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Sulfhydryl Modification and Activation of Phenylalanine Hydroxylase by Dinitrophenyl Alkyl Disulfide

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Received April 9, 1987; Revised Manuscript Received June 3, 1987

ABSTRACT: A new family of asymmetric thiol–disulfide exchange reagents, the dinitrophenyl alkyl disulfides (DNPSSR), was used to modify rat liver phenylalanine hydroxylase. The results indicate that the enzyme has two different types of reactive sulfhydryl (SH) residues per subunit. One SH residue was modified selectively by a DNPSSR having a neutral and hydrophilic alkyl group, and this modification was accompanied by appreciable activation of enzyme; the other SH residue was modified only by an anionic DNPSSR, and this modification did not result in activation. The catalytic properties of phenylalanine hydroxylase activated by DNPSSR were similar to those of the *N*-ethylmaleimide- (NEM-) modified enzyme, but the process of activation by DNPSSR was quite different from modification with NEM. An analysis of the reaction kinetics of the modification and of catalysis by the modified enzyme suggests that DNPSSR modification causes a change in the subunit interaction leading to a loss of the negative cooperativity normally seen with phenylalanine hydroxylase.

Specific sulfhydryl (SH)[1] residues in some proteins have important roles in the function and/or the structure of that protein. There have been many reports of SH modifications inactivating various enzymes or weakening the subunit interaction in the proteins but only a few reports on the activation of enzyme by SH modification (Sekine et al., 1962, 1984;

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[1] Abbreviations: DNPS⁻, dinitrothiophenolate anion; DNPSCl, dinitrobenzenesulfonyl chloride; DNPSSR, dinitrophenyl alkyl disulfides; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; SH, sulfhydryl.

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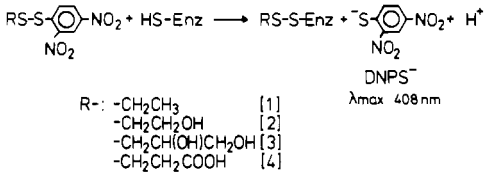

FIGURE 1: Reaction of DNPSSR with an SH compound.

Table I: Kinetic Parameters of Native and Modified Phenylalanine Hydroxylase[a]

DNPSSR	(6*R*)-tetrahydrobiopterin	
	K_m	V_{max}
native	9.3 ± 0.9	0.34 ± 0.01
3, $-CH_2CH(OH)CH_2OH$	9.2 ± 0.7	3.4 ± 0.1
4, $-CH_2CH_2COOH$	6.3 ± 0.6	2.2 ± 0.1

[a] The values were calculated with Wilkinson's program (Wilkinson, 1961) from the plots of $1/v$ vs. $1/[S]$. The K_m for (6*R*)-tetrahydrobiopterin is shown as micromolar. The V_{max} values are shown as $\mu mol \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$. (6*R*)-L-*erythro*-Tetrahydrobiopterin was used as a cofactor and phenylalanine (0.2 mM) as a substrate.

Motion et al., 1984). Rat liver phenylalanine hydroxylase [L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] is composed of four identical subunits, each with five SH residues, and the modification of one of the sulfhydryls with *N*-ethylmaleimide (NEM) or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) markedly increases the catalytic activity of the enzyme (Parniak & Kaufman, 1981). This pteridine-dependent monooxygenase is an allosteric enzyme (Shiman & Gray, 1980) that catalyzes the conversion of L-phenylalanine to L-tyrosine, an obligatory step for the degradation of phenylalanine in mammals (Kaufman, 1959, 1971). Since an absence of this enzyme in humans causes classical phenylketonuria (Scriver & Clow, 1980), the regulatory mechanism of its catalytic activity has been of interest both enzymologically and clinically. The activation of phenylalanine hydroxylase by SH modification in vitro may suggest the possibility of comparable regulation of catalytic activity in vivo for therapeutic purposes.

In this paper, we describe the use of a series of new disulfide exchange reagents, 2,4-dinitrophenyl alkyl disulfides (DNPSSR) (Figure 1), for the modification of rat liver phenylalanine hydroxylase. We examined the reactivity of the SH residues in the enzyme and some properties of the modified enzyme.

Materials and Methods

Materials. Phenylalanine hydroxylase was purified from rat liver by the method of Shiman et al. (1979). The purity of the enzyme was confirmed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The preparation gave a single band at M_r 50K, and the specific activity (V_{max}) is shown in Table I. (6*R*)-L-*erythro*-Tetrahydrobiopterin was kindly synthesized by Dr. Sadao Matsuura (Nagoya University, Nagoya, Japan) according to the method of Matsuura et al. (1985). All DNPSSR for chemical modification were prepared from corresponding thiol compounds (RSH) and 2,4-dinitrobenzenesulfonyl chloride (DNPSCl) according to the method of Parker and Kharasch (1960). The analytical data on these compounds are summarized in Table II. Reagents **3** and **4** are new compounds and have not been reported previously.

Assay of Phenylalanine Hydroxylase. The standard reaction mixture (200 μL) contained 0.2 mM L-phenylalanine, 40 μM (6*R*)-L-*erythro*-tetrahydrobiopterin, 5 mM dithiothreitol, 60 μg of catalase, and 2.14 μg of native or modified phenylalanine hydroxylase, in 0.1 M potassium phosphate buffer, pH 6.8. The reaction was started by the addition of enzyme at 25 °C and terminated after incubation for 2 min by the addition of 50 μL of 30% trichloroacetic acid. The reaction was linear with time and enzyme concentration for both the native and the modified enzyme. The amount of tyrosine produced was assayed by high-performance liquid chromatography (Shimadzu LC-3A HPLC) with fluorometric detection (Bailey & Ayling, 1980). A reversed-phase Unisil F3-50A column (4.6-mm i.d. × 50 mm, Gasukuro Kogyo Inc., Tokyo, Japan) and 0.1 M ammonium acetate buffer, pH 4.5, were used for the separation of tyrosine, and the amount was calculated from the native fluorescence at 300 nm after excitation at 282 nm in a Shimadzu RF-500L spectrofluorophotometer.

Modification of Phenylalanine Hydroxylase. The reaction mixture for modification (600 μL) contained 4.27 μM (as a subunit) purified rat liver phenylalanine hydroxylase and 128 μM DNPSSR in 20% glycerol-containing 50 mM sodium phosphate and 25 mM sodium borate buffers, pH 8.0. The incubation was carried out at 30 °C and was monitored by measuring the absorption of the released dinitrothiophenolate anion ($DNPS^-$) at 408 μm. The amount of SH group modified was calculated from the amount of $DNPS^-$ released, with a molar absorbance of 14 300 being used. As a blank, the same incubation was carried out without enzyme.

Results

Reaction of Phenylalanine Hydroxylase with DNPSSR. The time courses of SH modification of phenylalanine hydroxylase with four different reagents, $DNPSSCH_2CH_3$ (**1**), $DNPSSCH_2CH_2OH$ (**2**), $DNPSSCH_2CH(OH)CH_2OH$ (**3**), and $DNPSSCH_2CH_2COOH$ (**4**), are shown in Figure 2. All four reagents reacted at pH 8.0 releasing $DNPS^-$, but the time courses were markedly different depending upon the properties of the R groups. The three neutral reagents, **1**–**3**, reacted with only one of the five SH groups in each hydroxylase subunit, although the reaction velocities differed from one another. The reaction with reagents **2** and **3** proceeded according to second-order kinetics:

$$v = k[\text{DNPSSR}][\text{SH}] \quad (1)$$

The anionic reagent **4**, having a carboxyl group in the alkyl chain, reacted with an additional SH residue, and the reaction could be described as the sum of two different second-order reactions:

$$v = [\text{DNPSSR}](k_1[\text{SH1}] + k_2[\text{SH2}]) \quad (2)$$

In these equations, [SH], [SH1], and [SH2] are the concentrations of the reactive SH residues in phenylalanine hydroxylase and k, k_1, and k_2 are their apparent reaction velocity

Table II: Analytical Data for Several DNPSSR

reagent	mp (°C)	MS (*m/e*)	^{1}H NMR (DMSO-*d*, δ)
1	85–86	260 (M^+)	1.27 (t, 3 H), 2.90 (dd, 2 H), 8.44 (d, 1 H), 8.58 (d, 1 H), 8.84 (s, 1 H)
2	107–108	276 (M^+)	3.10 (t, 2 H), 4.24 (t, 2 H), 5.84 (s, 1 H), 8.48 (d, 1 H), 8.62 (d, 1 H), 8.84 (s, 1 H)
3	100–101	306 (M^+)	2.98 (d, 2 H), 3.38 (d, 2 H), 3.64 (m, 1 H), 4.20–5.28 (m, 2 H), 8.50 (d, 1 H), 8.61 (d, 1 H), 8.84 (s, 1 H)
4	122–123	304 (M^+)	2.65 (t, 2 H), 3.04 (t, 2 H), 8.44 (d, 1 H), 8.58 (d, 1 H), 8.85 (d, 1 H), 12.45 (s, 1 H)

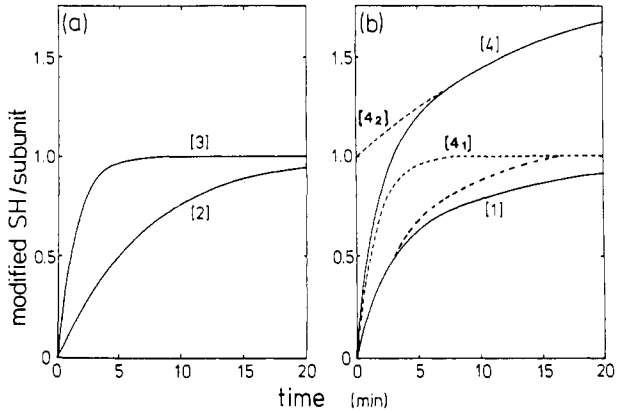

FIGURE 2: Time course of SH modification of phenylalanine hydroxylase with DNPSSR. Purified phenylalanine hydroxylase (4.27 μM) was incubated with 128 μM DNPSSR (1:30); R = $-CH_2CH_3$ (**1**), $-CH_2CH_2OH$ (**2**), $-CH_2CH(OH)CH_2OH$ (**3**), or $-CH_2CH_2COOH$ (**4**). The reaction was monitored by continuous measurement of the release of $DNPS^-$ at 408 nm. The two broken lines for $\mathbf{4}_1$ and $\mathbf{4}_2$ in (b) show the reaction calculated as second-order with $k_1 = 5.0 \times 10^{-3}$ $M^{-1}\cdot min^{-1}$ and $k_2 = 0.44 \times 10^{-3}$ $M^{-1}\cdot min^{-1}$, respectively. The broken line for **1** is calculated as second-order with $k = 1.7$.

Table III: Reactivity of DNPSSR with SH in Phenylalanine Hydroxylase, Given as Apparent Second-Order Rate Constants at pH 8.0 and 30 °C

DNPSSR	R	k ($\times 10^{-3}$ $M^{-1}\cdot min^{-1}$)
1	$-CH_2CH_2$	1.7[a]
2	$-CH_2CH_2OH$	1.1
3	$-CH_2CH(OH)CH_2OH$	5.5
4	$-CH_2CH_2COOH$	5.0,[a] 0.44[a]

[a] See Figure 2b.

constants; these are shown in Table III. The reaction curve with reagent **1** did not fit a linear plot for second-order kinetics; the reaction curve with other reagents did exhibit a linear plot.

Activation of Phenylalanine Hydroxylase by SH Modification. Modification with any of the DNPSSR brought about the activation of phenylalanine hydroxylase, and the final increases in the catalytic activity with each were similar (approximately 10 times). But the time courses of the activations (Figure 3) were quite different, and the activation rates were not proportional to the rates of SH modification. Thus the activation by three different reagents proceeded in the order **3** > **2** > **4**, while the velocity of SH modification was in the order **4** > **3** > **2**. To investigate the relation between the activation and the SH modification in more detail, the data in Figures 2 and 3 were replotted as Figure 4. In all cases, substantial activation was observed only after some portion of the SH group had been modified. The difference between the sigmoidal curves for reagent **2** or **3** and that for reagent **4** seems to suggest that the anionic reagent **4** reacts rapidly (at the rate $k_1 = 5.0 \times 10^{-3}$ $M^{-1}\cdot min^{-1}$; see Figure 2b) with the one SH that involves no activation of the enzyme and slowly (at the rate $k_2 = 0.44 \times 10^{-3}$ $M^{-1}\cdot min^{-1}$) with the one SH involved in enzyme activation, while the neutral reagents **2** and **3** attack only the latter SH. A difference between the activations seen with reagents **2** and **3** was also observed. Phenylalanine hydroxylase partially modified with reagent **2** always showed higher activity than enzyme modified to the same degree with the more hydrophilic reagent (**3**), although both modifications resulted in the same degree of activation when the modification was complete (1 mol of SH/subunit).

Properties of the Modified Enzyme. As the several DNPSSR showed different reactivity for the SH groups in phenylalanine hydroxylase depending on the properties of R,

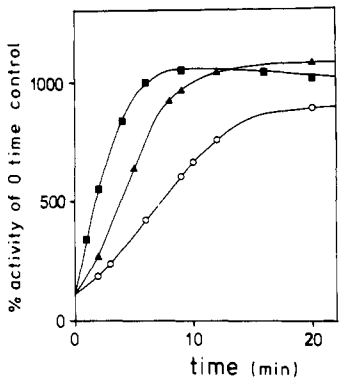

FIGURE 3: Time course of activation of phenylalanine hydroxylase with DNPSSR. The reaction conditions were the same as in the legend of Figure 2. For DNPSSR, R = $-CH_2CH_2OH$ (**2**) (▲), $-CH_2CH(OH)CH_2OH$ (**3**) (■), or $-CH_2CH_2COOH$ (**4**) (○). At each time interval indicated, the hydroxylase activity was measured on a portion (20 μL each) of the reaction mixture in a medium containing 0.2 mM phenylalanine and 40 μM (6*R*)-L-*erythro*-tetrahydrobiopterin.

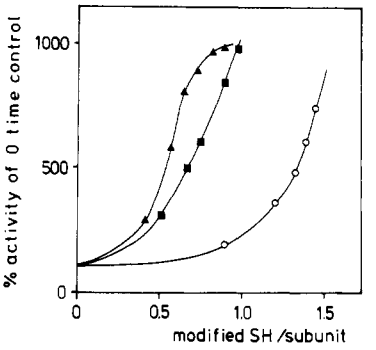

FIGURE 4: Relation between the activation of phenylalanine hydroxylase and the modification with DNPSSR. The data in Figures 2 and 3 were replotted. For DNPSSR, R = $-CH_2CH_2OH$ (**2**) (▲), $-CH_2CH(OH)CH_2OH$ (**3**) (■), or $-CH_2CH_2COOH$ (**4**) (○).

we investigated the properties of the enzyme modified with the neutral reagent (**3**) and compared them with those of enzyme modified with the anionic reagent (**4**). The modified enzymes showed normal Michaelis–Menten-type kinetics with (6*R*)-L-*erythro*-tetrahydrobiopterin as the cofactor (Figure 5a). A marked increase of V_{max} was observed after modification with either reagent **3** or reagent **4**, whereas the K_m value did not change significantly.

As shown in Figure 5b, both disulfide modifications changed the kinetic response to phenylalanine from a sigmoidal type to a hyperbolic one with a remarkable increase of the V_{max} value, and a substrate inhibition was observed on the modified enzymes. Except for the changes in their V_{max} values, no significant differences were observed between the enzymes modified with reagents **3** and **4**. The kinetic constants obtained with the native and with the modified phenylalanine hydroxylases are summarized in Table I.

In the presence of 0.2 mM L-phenylalanine, the native phenylalanine hydroxylase showed higher activity with 500 μM (6*RS*)-6-methyltetrahydropterin, a synthetic cofactor, at a saturating concentration, than with 40 μM (6*R*)-tetrahydrobiopterin, the natural cofactor, and the specific activities were 0.65 and 0.27 $\mu mol\cdot min^{-1}\cdot(mg\ of\ protein)^{-1}$, respectively. SH modification increased the enzyme activity about 2-fold with the synthetic cofactor and about 10-fold with the natural cofactor. Thus, after modification, the specific activity with the natural cofactor became higher than that with the synthetic cofactor.

An increase in the thermal stability of the enzyme was also observed after reaction with reagent **3**. When the native and

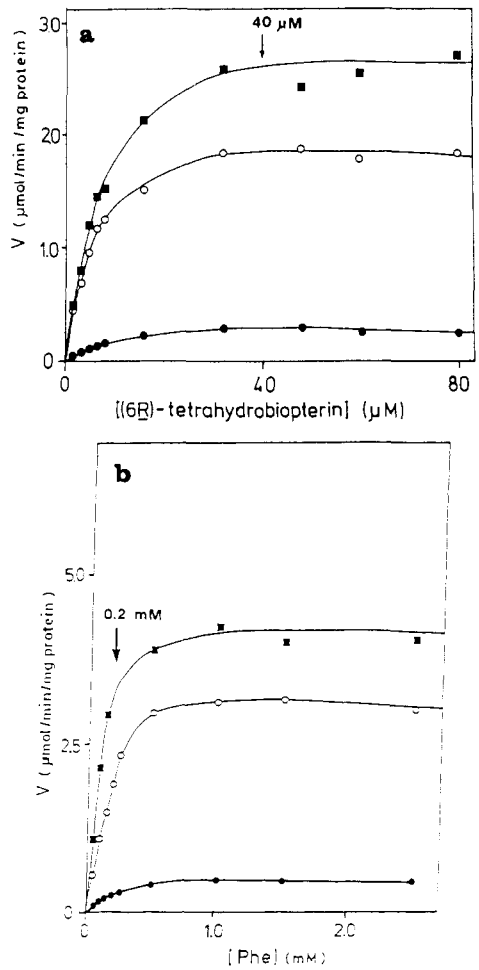

FIGURE 5: Kinetic properties of native or DNPSSR-modified phenylalanine hydroxylase. (a) With respect to (6*R*)-L-*erythro*-tetrahydrobiopterin. Native enzyme (●) was modified at 30 °C for 20 min with DNPSS-$CH_2CH(OH)CH_2OH$ (**3**) (■) or DNPSS-CH_2C-H_2COOH (**4**) (○) as shown in Figure 2. The enzymatic activity was measured at 25 °C for 2 min with 0.2 mM L-phenylalanine as a substrate [at the arrow in (b)]. (b) With resepct to L-phenylalanine. The enzymatic activity was measured at 25 °C for 2 min with 40 μM (6*R*)-L-*erythro*-tetrahydrobiopterin as a cofactor [at the arrow in (a)].

the modified enzymes were incubated at 40 °C, the $t_{1/2}$ for inactivation of native enzyme was 5 min, while the $t_{1/2}$ for inactivation of the modified enzyme was 15 min.

Discussion

The two NO_2 groups at the ortho and para positions of the benzene ring of the asymmetric disulfide DNPSSR impart to the reagent a high reactivity with SH residues as compared with DTNB, which is widely used for the assay of protein SH residues. The thiophenolate anion, $DNPS^-$, liberated quantitatively from DNPSSR by the disulfide exchange reaction, is much more stable to autoxidation to the corresponding disulfide, DNP–SS–DNP, that liberated that from DTNB (Satake and Takahashi, unpublished results). Furthermore, an alteration in the structure of the R group would be expected to give not only a different reactivity to the reagent but also different properties to the modified enzyme.

The SH residues of rat liver phenylalanine hydroxylase were modified by these asymmetric disulfide DNPSSR. The chemical properties of the R group were found to determine at least three things: (1) the rate at which the reagents react with SH groups; (2) which of the five SH groups of phenylalanine hydroxylase are attacked preferentially; (3) the rate at which the putative conformational change of the enzyme takes place. One reactive SH residue, which selectively reacts with the anionic reagent (**4**), may be localized in an electropositive environment. The other reactive SH residue may exist in a hydrophilic environment, since its reactivity to the neutral reagents (**1**–**3**) was increased according to the increase of the hydrophilicity of the reagents, **1** < **2** < **3**.

When the enzyme was partially modified, reagent **2** caused a greater activation than reagent **3**, but after complete modification, a similar degree of activation was observed with the two reagents. This indicates that the process of conformational change is affected by the structure of the R group introduced, although the rate could not be estimated quantitatively.

The reaction with reagent **1** did not conform to second-order kinetics and may be composed of two reactions, fast and slow. This result indicates that the conformational change increases the hydrophilicity of the environment around the target SH residue and also supports the hypothesis that the modification of two subunits of the tetramer is critical in causing the conformational change.

It has been reported that the activation of phenylalanine hydroxylase by modification with NEM is proportional to the incorporation of NEM (Parniak & Kaufman, 1981). This difference, the concordance with NEM, and the discordance with DNPSSR may be accounted for by the different reaction mechanisms governing alkylation (NEM) and disulfide formation (DNPSSR). The reaction rate with NEM was slower than that with DNPSSR. Full activation with DNPSSR took only 10–20 min when a 30-fold more excess of reagent to enzyme was used, whereas full activation with NEM at a 500-fold ratio of reagent to enzyme needed 120 min to modify 1 mol of SH/subunit (Parniak & Kaufman, 1981). Therefore, only the modification step could be the rate-limiting step for the activation with NEM, whereas the activation step can also be rate limiting in the case of modification with DNPSSR.

The modified phenylalanine hydroxylase showed a marked increase in V_{max} (Figure 5, Table I). The change in K_m for both phenylalanine and (6*R*)-L-*erythro*-tetrahydrobiopterin is considered to be a secondary effect of the conformational change to the activated form. The change of a sigmoidal progress curve to a hyperbolic progress curve with respect to the concentration of phenylalanine is similar to that seen upon activation by allosteric effectors (Shiman & Gray, 1980), by limited proteolysis (Fisher & Kaufman, 1973), or by SH modification with NEM (Parnak & Kaufman, 1981). Since enzyme fully modified with reagent **3** could not be further activated when it was preincubated with phenylalanine (data not shown), SH modification and phenylalanine preincubation may result in the same activated conformation of the enzyme. Comparison of enzyme modified with reagent **3** to that modified with reagent **4** indicates that the final state of enzyme modified with either of the two reagents may be similar, because the kinetic response with respect to substrate and cofactor was similar except for the extent of activation (Table I).

Phenylalanine, lysolecithin, and limited proteolysis are known to cause the same type of activation as that observed with DNPSSR, and the activation by limited proteolysis is accompanied by dissociation of the subunit from tetramer to dimer (Fisher & Kaufman, 1973). Recently, rat kidney enzyme isolated in an activated state was shown to have a dimeric structure (Rao & Kaufman, 1986). Considering the tetrameric structure of the liver enzyme, these activations seem to be caused by a disappearance of negative cooperativity between the subunits. The SH modification by DNPSSR may also weaken the subunit interaction and cause a conformational

change of enzyme.

The degree of activation observed with a synthetic cofactor, (6*RS*)-6-methyltetrahydropterin, was less than that with the natural cofactor, (6*R*)-L-*erythro*-tetrahydrobiopterin. Similar results are reported upon activation by phenylalanine (Kaufman & Mason, 1982), phosphorylation (Abita et al., 1976), limited proteolysis (Abita et al., 1984), or chemical modification (Parniak & Kaufman, 1981).

It is not known if phenylalanine hydroxylase is regulated via modification of SH residues in vivo. Since the disulfide formation is likely to occur in vivo, SH groups could have a regulatory role in the activation of phenylalanine hydroxylase in vivo. Furthermore, the stabilization against the thermal denaturation with reagent **3** indicates the possibility of producing a stable enzyme preparation by chemical modification.

In conclusion, a new family of asymmetric thiol–disulfide exchange reagents, DNPSSR, were shown to have considerable potential as active site probes. The reagents were used to further characterize the activation of phenylalanine hydroxylase by SH modification.

ACKNOWLEDGMENTS

We are grateful to Dr. Gordon Guroff (National Institutes of Health, Bethesda, MD) for his kind help in the preparation of the manuscript.

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Determination of the Energetics of the UDP-glucose Pyrophosphorylase Reaction by Positional Isotope Exchange Inhibition†

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Received April 10, 1987

ABSTRACT: A method has been developed for obtaining qualitative information about enzyme-catalyzed reactions by measuring the inhibitory effects of added substrates on positional isotope exchange rates. It has been demonstrated for ordered kinetic mechanisms that an increase in the concentration of the second substrate to add to the enzyme will result in a linear increase in the ratio of the chemical and positional isotope exchange rates. The slopes and intercepts from these plots can be used to determine the partitioning ratios of binary and ternary enzyme complexes. The method has been applied to the reaction catalyzed by UDP-glucose pyrophosphorylase. A positional isotope exchange reaction was measured within oxygen-18-labeled UTP as a function of variable glucose 1-phosphate concentration in the forward reaction. In the reverse reaction, a positional isotope exchange reaction was measured within oxygen-18-labeled UDP-glucose as a function of increasing pyrophosphate concentration. The results have been interpreted to indicate that the interconversion of the ternary central complexes is fast relative to product dissociation in either direction. In the forward direction, the release of UDP-glucose is slower than the release of pyrophosphate. The release of glucose 1-phosphate is slower than the release of UTP in the reverse reaction.

The positional isotope exchange (PIX)[1] technique, first developed by Midelfort and Rose (1976), has become a widely used technique in mechanistic enzymology. The technique can be applied to any enzyme system in which functionally nonequivalent groups become torsionally equivalent via a reaction intermediate or product, thus allowing scrambling of isotopically labeled substituents within a substrate. Traditionally,

† This work was supported by the National Institutes of Health (GM-33874) and the Robert A. Welch Foundation (A-840). F.M.R. is the recipient of NIH Research Career Development Award DK-01366. The authors acknowledge with thanks financial support by the Board of Regents of Texas A&M University.

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[1] Abbreviations: HPLC, high-performance liquid chromatography; PIX(E), positional isotope exchange (enhancement); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-6465$01.50/0