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Pyridoxal 5′-phosphate binds to a lysine residue in the adenosine 3′-phosphate 5′-phosphosulfate recognition site of glycolipid sulfotransferase from human renal cancer cells

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In the course of characterization of glycolipid sulfotransferase from human renal cancer cells, the manner of inhibition of sulfotransferase activity with pyridoxal 5′-phosphate was investigated. Incubation of a partially purified sulfotransferase preparation with pyridoxal 5′-phosphate followed by reduction with $NaBH_4$ resulted in an irreversible inactivation of the enzyme. When adenosine 3′-phosphate 5′-phosphosulfate was co-incubated with pyridoxal 5′-phosphate, the enzyme was protected against this inactivation. Furthermore, pyridoxal 5′-phosphate was found to behave as a competitive inhibitor with respect to adenosine 3′-phosphate 5′-phosphosulfate with a K_i value of 287 μM. These results suggest that pyridoxal 5′-phosphate modified a lysine residue in the adenosine 3′-phosphate 5′-phosphosulfate-recognizing site of the sulfotransferase.

Keywords: sulfatides, sulfotransferase, pyridoxal 5′-phosphate, protein modification

Introduction

In human renal cancer cells the elevation of glycolipid sulfotransferase activities is associated with an increment in sulfoglycolipids [1, 2]. To elucidate the mechanism of the elevation in sulfotransferase activity, characterization of the enzyme in human renal cancer cells is indispensable.

Recently, purifications of glycolipid sulfotransferases from rat kidney [3], rat testis [4] and mouse brain [5] were reported by three groups. However, their properties, including molecular masses and the manner of regulation of enzyme activities differed.

Pyridoxal 5′-phosphate (PLP) has been frequently used as a protein modifier binding to the amino group of lysine residues with the formation of the Schiff base. For instance, PLP binds covalently to the phosphate-binding sites of many enzymes and irreversibly inactivates them (reviewed in [6]). In the course of characterization of human renal cancer-cell glycolipid sulfotransferase, we surveyed various adenosine 3′,5′-diphosphosulfate (PAPS) analogues with respect to their inhibitory activities against the enzyme. Among them, PLP was found to inhibit the sulfotransferase activity as observed in the mouse brain enzyme [7]. However, the fine mechanism of the inhibition by PLP has not been investigated. Here, we describe how PLP inhibits human renal cancer-cell glycolipid sulfotransferase.

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Materials and methods

Materials

ATP, adenosine 5′-phosphosulfate (APS), adenosine 3′,5′-diphosphate (PAP) and PAPS were purchased from Sigma. [^{35}S]PAPS (~2 Ci mmol^{-1}) was from New England Nuclear. AMP and ADP were from Oriental Yeast (Tokyo, Japan) and Yamasa Shoyu (Chiba, Japan), respectively. PLP was obtained from Seikagaku Kogyo (Tokyo, Japan). DE-52 was from Whatman. DEAE Sephadex A-25 and Heparin-Sepharose CL-6B were from Pharmacia. Adenosine diphosphopyridoxal (AP_2PL) [8] was a generous gift from Dr T. Fukui (Osaka University, Japan). Galactosylceramide (GalCer) was prepared in our laboratory from bovine brain.

Preparation of PAPS

PAPS obtained from Sigma was further purified by reverse-phase paired-ion HPLC as described previously [9]. Separation was carried out on liquid chromatography equipment (LC9A and SPD-6A, Shimadzu Assoc., Japan) with a 4.6 × 250 mm Wakosil 5C18-200 (Wako Pure Chemical, Japan) column. AMP, ADP, ATP, APS, PAP and PAPS were dissolved in the same buffer as described [9], and were applied on the HPLC column with a flow rate of 0.8 ml min^{-1}, after which the absorbance at 254 nm was monitored. The retention times of AMP, ADP, ATP, APS, PAP and PAPS were 5.6, 6.8, 8.4, 7.2, 8.0 and 10.2 min, respectively.

Enzyme preparation

SMKT-R3 cells [10] were cultured and harvested as described [2]. The cells were re-suspended in the same volume of Tris-buffered saline, an equal volume of 2 × solubilization buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 2% Lubrol PX, 40% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4) was added and the cells were sonicated for 10 min on ice. The solution was then centrifuged at 100 000 × **g** for 1 h and the supernatant was dialysed against buffer A (10 mM triethanolamine-HCl, 10% glycerol, 0.05% Lubrol PX, and 5 mM $MnCl_2$, pH 7.0). The dialysate was applied on a DE-52 column which had been equilibrated with buffer A and the pass-through fractions were subjected to a Heparin-Sepharose column. After washing the column with buffer A, GalCer sulfotransferase was eluted with buffer B (20 mM triethanolamine-HCl, 20% glycerol, 0.1% Lubrol PX, 10 mM $MnCl_2$, and 0.2 M NaCl, pH 7.0). The effluent was stored at −20 °C and used as the enzyme source for the following experiments. Intrinsic GalCer and lactosylceramide (LacCer) associated with the crude enzyme preparation were removed by Heparin-Sepharose chromatography.

Enzyme assay

PAPS:GalCer sulfotransferase activity was assayed as described previously [11] with a slight modification. The reaction mixture contained 5 nmol GalCer, 0.5 μmol $MnCl_2$, 1 nmol [^{35}S]PAPS (100 cpm $pmol^{-1}$), 0.5 mg Lubrol PX, 12.5 nmol dithiothreitol, 0.25 μmol NaF, 0.1 μmol ATP, 20 μg BSA and enzyme protein in 25 mM Na cacodylate, pH 6.4, in a total volume of 50 μl. One unit of the activity is defined as the amount of enzyme that transferred 1 μmol of sulfate per minute.

Inactivation of the sulfotransferase by PLP or AP_2PL

The GalCer sulfotransferase preparations were incubated with various concentrations of PLP or AP_2PL in a mixture containing 25 mM HEPES-NaOH, pH 7.0, 0.15 M NaCl and 20% glycerol for 60 min at 4 °C. Reductive fixation of reagents to the enzyme was performed by treatment with 1 mg ml^{-1} $NaBH_4$ for 15 min at 0 °C. Modified enzyme preparations were dialysed against 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 20% glycerol overnight and the residual enzyme activity was assayed. For the experiments on protection against inactivation, reaction mixtures were supplemented with PAPS or GalCer during the PLP or AP_2PL treatment.

Effects of PLP, AP_2PL, 5'-AMP, 3'-ADP or ATP on the sulfotransferase activity

The sulfotransferase activities were assayed with various concentrations of PAPS in the presence of fixed concentrations of PLP, AP_2PL, 5'-AMP, 3'-AMP, ADP or ATP, which had been neutralized with NaOH. The apparent K_m and V_{max} values were calculated by the direct linear plot of Eisenthal and Cornish-Bowden [12] and the results were presented by Lineweaver-Burk plots.

Results

Inactivation of GalCer sulfotransferase by PLP

Irreversible inactivation of GalCer sulfotransferase from SMKT-R3 cells was achieved when the enzyme was incubated with PLP followed by reduction with $NaBH_4$. Inactivation of the sulfotransferase depended on the concentration of PLP (Fig. 1, closed circles). After reduction, the enzyme activity was not reversible by dialysis. $NaBH_4$ treatment alone had no effect on the enzyme activity. PLP and AP_2PL, reduced prior to incubation, could not inactivate the sulfotransferase

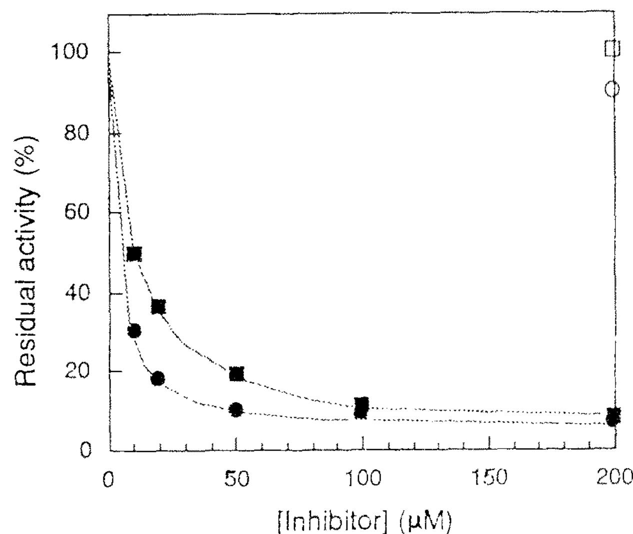

Figure 1. Effects of PLP- and AP_2PL-treatment on GalCer sulfotransferase activity. The enzyme, 0.15 mU, was incubated with various concentrations of PLP (●) or AP_2PL (■), followed by reduction and dialysis and assayed as described under Materials and methods. PLP (○) and AP_2PL (□), reduced prior to incubation, did not inactivate the enzyme activity.

(Fig. 1, open circle and open square). This means that covalent binding through the aldehyde groups is essential for the inactivation of the enzyme. A significant reduction in GalCer sulfotransferase activity by AP_2PL was also observed (Fig. 1, closed squares), whereas a higher concentration of AP_2PL was necessary to inactivate the enzyme to the same extent as PLP.

Protection of the sulfotransferase against PLP- or AP_2PL-inactivation by PAPS

To elucidate which substrate-binding site of the sulfotransferase PLP binds to, the effects of PAPS and GalCer on the PLP- or AP_2PL-inactivation were examined. Addition of PAPS at a concentration of 0.5 μM resulted in significant protection against the inactivation caused by 200 μM PLP or AP_2PL (Fig. 2). The inactivation by AP_2PL was more easily prevented than that by PLP, indicating that AP_2PL was a less potent inhibitor. In contrast, GalCer did not protect the enzyme against inactivation (Fig. 3). These results indicate that PAPS occupied the PAPS-recognizing site and neither PLP nor AP_2PL could reach the lysine residue in the site or that a conformational change caused by the binding of PAPS covered the lysine residue on which PLP and AP_2PL acted.

Effect of AMP, ADP or ATP on the sulfotransferase activity

To further define the interactions of PLP and AP_2PL with the sulfotransferase, the effects of PAPS analogues on the sulfotransferase activity were studied. In the case of oestrogen sulfotransferase, ADP was shown to be a noncompetitive inhibitor with respect to PAPS, but 3′-AMP was a competitive inhibitor [13]. Therefore, we examined whether such adenine nucleotides could inhibit GalCer sulfotransferase activity. Contrary to expectations, 3′-AMP, 5′-AMP, ADP and ATP decreased the apparent K_m value for PAPS and the V_{max} value (Fig. 4, Table 1). Although the reason why they facilitated the access of PAPS to the enzyme remains to be solved, we

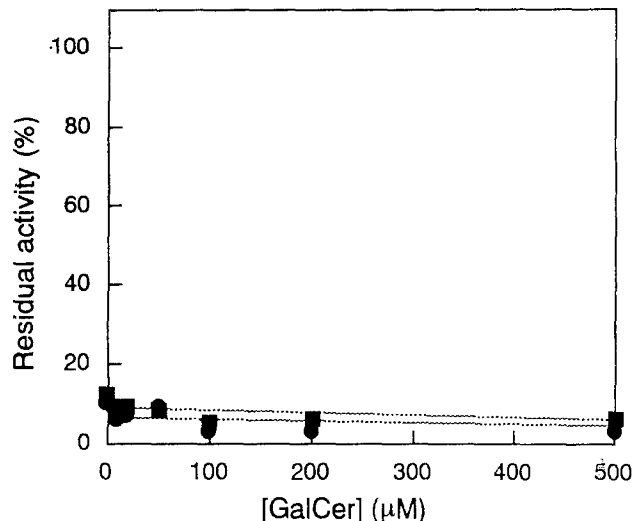

Figure 3. Protection of the sulfotransferase against PLP- or AP_2PL-inactivation by GalCer. The enzyme, 0.15 mU, was incubated in the presence of a 0.2 mM final concentration of PLP (●) or AP_2PL (■) and indicated concentrations of GalCer before $NaBH_4$ reduction. After dialysis, the activity remaining was determined.

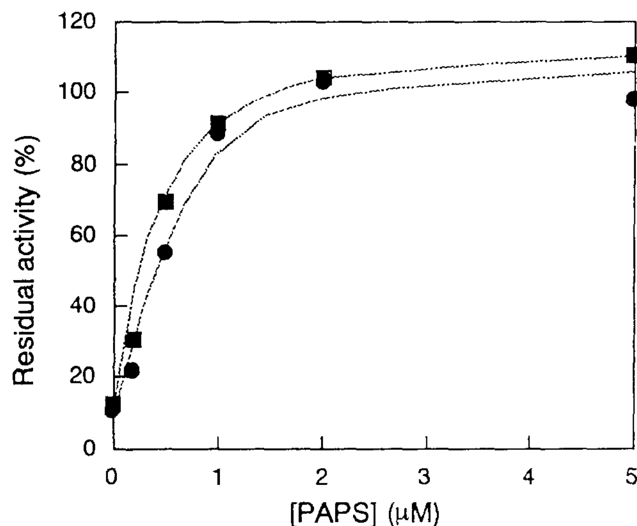

Figure 2. Protection of the sulfotransferase against PLP- or AP_2PL-inactivation by PAPS. The enzyme, 0.15 mU, was incubated in the presence of a 0.2 mM final concentration of PLP (●) or AP_2PL (■) and indicated concentrations of PAPS before $NaBH_4$ reduction. After dialysis, the activity remaining was determined.

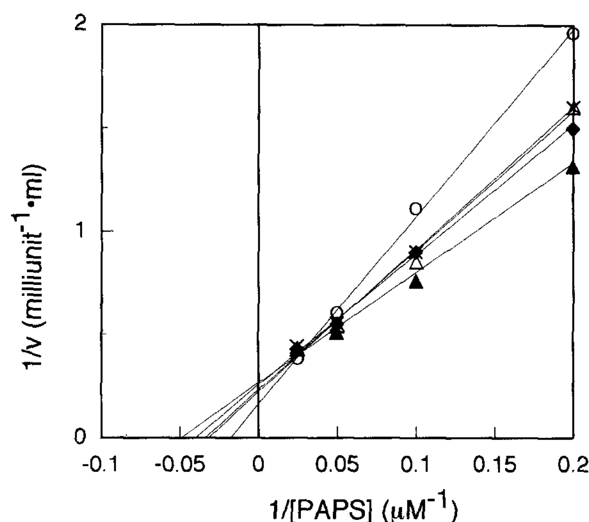

Figure 4. Effect of adenine nucleotides on the sulfotransferase. Enzyme reactions were carried out using standard reaction mixtures containing no ATP and various concentrations of PAPS, supplemented without (○) or with 1 mM 3′-AMP (△), 5′-AMP (×), ADP (▲) or ATP (◆).

Table 1. Effects of 3'-AMP, 5'-AMP, ADP and ATP on the K_m value for PAPS and the V_{max} value.

	Apparent K_m for PAPS (μM)	V_{max} ($mU ml^{-1}$)
None	74	6.7
3'-AMP	32	4.1
5'-AMP	36	4.3
ADP	22	3.5
ATP	28	3.6

employed ATP in the standard reaction mixture to save PAPS.

Effects of PLP and AP$_2$PL on the sulfotransferase activity

Figure 5 shows Lineweaver-Burk plots of PAPS-saturation curves in the presence or absence of PLP and AP$_2$PL, in the presence of 2 mM ATP. All lines crossed at the same *y* intercept, indicating that PLP and AP$_2$PL behave as competitive inhibitors with respect to PAPS. When a similar kinetic study was performed in the absence of ATP, PLP also inhibited the sulfotransferase activity competitively against PAPS (data not shown). On the basis of the data in Fig. 5, the apparent K_m value for PAPS was calculated to be 28 μM, and the K_i values for PLP and AP$_2$PL were determined to be 287 and 1170 μM, respectively. On the other hand, PAP also inhibited the enzyme activity competitively with PAPS and the K_i value was 52.6 μM (data not shown). These observations confirmed that PLP and AP$_2$PL bound to the PAPS-recognizing site of GalCer sulfotransferase.

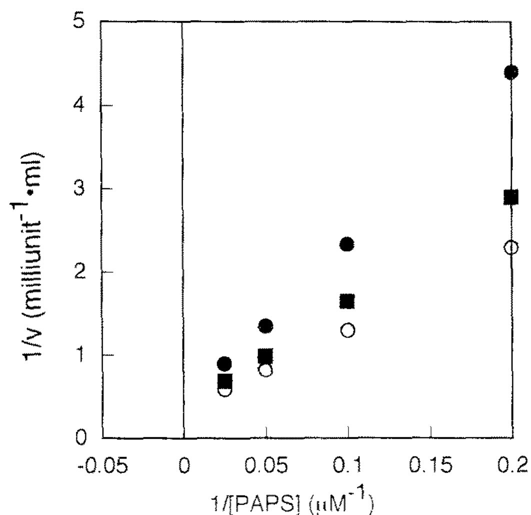

Figure 5. Inhibition of the sulfotransferase by PLP or AP$_2$PL. Standard reaction mixtures containing 2 mM ATP and various concentrations of PAPS were utilized in the absence (○) or presence of 250 μM PLP (●) or AP$_2$PL (■).

Discussion

The present paper suggests the presence of a catalytically essential lysine residue associated with binding of PAPS to GalCer sulfotransferase from SMKT-R3 cells. Incubation of the enzyme with PLP followed by $NaBH_4$ reduction resulted in its irreversible inactivation. Although AP$_2$PL had been synthesized to be a more specific and effective binding reagent to the adenine nucleotide-binding site than PLP [8], the affinity of AP$_2$PL towards human renal cancer-cell GaCer sulfotransferase was lower. The 3'-phosphate attached to adenosine, existing in PAP and PAPS, may be essential for the binding. Alternatively, AP$_2$PL may be too bulky to access the PAPS-binding site of the sulfotransferase. AP$_2$PL is reported to be a poor inhibitor in terms of potency and specificity to the ATP-binding site of rabbit muscle pyruvate kinase and of beef heart mitochondrial F1-ATPase [14].

In this study, ATP, ADP and AMP modulated the sulfotransferase activity, decreasing the apparent K_m value for PAPS and the V_{max} value. The ATP requirement of glycolipid sulfotransferase of rat brain was demonstrated previously [15] and it is suggested that ATP is utilized to convert PAP back to PAPS or to prevent PAPS-degrading enzymes from attaining an active form. Glycolipid sulfotransferase of rat testis is stimulated by phosphorylation with a testicular protein kinase [4] and cAMP dependent protein kinase [16]. Mouse brain sulfotransferase is also suggested to be phosphorylated and stimulated by ATP and Pi [7]. However, since ADP and AMP also demonstrated effects similar to that of ATP in the present study, it is unlikely that phosphorylation was involved in the effect of ATP observed in this study. Although PAP inhibited the sulfotransferase competitively with respect to PAPS, neither 3'-AMP nor 5'-AMP competed with PAPS. These results suggest that both 3'- and 5'-phosphate in the ribose of adenosine are required to be recognized at the PAPS-binding site. There is a possibility that glycolipid sulfotransferase has another nucleotide-binding site to which AMP, ADP and ATP are all able to bind and that their binding stimulates the binding of PAPS to the specific site. Alternatively, the adenine nucleotides may not act on the enzyme directly and a contaminant protein may behave as a modulator in their presence. This possibility must be examined by using a purified sulfotransferase.

In the future, characterization of lysine residues involved in the interaction with PLP should provide information on the PAPS-binding site of the sulfotransferase.

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