammonia at C-4 of UTP to form the carbinolamine or, alternatively, the phosphorylation of the carbonyl oxygen of UTP by the ATP. von der Saal *et al.* have shown that the enzyme will catalyze the exchange of label from the $\beta\gamma$ -bridge position to the β -nonbridge position in the presence of UTP.³¹ No ammonia or glutamine is

²⁷ M. B. Bass, H. J. Fromm, and F. B. Rudolf, *J. Biol. Chem.* **259**, 12330 (1984).

²⁸ G. Lowe, B. S. Sproat, G. Tansley, and P. M. Cullis, *Biochemistry* **22**, 1229 (1983).

²⁹ G. Lowe, B. S. Sproat, and G. Tansley, *Eur. J. Biochem.* **130**, 341 (1983).

³⁰ G. Lowe and G. Tansley, *Tetrahedron* **40**, 113 (1984).

³¹ W. von der Saal, P. M. Anderson, and J. J. Villafranca, *J. Biol. Chem.* **260**, 14993 (1985).

required for the reaction to be observed. This result is consistent only with the phosphorylated UTP intermediate, and thus the carbinolamine intermediate can be discarded.

The enzymatic synthesis of GMP follows a reaction scheme that is analogous to the synthesis of CTP. The enzyme GMP synthetase utilizes ATP, XMP, and glutamine to construct the final bond in the formation of GMP. The proposed reaction mechanism involves the adenylation the carbonyl oxygen of XMP by ATP. von der Saal *et al.* were able to demonstrate that on incubation of ^{18}O -labeled ATP and XMP a PIX reaction occurred which did not require the presence of glutamine or ammonia.³²

The enzyme pyruvate-phosphate dikinase catalyzes the formation of phosphoenolpyruvate (PEP) from ATP, phosphate, and pyruvate. The other two products of the reaction are AMP and pyrophosphate. The proposed reaction mechanism is thought to involve at least three separate reactions. ATP pyrophosphorylates the enzyme in the first reaction, and this intermediate subsequently phosphorylates phosphate to produce pyrophosphate and a phosphorylated enzyme intermediate. In the last step the phosphorylated enzyme transfers the phosphoryl group to pyruvate to form the ultimate product, PEP. Wang *et al.* used a variety of ^{18}O -labeled ATP molecules to demonstrate that the observed PIX reactions were consistent with the proposed reaction mechanism.³³

The enzyme PEP carboxykinase catalyzes the formation of PEP and CO_2 from oxaloacetate and GTP. Chen *et al.* have utilized PIX methodology to examine the partitioning of enzyme-product complexes.³⁴ No positional isotope exchange was observed within the labeled GTP under initial velocity conditions when enzyme was mixed with oxaloacetate and $[\beta, \gamma\text{-}^{18}\text{O}]\text{GTP}$. These results have been interpreted to indicate that at least one of the products dissociates rapidly from the enzyme-GDP-PEP- CO_2 complex relative to the net rate of GTP formation from the complex.

Summary

The positional isotope exchange technique has been found to be quite useful for the identification of reaction intermediates in enzyme-catalyzed reactions. For reactions where intermediates are not expected the method can be used with great utility for the quantitative determination of the partitioning of enzyme-product complexes. However, it must be remem-

³² W. von der Saal, C.S. Crysler, and J. J. Villafranca, *Biochemistry* **24**, 5343 (1985).

³³ H. Ch. Wang, L. Ciskanik, D. Dunaway-Mariano, W. von der Saal, and J. J. Villafranca, *Biochemistry* **27**, 625 (1988).

³⁴ C. Y. Chen, Y. Sato, and V. L. Schramm, *Biochemistry* **30**, 4143 (1991).

bered that it has been explicitly assumed that the functional group undergoing positional exchange is free to rotate. This assumption is not always valid since examples have been discovered where the functional group rotation is indeed hindered. For instance, in the reaction catalyzed by argininosuccinate synthetase a PIX reaction was not observed on incubation of ATP and citrulline even though a citrulline-adenylate complex has been identified from rapid quench experiments.³⁵

Acknowledgments

The authors are grateful for financial support from the National Institutes of Health (DK30343, GM33894, and GM49706).

³⁵ L. W. Hilscher, C. D. Hanson, D. H. Russel, and F. M. Rauschel, *Biochemistry* **24**, 5888 (1985).

[16] Manipulating Phosphorus Stereospecificity of Adenylate Kinase by Site-Directed Mutagenesis

By MING-DAW TSAI, RU-TAI JIANG, TERRI DAHNKE, and
ZHENGTAO SHI

Introduction

Nucleoside phosphorothioates^{1,2} occupy an important niche in the fields of molecular biology, biochemistry, and mechanistic enzymology. The applications in molecular biology, which include DNA sequencing and oligonucleotide-directed mutagenesis, have been reviewed.³ The applications of nucleoside phosphorothioates to various biochemical problems have also been reviewed by Eckstein.⁴ Many applications take advantage of the stereospecificity of enzymes toward specific isomer(s) of nucleoside phosphorothioates, a property uncovered by mechanistic enzy-

¹ ADP, Adenosine 5'-diphosphate; ADP α S, adenosine 5'-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5'-monophosphate; AMPS, adenosine 5'-monothiophosphate; AP $_5$ A, P¹,P⁵-bis(5'-adenosyl)pentaphosphate; ATP, adenosine 5'-triphosphate; ATP α S, adenosine 5'-(1-thiotriphosphate); ATP β S, adenosine 5'-(2-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

² D. A. Usher, E. S. Erenrich, and F. Eckstein, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 115 (1972).

³ F. Eckstein and G. Gish, *Trends Biochem. Sci.* **14**, 97 (1989).

⁴ F. Eckstein, *Annu. Rev. Biochem.* **54**, 367 (1985).