[Chem. Pharm. Bull. 31(12)4565—4567(1983)]

p-Nitrophenol Sulfate Conjugation with Substrate Inhibition in Rat Liver Cytosol Fraction

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(Received April 19, 1983)

p-Nitrophenol (PNP) sulfate conjugation in rat liver cytosol fraction was investigated over a wide PNP concentration range (1.25 μM—5 mM). The PNP sulfate synthesis rate in the liver cytosol decreased in the low PNP concentration range (10—500 μM) and increased again at concentrations above approximately 500 μM, as was previously found in isolated liver cells (*J. Pharm. Dyn.*, **5**, 811 (1982)). This apparent substrate at low PNP concentration was found in the sulfotransferase reaction when the cosubstrate, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), had been generated in advance as well as in the overall reaction (*i.e.*, including the generation process of PAPS), suggesting that the inhibition was due to direct interaction between PNP and aryl sulfotransferase, and not to inhibition of PAPS generation by PNP.

Keywords——*p*-nitrophenol; sulfate conjugation; rat liver cytosol fraction; aryl sulfotransferase; substrate inhibition; 3′-phosphoadenosine-5′-phosphosulfate

The sulfate conjugation reaction is considered to be a major route of detoxication for substrates bearing a hydroxyl or phenolic functional group, together with the glucuronide conjugation reaction. Banerjee and Roy reported kinetic studies on the separated aryl sulfotransferase from guinea-pig liver using *p*-nitrophenol (PNP) as a substrate.[1,2] Sekura and Jakoby,[3,4] and Duffel and Jakoby,[5] noted the multiplicity of aryl sulfotransferase and succeeded in the separative purification of the enzyme into four types.

On the other hand, the authors found that PNP glucuronidation in isolated rat liver cells showed Michaelis–Menten type kinetics, but the sulfation rate decreased at 10—200 μM PNP.[6] The possible mechanisms for such apparent substrate inhibition in the isolated liver cells were considered to be as follows: (1) direct interaction between PNP and aryl sulfotransferase, (2) decrease of cell viability caused by PNP (this was ruled out in the previous paper[6]), (3) deficiency of the cosubstrate in sulfation, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), caused by the inhibition of biosynthesis. In the present paper, the PNP sulfate conjugation in liver cytosol fraction was studied in order to clarify the actual inhibition mechanism in the isolated liver cells.

Experimental

Materials——PNP, monopotassium salt of PNP sulfate and disodium salt of adenosine-5′-triphosphate (ATP) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Preparation of Liver Cytosol Fraction——Male Wistar rats (255—300 g) were purchased from Matsumoto Animals Laboratory, Chiba, Japan. The liver was freshly excised in the same manner as in Baur *et al.* 's preparation method for isolated liver cells,[7] and homogenized in 0.154 M cold KCl solution using a Teflon-glass homogenizer to give a 10% (w/v) homogenate. The homogenate was centrifuged at 10000*g* for 10 min and the resulting supernatant was further centrifuged at 105000*g* for 60 min. The final supernatant was diluted four times with 0.4 M Tris-HCl buffer (pH 7.4) to make a cytosol fraction.

Conditions of Sulfate Conjugation Reaction——The overall reaction and the sulfotransferase reaction according

to a modification of Wong's procedure[8] were carried out. (i) Overall Reaction: After the incubation of the cytosol fraction (2 ml) prepared as above for 5 min at 37 °C, the reaction was started by adding 2 ml of the reaction medium containing 12.5 mM ATP, 12.5 mM Na_2SO_4 and 12.5 mM $MgCl_2$ in 0.4 M Tris-HCl buffer (pH 7.4) and simultaneously adding 1 ml of 0.4 M Tris-HCl buffer solution (pH 7.4) of PNP. The reaction was terminated by the addition of 25% (v/v) $HClO_4$ (1 ml) after 5 min. (ii) Sulfotransferase Reaction: After the incubation of the cytosol fraction (2 ml) for 5 min at 37°C, PAPS was first generated by adding 1 ml of the medium containing 15 mM ATP, 15 mM Na_2SO_4 and 15 mM $MgCl_2$ in 0.4 M Tris-HCl buffer (pH 7.4) and by extending the incubation time to 1 h. The generation reaction was stopped by adding 1 ml of 50 mM ethylenediaminetetraacetic acid. Thereafter the sulfotransferase reaction was initiated by adding 1 ml of PNP solution and terminated by adding 1 ml of 25% (v/v) $HClO_4$ after 3 min. The sulfotransferase reaction at pH 5.6 was performed in 0.1 M sodium acetate–acetic acid buffer in place of 0.4 M Tris-HCl buffer.

Assay for PNP Sulfate——The reaction solution treated with 25% (v/v) $HClO_4$ (containing 1.34 mM *p*-fluorophenol as an internal standard) was centrifuged for 10 min at 3000 rpm and the supernatant was used for the reversed-phase high performance liquid chromatography assay reported by Machida *et al.* for the separative determination of PNP glucuronide and sulfate.[6,9] The composition of the mobile phase was slightly changed to water–methanol–acetic acid (68.5 : 30 : 1.5, v/v/v) containing 0.1 g/l KNO_3 and 13 mg/l tetrabutylammonium bromide. The capacity factors (k' values) of PNP and PNP sulfate were 5.1 and 2.5, respectively.

Results

Figures 1a and b show the results in the overall reaction and sulfotransferase reaction at low PNP concentration (1.25—100 μM) and at high PNP concentration (150 μM—5 mM), respectively. In the overall reaction including the PAPS generating process, a decrease of PNP sulfate conjugation rate was observed at more than 5 μM PNP (Fig. 1a) but the rate increased again at more than 500 μM PNP (Fig. 1b). In the sulfotransferase reaction with PAPS previously generated, the rate *vs.* PNP concentration profile was similar, though the rates were much larger than in the overall reaction.

Discussion

Since the inhibition of PNP sulfate conjugation observed in isolated liver cells[6] was also found in both the overall reaction and the sulfotransferase reaction using liver cytosol fraction, such inhibition is considered to be due to the interaction between PNP and aryl sulfotransferase.

Banerjee and Roy studied PNP sulfate conjugation at pH 5.6 by aryl sulfotransferase

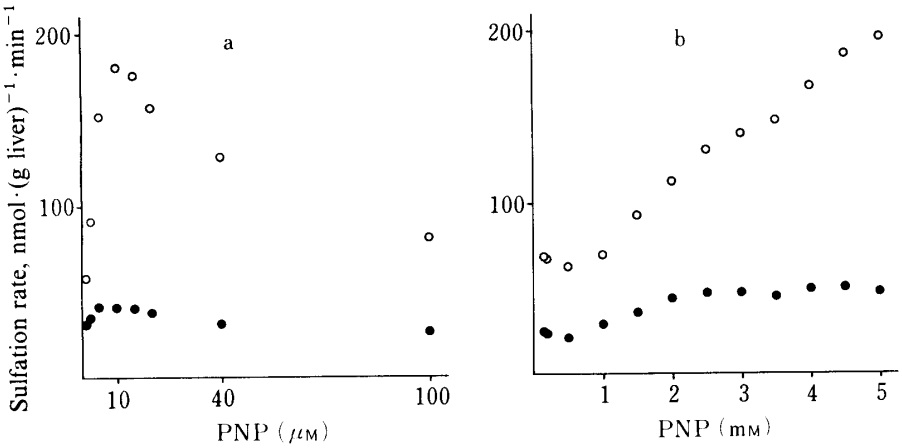

Fig. 1. PNP Sulfate Conjugation Rates at Low (a) and High (b) Concentration of PNP in the Overall Reaction (Closed Circles) and Sulfotransferase Reaction (Open Circles)

The results are those of a representative experiment. Reaction conditions are described in detail in Experimental.

partially purified from guinea-pig liver but did not observe such an inhibition reaction at less than 100 μM PNP.[2] However, we found in the present study that the PNP concentration at which the inhibition starts to occur is dependent on pH and that this concentration (approximately 10 μM at pH 7.4) shifts to 200 μM at pH 5.6 (not shown in figure). Considering the species difference of aryl sulfotransferase as well as the above finding, the inhibition found in the present study is not necessarily in conflict with Banerjee and Roy's report.[2] Similar inhibition of the sulfotransferase reaction (pH 7.4) was recently found to be caused by 7-hydroxycoumarin, 2-hydroxybiphenyl and 4-hydroxybiphenyl in the cytosol fraction of guinea-pig intestinal cells.

Consequently, it was concluded that the substrate inhibition of PNP sulfate conjugation observed in rat liver cells[6] was caused by the interaction between PNP and aryl sulfotransferase in the cytosol (mechanism (1) cited in the introduction). The increase of the PNP sulfate conjugation rate observed after the inhibition might be due to the multiplicity of aryl sulfotransferase recently reported.[3,4]

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