THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 262, No. 25, Issue of September 5, pp. 12092-12095, 1987
Printed in U.S.A.

Analysis of Ping-Pong Reaction Mechanisms by Positional Isotope Exchange

APPLICATION TO GALACTOSE-1-PHOSPHATE URIDYLTRANSFERASE*

(Received for publication, May 15, 1987)

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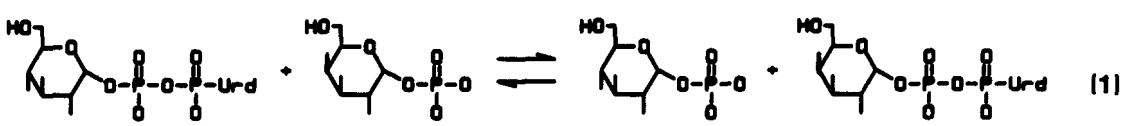
A new positional isotope exchange method has been developed that can be used for the analysis of enzyme-catalyzed reactions which have ping-pong kinetic mechanisms. The technique can be used to measure the relative rates of ligand dissociation from enzyme-product complexes. Enzyme is incubated with the labeled substrate and an excess of the corresponding unlabeled product. The partitioning of the enzyme-product complex back toward free enzyme is determined from the rate of positional isotope exchange within the original labeled substrate. The partitioning of the enzyme-product complex forward toward free enzyme is determined from the rate of formation of totally unlabeled substrate. It has been shown that the ratio of the two rates provides a lower limit for the release of product from the enzyme-product complex. The technique has been applied to the reaction catalyzed by galactose-1-phosphate uridyltransferase. The lower limit for the release of glucose 1-phosphate from the uridyl-enzyme relative to the maximal velocity of the reverse reaction was determined to be 3.4 ± 0.5.

The positional isotope exchange technique, introduced by Midelfort and Rose (1) in 1976, has become a valuable probe of mechanisms in molecular enzymology. The PIX[1] experiments have been used for the identification of reaction intermediates (2-5) and for the determination of the partition ratios of enzyme-substrate and enzyme-product complexes during the catalytic process (6, 7). In general, the PIX technique can be used to probe any reversible enzyme-catalyzed reaction in which functionally nonequivalent groups become torsionally equivalent via a reaction intermediate or product. To date, the positional scrambling of oxygen and nitrogen labels within phosphoryl (1-5), carboxyl (2), and guanidino (6) groups has been successfully used in the analysis of both random and ordered kinetic mechanisms.

One of the more common misconceptions about the PIX technique is that it should be ideal for the analysis of ping-pong reaction mechanisms. This expectation exists because the positionally labeled substrate should be able to bind and react covalently with the enzyme in the total absence of the second substrate and form a stable but modified enzyme and first product complex. Positional isotope exchange could occur by reformation of the initial substrate via a reaction between the newly formed product and the modified enzyme. However, no significant PIX reaction is to be expected because of the facile dissociation of the product from the modified enzyme in the classic ping-pong reaction mechanism. At catalytic concentrations of the enzyme, the amount of product produced would be so low (essentially 1 enzyme eq) as to prevent any significant rebinding to the enzyme after dissociation into the bulk solution. Therefore, after the first turnover of the enzyme all of the protein would be in the modified form that is functionally unable to process any more of the labeled substrate.

In this paper we have developed a method for measuring PIX reactions with enzymes having ping-pong kinetic mechanisms. The new technique recycles the modified enzyme form by inclusion of the unlabeled reaction product into the initial reaction mixture. This methodology has been applied to the reaction catalyzed by galactose-1-phosphate uridyltransferase. This enzyme catalyzes the reaction indicated below.



[1]

The steady-state kinetic analysis and the stereochemical outcome of the reaction at the α-phosphorus of UDP-glucose clearly show that this enzyme has a ping-pong reaction mechanism in which a covalent uridyl-enzyme intermediate forms (8, 9).

MATERIALS AND METHODS

Sucrose synthetase was partially purified from wheat germ according to the procedure of Singh *et al.* (10). Galactose-1-phosphate uridyltransferase, carbamate kinase, and nucleoside monophosphate kinase were purchased from Sigma. Oxygen-18 labeled water (97%) was purchased from Cambridge Isotope Laboratories. Oxygen-18 labeled potassium phosphate was made according to the procedure of Riseley and Van Etten (11).

* This work was supported by National Institutes of Health Grant GM-33874 and Robert A. Welch Foundation Grant A-840. We acknowledge with thanks financial support by the Board of Regents of Texas A&M University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PIX, positional isotope exchange; MES, 2-(*N*-morpholino)ethanesulfonate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Preparation of [β-$^{18}O_3$]UDP-Glucose—UDP-glucose labeled with oxygen-18 at the β-phosphoryl group was synthesized enzymatically with carbamate kinase, nucleoside monophosphate kinase, and sucrose synthetase in two steps. The first step involved the formation of [β-$^{18}O_3$]UDP, and the second step involved the formation of the [β-$^{18}O_3$]UDP-glucose. The first reaction mixture contained 10 mM UMP, 7.5 mM ADP, 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 10 mM carbamoyl [$^{18}O_4$]phosphate, 50 units of carbamate kinase, and 12 units of nucleoside monophosphate kinase in a volume of 100 ml. The reaction was quenched by lowering the pH to 3.0 and the enzymes removed by passage through a YM-30 ultrafiltration membrane (Amicon Corp.). The nucleotides were separated by passage through a DE52 anion exchange column. The eluting buffer was a 4.0-liter gradient of 10–250 mM triethylamine-bicarbonate, pH 7.5. [β-$^{18}O_3$]UDP was isolated (500 μmol) and used in the synthesis of [β-$^{18}O_3$]UDP-glucose. The reaction mixture contained 10 mM [β-$^{18}O_3$]UDP, 50 mM sucrose, 25 mM MES, pH 6.0, and 6.0 units of sucrose synthetase in a volume of 50 ml. The reaction was quenched and the product isolated by chromatography on DE52. The yield was 400 μmol of [β-$^{18}O_3$]UDP-glucose. The oxygen-18 incorporation at the indicated positions was 90%.

Positional Isotope Exchange Reaction—The positional isotope exchange reactions were monitored by ^{31}P NMR until the reaction reached isotopic equilibrium. The positional isotope exchange rate was monitored by following the interchange of the β-nonbridge and α,β-bridge oxygens of [β-$^{18}O_3$]UDP-glucose. The chemical rate was followed by the appearance of [β-$^{16}O_3$]UDP-glucose. The reaction conditions were: 5 mM [β-$^{18}O_3$]UDP-glucose, 25 mM BisTris propane, pH 8.5, 0.1 mM EDTA, 25% D_2O, galactose-1-phosphate uridyltransferase, and variable amounts of glucose-1-P in a total volume of 3.0 ml.

^{31}P Nuclear Magnetic Resonance Measurements—^{31}P NMR spectra were obtained on a Varian XL-400 multinuclear spectrometer operating at a frequency of 162 MHz. The kinetic data were acquired by either a number of transient array or a preacquisition delay array. Typical acquisition parameters were: 4500-Hz sweep width, 3-s acquisition time, no delay between pulses, 15-μs pulse width (pulse width 90° = 25 μs), and Waltz decoupling (4 db).

THEORY

The minimal scheme for the first half of a classic ping-pong kinetic mechanism is as follows:

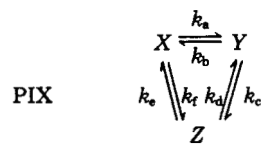
$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FP \underset{k_6 P}{\overset{k_5}{\rightleftharpoons}} F$$

SCHEME I

where E = enzyme, A = substrate, P = product, and F is the stable enzyme form which contains the portion of A which will eventually be transferred to the second substrate. If the substrate A is isotopically labeled and incubated with enzyme then a PIX reaction can occur via the partitioning of the complex FP back to free enzyme and positionally exchanged substrate. However, if the enzyme form F is chemically stable no significant PIX reaction is to be expected after the first turnover because all of the enzyme will be in the enzyme form F which is unable to catalyze any PIX reaction. Since the maximum concentration of the product P to be produced can only equal the concentration of the enzyme used, there cannot be a return to free enzyme from F because the dissociation constant of P from FP is expected to be significantly greater than the catalytic concentration of enzyme used in the experiment.

What is required for the observation of PIX reactions in ping-pong mechanisms is a method for reconverting enzyme form F back to free enzyme E. The ideal method would be to cause the hydrolysis of the covalent bond between the enzyme and the transferred piece from the initial substrate. An alternative approach would be to add a large excess (relative to the labeled substrate) of the unlabeled product P to the reaction mixture. The large excess of unlabeled P would serve two functions. The first would be to return the modified enzyme form F back to free enzyme E. This return back to free enzyme also produces unlabeled substrate A which would provide a convenient measure of the partitioning of FP to F. The second function would be to dilute the relative concentration of the labeled product P. This latter function is critical since the primary objective of the PIX study is to determine the partitioning of enzyme form FP. Therefore, it is absolutely essential that the formation of positionally exchanged substrate not occur from labeled P that has dissociated and then reassociated with the enzyme. This latter exchange mechanism can be termed "pseudo-PIX." Thus, the partitioning of FP directly back to E can be determined by the rate of conversion of the labeled substrate A to the positionally exchanged substrate.

An analysis of this PIX experiment is modeled below



SCHEME II

where X, Y, and Z represent the concentrations of positionally labeled, unlabeled, and positionally exchanged substrates, respectively. The interconversion of $X \rightarrow Y \rightarrow Z$ (involving only the rate constants k_a through k_d) is simply the molecular isotope exchange for the equilibration of the labeled and unlabeled substrates. Any formation of the positionally exchanged material (Z) via this route would be a "pseudo-PIX" reaction. The true PIX rate involves the direct interconversion of X and Z without the intermediate formation of unbound labeled product.

The rate constants in Scheme II (k_a through k_f) have the following relative values

$$k_a = xy(1 + w) \quad (2)$$

$$k_b = x \quad (3)$$

$$k_c = xw \quad (4)$$

$$k_d = xy(1 + w) \quad (5)$$

$$k_e = xz \quad (6)$$

$$k_f = xzw \quad (7)$$

where x is proportional to the amount of enzyme used and thus 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 positional isotope exchange experiment can be conducted via the following protocol. The reaction is initiated by the incubation of labeled substrate A and unlabeled product P with enzyme. The partitioning of FP to F will be determined by the first-order equilibration of the label between the substrate and the product. 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. Therefore, the rate of formation of the positionally exchanged substrate via the "pseudo-PIX" mechanism can be calculated from the exchange rate of the total label between product P and total substrate. The overall rate of formation of the positionally exchanged substrate can thus be corrected to give the actual PIX rate due to the direct interchange of X

and Z. The correction factor will be smaller at larger ratios of unlabeled product relative to labeled substrate.

A numerical solution for the approach to equilibrium of X, Y, and Z in terms of the rate constants k_a through k_f can be made using the KINSIM program of Frieden (12). The factor x can be determined by obtaining the best fit to a plot of $([X] + [Z])/([X] + [Y] + [Z])$ *versus* time since the values for y and w would be known in advance. After the value of x is determined then the value for z can be determined from a fit to a plot of $[Z]/([X] + [Z]$ *versus* time.

The partitioning of the complex FP in terms of the rate constants presented in Scheme I is as follows.

$$\frac{v_{chem}}{v_{pix}} = \frac{k_5\,(k_2 + k_3)}{k_2 k_4} \tag{8}$$

The initial rate for the conversion of labeled substrate to products (v_{chem}) is $[X_0]k_a$, and the initial rate for the positional isotope exchange rate (v_{pix}) is $[X_0]\ (k_f + k_e)$. Therefore, the partitioning of the enzyme form FP can be determined from Equation 9.

$$\frac{v_{chem}}{v_{pix}} = \frac{y}{z} \tag{9}$$

Since the maximal velocity in the reverse direction (V_2) is less than or equal to $k_2k_4/(k_2 + k_3)$ then the lower limit of k_5 relative to V_2 can be obtained from Equation 10 as indicated below.

$$\frac{k_5}{V_2} \geq \frac{v_{chem}}{v_{pix}} \tag{10}$$

RESULTS AND DISCUSSION

The positional isotope exchange technique has been applied to the reaction catalyzed by galactose-1-phosphate uridyltransferase. This enzyme catalyzes the transfer of the uridyl group from UDP-glucose to galactose 1-phosphate. A covalent uridyl-enzyme adduct intermediate has been shown to be an intermediate in this reaction (8, 9). The PIX analysis of the reaction mechanism was initiated by the incubation of labeled [β-$^{18}O_3$]UDP-glucose (X), enzyme, and variable amounts of unlabeled glucose 1-phosphate. The partitioning of the uridyl-enzyme·glucose 1-phosphate complex (FP) forward toward free enzyme through the unbound uridyl-enzyme (F) was determined by monitoring the formation of totally unlabeled UDP-glucose (Y) as a function of time. The partitioning backwards from this same complex toward free enzyme was determined by measuring the rate of positional isotope exchange within the labeled UDP-glucose to form the positionally exchanged substrate (Z).

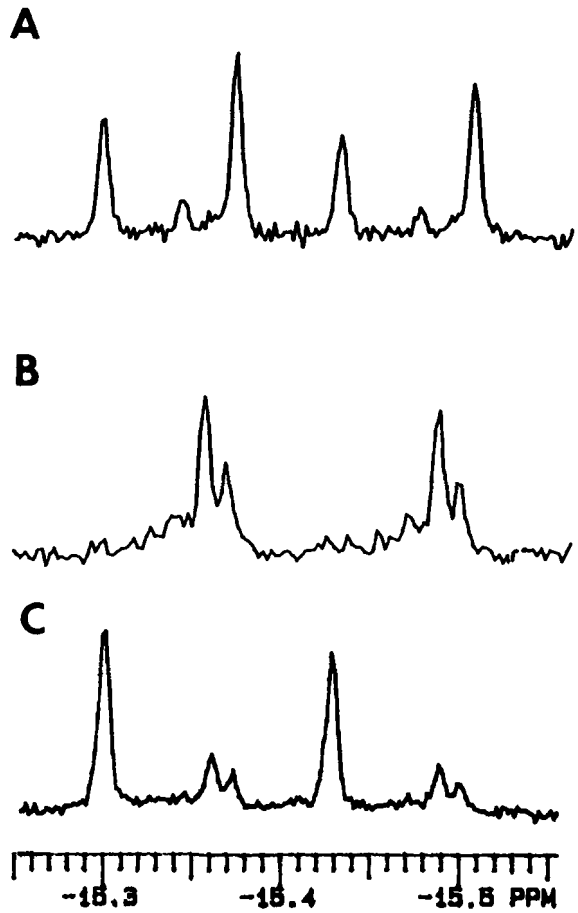
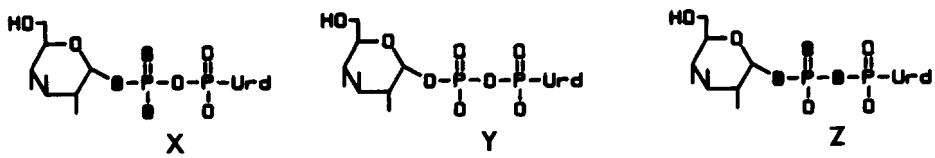


FIG. 1. A, ^{31}P NMR spectrum of the β-phosphorus for a mixture of unlabeled UDP-glucose (Y) and [β-$^{18}O_3$]UDP-glucose (X). B, ^{31}P NMR spectrum of the β-phosphorus of a mixture of [$\alpha\beta$-^{18}O,β-$^{18}O_2$] UDP-glucose (Z) and [β-$^{18}O_3$]UDP-glucose (X). C, ^{31}P NMR spectrum of the β-phosphorus of a mixture of [β-$^{18}O_3$]UDP-glucose (X), unlabeled UDP-glucose (Y), and [$\alpha\beta$-^{18}O,β-$^{18}O_2$]UDP-glucose (Z). Additional details are given in the text.



The relative concentrations of the labeled and unlabeled forms of UDP-glucose (X, Y, and Z) were determined by intergration of the ^{31}P NMR signals for the β-phosphorus of UDP-glucose. Shown in Fig. 1A is a ^{31}P NMR spectrum of the β-phosphorus of a sample containing a 3:2 mixture of X and Y. The doublet for the β-phosphorus of X appears 0.071 ppm upfield from the comparable resonance signal for Y because of the chemical shift difference induced by the incorporation of the three atoms of oxygen-18 directly bonded to the β-phosphorus (13). Shown in Fig. 1B is an equilibrium mixture of X and Z. The doublet for the β-phosphorus of X appears 0.013 ppm upfield from the signal for the β-phosphorus of Z because the induced oxygen-18 chemical shift difference is larger for those phosphorus-oxygen-18 bonds with more double bond character (13). When the PIX reaction is initiated by the addition of enzyme, labeled UDP-glucose (X), and unlabeled glucose 1-phosphate the signal for the β-phosphorus of X decreases while those for the β-phosphorus of Y and Z increase. The concentrations of the various labeled forms of UDP-glucose can, therefore, be continuously measured as a function of time by ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum of a reaction mixture that has come to isotopic equilibrium among the three equilibrating species is shown in Fig. 1C.

Shown in Fig. 2 is a plot of the interconversion of the labeled and unlabeled UDP-glucose molecules as a function of time. The reaction was initiated by the incubation of 5 mM labeled UDP-glucose (X) and 61 mM unlabeled glucose 1-phosphate with enzyme. The equilibration of the total labeled

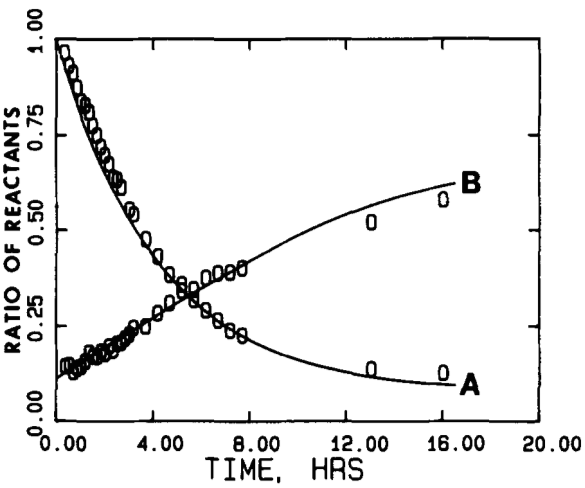


FIG. 2. **Time course for the interconversion of the labeled and unlabeled forms of UDP-glucose.** *A*, plot of $([X] + [Z])/([X] + [Y] + [Z])$ *versus* time. *B*, plot of $[Z]/([X] + [Z])$ *versus* time. The *solid lines* are for a fit of the data to the model presented in Scheme II. See text for additional details.

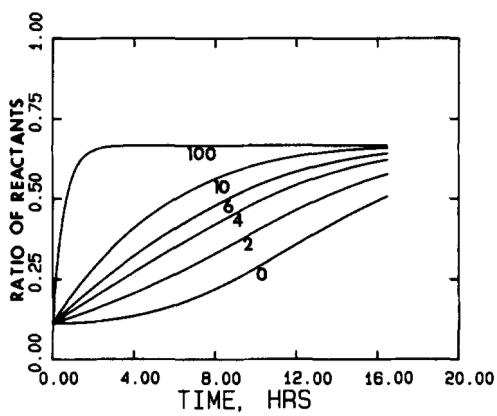


FIG. 3. **Simulations for the interconversion of the labeled UDP-glucose (*X*) and the positionally exchanged UDP-glucose (*Z*) as a function of time using the KINSIM program and the kinetic model presented in Scheme II.** The values for z are 0, 2, 4, 6, 10, and 100.

substrate ($X + Z$) with the total UDP-glucose pool is shown in curve A. As expected there is a first-order approach to isotopic equilibrium for these species. The *solid line* was obtained from a fit of the data using the KINSIM program of Frieden (12) and the model presented in Scheme II. With values of $y = 12.2$ and $w = 2$, the best fit for x is 6.0×10^{-3}/h. It should be noted that equilibration of the labeled and unlabeled forms of UDP-glucose is totally independent of the rate of interconversion between the two labeled forms of UDP-glucose. The equilibration of the labeled UDP-glucose (X) and the positionally exchanged UDP-glucose (Z) as a function of time is shown in curve B of Fig. 2. The solid line is drawn using the previously obtained values for w, x, and y and a value of z of 4.1. Thus, the ratio of the chemical and PIX rate can be calculated (using Equation 9) as 3.0.

The time course for the equilibration of the two possible labeled forms of UDP-glucose is dependent on the ratio of the

TABLE I

Ratios of the chemical and PIX rates at pH 8.5 and 25 °C

Concentration of glucose-1-P	y^a	v_{chem}/v_{pix}
mM		
0	0	No PIX reaction
18	3.6	4.3
38	7.8	2.8
61	12.2	3.0

[a] $[\text{Glucose-1-P}]_t/[\text{UDP-glucose}]_t$.

PIX and "pseudo-PIX" reaction pathways. The route through the "pseudo-PIX" reaction pathway can be hindered by initiating the reaction with a high ratio of unlabeled glucose 1-phosphate relative to the concentration of labeled UDP-glucose. Shown in Fig. 3 are simulations of fits to the data in curve B of Fig. 2. These curves are constructed using z values of zero to 100. It is readily apparent that in the absence of a pathway for the direct interconversion of X and Z that there would be an appreciable lag for the appearance of the positionally exchanged substrate via only the "pseudo-PIX" pathway.

The ratios of the net chemical and PIX rates for other concentrations of unlabeled glucose 1-phosphate are presented in Table I. As predicted by the model for a true ping-pong enzyme mechanism there is no significant effect on this ratio by varying the glucose 1-phosphate concentration. The average value for this ratio is 3.4 ± 0.5. No positional isotope exchange was observed in the absence of any added glucose 1-phosphate. As indicated in Equation 10 the lower limit for the release of glucose 1-phosphate from the uridyl-enzyme complex is 3.4 times faster than the maximal velocity in the reverse reaction. If the labeled UDP-galactose were available then the lower limit for the release of galactose 1-phosphate from the uridyl-enzyme intermediate could be determined relative to the maximal velocity of the forward reaction.

Acknowledgment—We thank Professor Donald Pettigrew for his help with the KINSIM program.

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