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ADENOSINE 5'-DIPHOSPHATE DIALDEHYDE: AN AFFINITY LABELING REAGENT FOR PHENOL-SULFOTRANSFERASE*

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SUMMARY: Adenosine 5'-diphosphate (5'-ADP) was oxidized with periodic acid to 2'-O-[(R)-formyl(adenin-9-yl)methyl]-3'-diphosphate-3'-deoxy-(S)-glyceraldehyde (ADP-dialdehyde). ADP-dialdehyde, but not 2',3'-acyclic ADP, inhibited phenol-sulfotransferase (PST). The inhibition of PST by ADP dialdehyde was irreversible. A kinetic analysis of the enzyme inactivation suggests the formation of a dissociable enzyme-inhibitor complex prior to the inactivation step. PST could be completely protected from inactivation by the inclusion of 3'-phosphoadenosine-5'-phosphosulfate in the preincubation mixture. These results are consistent with ADP-dialdehyde being an affinity labeling reagent for PST.

The detoxification of xenobiotic phenols and the metabolism of endogenous neurotransmitters (e.g. dopamine, norepinephrine, serotonin, etc.) are dependent on the enzyme phenol-sulfotransferase (PST)(EC 2.8.2.1)(1-4). PST catalyzes the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a phenolic acceptor substrate. Banerjee and Roy (5) have shown that adenosine 3',5'-diphosphate (3',5'-ADP), the product of this sulfate transfer reaction, is a potent, reversible inhibitor of PST. 3',5'-ADP and related nucleotides [e.g. adenosine 5'-diphosphate (5'-ADP)] inhibit PST by competing with PAPS for its binding site (5,6). In an effort to probe the PAPS binding site on PST, we have attempted to develop affinity labeling re-

*The abbreviations used are: 5'-ADP, adenosine 5'-diphosphate; 3',5'-ADP, adenosine 3',5'-diphosphate; ADP-dialdehyde, 2'-O-[(R)-formyl(adenin-9-yl)methyl]-3'-diphosphate-3'-deoxy-(S)-glyceraldehyde; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PST, phenol-sulfotransferase.

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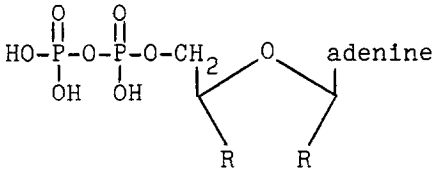
agents by incorporating chemically reactive functional groups into the 5'-ADP structure. Several related ribonucleosides have been converted to affinity labeling reagents by periodate oxidation of the 2',3'-cis diol functionality to form the corresponding ribonucleoside dialdehyde (7-10). An example is 6-methylmercaptopurine ribonucleoside dialdehyde which has been shown to inhibit E. coli DNA dependent DNA polymerase by covalently binding to an ε-amino group of a lysine residue at the enzyme's initiation site (9).

As a possible affinity labeling reagent for PST, we have synthesized 2'-O-[(R)-formyl(adenin-9-yl)methyl]-3'-diphosphate-3'-deoxy-(S)-glyceraldehyde (ADP-dialdehyde) by periodate oxidation of 5'-ADP. The effects of ADP-dialdehyde on PST activity are described in this communication.



ADP dialdehyde R=-CHO
2',3'-acyclic ADP R=-CH_2OH

Materials and Methods

PST was purified from rat liver (male, Sprague-Dawley, 150-175 gr) according to a previously reported procedure (11). The enzyme was purified through the $(NH_4)_2SO_4$ fractionation step resulting in a preparation which contained 33.8 mg of protein per milliliter with a specific activity of 0.25 pmol product formed/mg of protein/min using p-nitrophenol as a substrate. PST activity was determined using [^{35}S] PAPS (New England Nuclear, 1.02-2.03 Ci/mmole) and p-nitrophenol by a modification of a previously described radiochemical assay (12). The assay is based on the transfer of $^{35}SO_4$ from [^{35}S] PAPS to p-nitrophenol, then precipitation of unreacted [^{35}S] PAPS with $Ba(OH)_2$ and $ZnSO_4$, leaving the

product p-nitrophenyl [^{35}S] sulfate in solution. Radioactivity in aliquots (1 ml) of the resulting supernatant were determined by liquid scintillation counting. The results were corrected using appropriate p-nitrophenol blanks. Unlabeled PAPS was prepared using the enzymatic method of Roy (13) and purified by chromatography on Ecteola-cellulose (Sigma) (14).

ADP dialdehyde was prepared by oxidation of 5'-ADP using periodic acid in water (15) and purified by chromatography on a Biogel-2 (Bio-Rad) eluting with water. ADP dialdehyde was reduced to the corresponding 2',3'-acyclic ADP using sodium borohydride. For both ADP dialdehyde and 2',3'-acyclic ADP the nmr spectral data were consistent with the assigned structures.

A typical inactivation experiment was carried out in a total volume of 1.0 ml containing 5 mM phosphate buffer, pH 7.4 (DTT concentration = 1.0 mM), variable concentrations of ADP dialdehyde and the enzyme preparation. The preincubation step was started by the addition of enzyme and incubation was carried out at 28°. In protection experiments, varying amounts of PAPS, 3',5'-ADP or p-nitrophenol were included during the preincubation. After the appropriate preincubation time, aliquots were removed and assayed for residual enzyme activity. The percent activity remaining at any given time was calculated relative to zero-time activity. The pseudo-first order kinetic constants of inactivation, K_{app}, were calculated from the slopes of the plots of log percentage activity remaining vs. preincubation time (16,17).

Results and Discussion

As shown in Table 1, ADP dialdehyde was found to be a potent, irreversible inhibitor of the PST catalyzed reaction. The irreversible nature of this inhibition is evident from the fact that the activity could not be recovered after dialysis. In contrast,

Table 1

Effect of 5'-ADP, ADP Dialdehyde and 2',3'-Acyclic ADP on PST Activity[a]

Compound	Inhibition %[b]	
	Before Dialysis	After Dialysis
5'-ADP	32	0
ADP Dialdehyde	99	99
2',3'-Acyclic ADP	8	0

[a]The standard preincubation mixture consisted of the inhibitor (4.0 mM); dithiothreitol (1 mM); phosphate buffer, pH 7.4 (5 mM); and the enzyme preparation in a total volume of 1.0 ml. The preincubation was carried out for 30 min at 28°. An aliquot (100 µl) of the preincubation mixture was then assayed for PST activity as described in the text. Another aliquot was dialyzed against phosphate buffer, pH 7.4 (5 mM) for 2 hours and then assayed for residual enzyme activity.

[b]The standard assay mixture contained the following components (in µmole): pH 6.4, phosphate buffer (5), so that the final volume was 1.0 ml; p-nitrophenol (0.05); 0.7 µCi of [^{35}S] PAPS; and a 100 µl aliquot of the preincubation mixture. Incubation was carried out for 5 min at 37°. The unreacted [^{35}S] PAPS was precipitated with $Ba(OH)_2$ and $ZnSO_4$. After centrifugation, an aliquot (1 ml) of the supernatant was removed and the radioactivity determined. % Inhibition was calculated using as a control a preincubation mixture treated under identical conditions except no inhibitor was present.

both 5'-ADP and 2',3'-acyclic ADP showed weaker inhibitory activities, which were totally reversible upon dialysis. When ADP dialdehyde was preincubated with the enzyme for varying time periods, a time dependent loss of PST activity was observed. The time course for inactivation of PST by various concentrations of ADP dialdehyde is presented in Figure 1. The loss of PST activity follows pseudo-first order kinetics and the rate of inactivation is dependent upon the concentration of ADP dialdehyde. In contrast, 5'-ADP or 2',3'-acyclic ADP didn't produce a similar time dependent loss of enzyme activity. These results suggest that the

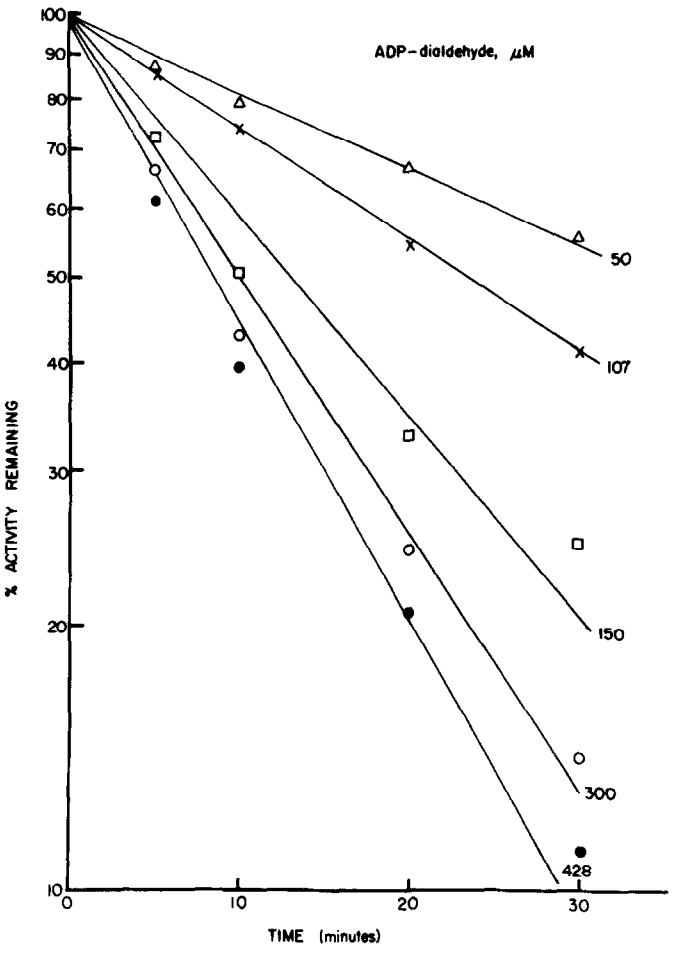


Figure 1: Effect of ADP-dialdehyde concentration on the the rate of inactivation of PST. Activity remaining after the appropriate preincubation time was determined as described in the text. The pseudo-first order rate constants of inactivation, K_{app}, were calculated from the slopes for each concentration of inhibitor.

aldehydic functionalities of ADP dialdehyde play a crucial role in the irreversible inactivation of PST.

One of the criteria of an affinity labeling reagent is that it exhibit a saturation effect on the rate of inactivation of the

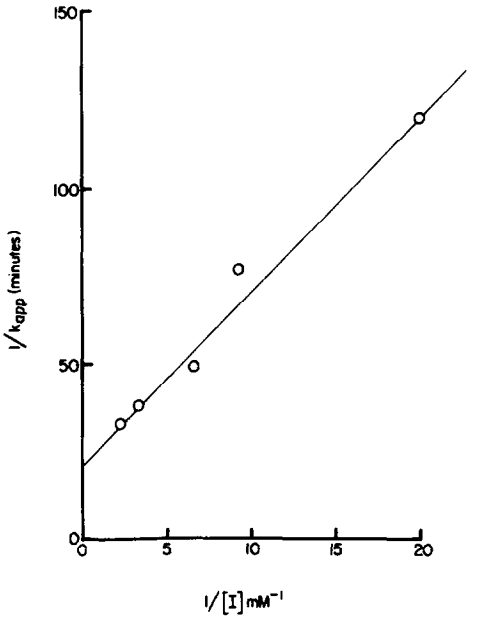


Figure 2: Double reciprocal plot of the pseudo-first order rate constant of inactivation, K_{app}, vs ADP dialdehyde concentration. k_2 and K_I were calculated from the y-intercept and slope respectively, using least squares method.

enzyme (18). Such a rate saturation effect suggests the inactivation occurs via a unimolecular reaction with a dissociable complex rather than via a non-specific bimolecular reaction. The model for a mechanism involving a dissociable complex is shown in eq. 1 and 2, where E•I is the reversible complex, E-I the inactive enzyme, K_I the steady state constant of inactivation (K_I = [E][I]/[E•I]) and k_2 the first order rate constant (16). For a system to fit this model, reciprocal plots of $1/k_{app}$ vs $1/[I]$ should be linear with the y intercept = $1/k_2$ and the slope = K_I/k_2.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\bullet I \xrightarrow{k_2} E\text{-}I \qquad (1)$$

$$\frac{1}{k_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \qquad (2)$$

Table 2

Substrate Protection of PST from Inactivation by ADP-Dialdehyde

Additions, μM[a]			%
PAPS	3',5'-ADP	p-nitrophenol	Residual Activity After 30 min, 28°[b]
--	--	--	14
50	--	--	75
205	--	--	100
--	50	--	85
--	--	1,000	49

[a]The standard preincubation mixture consisted of ADP dialdehyde (283 μM); phosphate buffer, pH 7.4 (5 mM); and the enzyme preparation in a total volume of 0.3 ml. The preincubation was carried out for 30 min at 28° after which residual PST activity was determined as described in Table 1.

[b]PST activity remaining was calculated using as a reference a preincubation mixture treated under similar conditions except ADP dialdehyde was not present.

When the data for PST inactivation by ADP dialdehyde (Figure 1) was treated in this manner, a linear relationship was observed as shown in Figure 2. The positive intercept on the abscissa indicates a saturation effect on the rate of inactivation of PST by ADP dialdehyde. From the data shown in Figure 2 the steady-state constant of inactivation K_I = 246 μM and the limiting rate constant of inactivation, k_2 = 0.0496 min^{-1} were calculated. The linearity and positive intercept observed in this reciprocal plot provide evidence for the formation of a dissociable enzyme inhibitor complex prior to enzyme inactivation.

Another criteria for an affinity labeling reagent is substrate or competitive inhibitor protection of the enzyme from inactivation (18). The results of substrate protection experiments are shown in Table 2. PST can be protected from inactivation by

ADP dialdehyde if PAPS, the sulfate donor, or 3',5'-ADP, a competitive inhibitor, are included in the preincubation mixture. Addition of very high concentrations of p-nitrophenol, the sulfate acceptor, to the preincubation mixture provides only partial protection of the enzyme from inactivation.

The results of these experiments suggest that ADP dialdehyde is an affinity labeling reagent for PST. The mechanism of enzyme inactivation would appear to involve the initial binding of ADP-dialdehyde to the PAPS binding site via a dissociable complex. This initial binding would be followed by covalent bond formation between the aldehydic functionalities of ADP dialdehyde and an amino acid residue in the proper juxtaposition at the enzyme's active site. Studies are presently underway to identify the modified amino acid residue on PST.

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