RESEARCH COMMUNICATION

Acetylation of *p*-aminobenzoylglutamate, a folic acid catabolite, by recombinant human arylamine *N*-acetyltransferase and U937 cells

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N-acetyl-*p*-aminobenzoylglutamate is a major urinary metabolite of folic acid. It is formed by acetylation of *p*-aminobenzoylglutamate following cleavage of the C₉–N₁₀ bond of folic acid. Using recombinant human type 1 (NAT1) and type 2 (NAT2) arylamine *N*-acetyltransferase, we have shown that *p*-aminobenzoylglutamate is a specific NAT1 substrate. At an acetyl-CoA concentration of 50 μ M, the *K_m* for *p*-aminobenzoylglutamate (pABG) acetylation by recombinant NAT1 was 130 $\pm$ 13 μ M. For the human pro-monocytic cell-line U937, the apparent *K_m* was slightly higher (333 $\pm$ 17 μ M). Inhibitor studies supported NAT1-dependent acetylation of pABG by U937 cell cytosols. These studies are the first to identify a potential endogenous substrate for human NAT1 and suggest that this enzyme may be important in the cellular clearance of pABG.

U.S.A. *N*-Ac-pABG was synthesized by the method of Baker et al. [11]. The structure of the product was confirmed by NMR and purity (> 99%) was determined by HPLC. rNAT1 and rNAT2 were expressed in monkey kidney COS-1 cells as previously described [12]. For rNAT1, the enzyme activity was 1400 nmol·min⁻¹·mg protein⁻¹ [*p*-aminobenzoic acid (1 mM) as substrate; acetyl-CoA concentration = 500 μ M] and for NAT2, the enzyme activity was 36 nmol·min⁻¹·mg protein⁻¹ [sulphamethazine (1 mM) as substrate; acetyl-CoA concentration = 500 μ M]. All other reagents were of the highest quality available.

INTRODUCTION

In humans, acetylation of arylamines is catalysed by two closely related enzymes, *N*-acetyltransferase type 1 (NAT1) and *N*-acetyltransferase type 2 (NAT2). The substrate specificity and tissue distribution of the two enzymes differ considerably despite an 87% sequence homology [1,2]. A notable difference between the two isoenzymes is the presence of NAT1 in fetal and neonatal tissue [3,4]. By contrast, NAT2 is not evident until about 12 months after birth [5]. NAT1 also is present in human placenta [6]. These differences suggest a role for NAT1 in addition to the metabolism of xenobiotics. However, to date, no candidate endogenous substrate has been reported.

Several studies have shown that NAT1 is inhibited by folic acid [7,8] although the intracellular concentration is much lower than that required to inhibit the enzyme *in vitro* [8]. Folic acid is an essential vitamin in humans and may be important in the prevention of neural tube defects as well as a variety of other malformations in developing embryos [9]. During the second and third trimester of pregnancy, folic acid requirement increases significantly due to a higher rate of folate turnover [10]. Urinary excretion profiles in pregnant and non-pregnant women show that folic acid is catabolized to *p*-aminobenzoylglutamate (pABG) by cleavage of the C₉–N₁₀ bond. pABG is further metabolized by *N*-acetylation to *N*-acetyl-*p*-aminobenzoylglutamate (*N*-Ac-pABG) which is the major folate urinary metabolite. These data suggest that pABG may be an endogenous substrate for arylamine *N*-acetyltransferase.

In this paper, we have used recombinant human NAT1 (rNAT1) and rNAT2 as well as U937 cells to investigate the acetylation of pABG. We show that pABG is an NAT1-specific substrate and propose that acetylation serves to enhance the cellular elimination of this folic acid breakdown product.

EXPERIMENTAL

Materials

pABG was purchased from Sigma Chemical Co., St. Louis, MO,

Cell culture

U937 cells were obtained from Dr. P. Holt, Princess Margaret Hospital, Western Australia, and were cultured in RPMI 1640 with 10% fetal calf serum, 50 μ g/ml gentamicin and 2.5 μ g/ml amphotericin B at 37 °C in an atmosphere of 5% CO₂ in air. Cell supernatants were prepared by sonicating cells (3 $\times$ 10⁶/ml) in 20 mM Tris/HCl (pH 7.4), 0.5 mM EDTA and 1 mM dithiothreitol on ice for 15 s and centrifuging the mixture at 10000 g for 3 min. Protein concentrations were determined by the Bradford method.

Acetylation of pABG

COS-1 cell supernatants (2.5–3.3 μ g of total protein) or U937 cell supernatants (20 μ g of total protein) were incubated with pABG (0–800 μ M) and 50 μ M acetyl-CoA in 20 mM Tris/HCl (pH 7.4), 0.5 mM EDTA and 1 mM dithiothreitol. After 15 min, the reaction was stopped with 20 μ l of glacial acetic acid and the reaction product (*N*-Ac-pABG) was quantified by HPLC. The rate of pABG acetylation was linear with time (up to 40 min) and with protein concentration (up to at least 50 μ g for U937 cell supernatants and at least 5 μ g for COS-1 cell supernatants). Mock-transfected COS-1 cell supernatants were used as controls. For the U937 cells, heat-inactivated protein or incubations in the absence of acetyl-CoA were used as controls. In those studies

where inhibitors were included, the inhibitor, in Tris/HCl buffer, was added to give a final concentration of 200 μM .

HPLC assay for N-Ac-pABG

Reaction supernatants (20–50 μl) were analysed by HPLC using a Beckman 5 μC_{18} reverse phase column (4.6 $\times$ 150 mm) and a mobile phase of 12% methanol in 1% aqueous acetic acid. N-Ac-pABG was detected by absorption at 260 nm. At a flow rate of 0.8 ml/min, the retention times for pABG and N-Ac-pABG were 6.7 and 15.8 min, respectively. N-Ac-pABG was stable in acid for at least 6 h.

Data analysis

The estimates for K_m and V_{max} were determined by fitting the Michaelis–Menten equation to the primary data by non-linear least squares regression analysis using SigmaPlot software (Jandel Scientific). Parameter optimization was confirmed by commencing the iterative process from at least three independent initial estimates.

RESULTS AND DISCUSSION

Figure 1 shows representative HPLC traces for reaction products of pABG incubated with rNAT1 (b) or rNAT2 (c). N-Ac-pABG was only present in incubations containing rNAT1. Increasing the time of reaction to 1 h, the concentration of acetyl-CoA to 500 μM or the amount of total protein to 20 μg did not result in acetylation of pABG by rNAT2 (results not shown). When cytosols from mock-transfected COS-1 cells were used, no acetylation was seen. These results show that pABG is acetylated by rNAT1 but not by rNAT2.

The kinetics of pABG metabolism by rNAT1 and U937 cells

Figure 1 HPLC traces of N-Ac-pABG (a) and the reaction products from incubations of pABG with rNAT1 (b) or rNAT2 (c)

pABG (200 μM) was incubated with 2.5 μg (b) or 3.3 μg (c) of COS-1 cell supernatant protein expressing rNAT1 and rNAT2 respectively, in a final volume of 25 μl . After 20 min, the reaction was stopped with 5 μl of glacial acetic acid and 20 μl of the resulting mixture was analysed by HPLC.

Figure 2 Effect of pABG concentration on the rate of N-Ac-pABG formation by rNAT1 (●) or U937 cells (○)

Reactions were carried out for 15 min in the presence of 50 μM acetyl-CoA. Each point is the mean from at least two independent experiments.

Table 1 Effect of various arylamines on pABG acetylation by rNAT1 and U937 cell supernatant

Data are expressed as the mean of three experiments with a maximum range of 15% of the respective means.

Inhibitor	Acetyltransferase activity (% of control)	
	rNAT1	U937
Sulphacetamide	99	96
Sulphamethazine	92	100
Sulphaguanidine	102	100
Sulphaethidole	64	57
<i>p</i> -Aminohippuric acid	36	34
<i>p</i> -Aminophenol	34	22
Sulphamethoxypyridazine	11	14
<i>p</i> -Aminosalicylic acid	0	2
<i>p</i> -Aminobenzoic acid	1	2
Sulphaquinoxaline	0	0

are illustrated in Figure 2. The rates of acetylation exhibited apparent Michaelis–Menten kinetics with respect to pABG concentration. From three independent experiments, K_m s (mean $\pm$ S.E.M.) of $130 \pm 13 \mu\text{M}$ and $333 \pm 17 \mu\text{M}$ were estimated for rNAT1 and U937 cells respectively. The maximum rates of acetylation were 170 and 83 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ respectively. Because arylamine *N*-acetylation proceeds via a ‘Ping Pong Bi Bi’ mechanism, the apparent K_m is dependent on cofactor concentration [1]. Cellular cytosol acetyl-CoA levels have been estimated in the low micromolar range [13], suggesting that the K_m for pABG *in vivo* is probably lower than that determined in the present study.

The higher K_m for the U937 cells compared with that for rNAT1 suggests that pABG may be acetylated by an enzyme other than NAT1. Alternatively, it may indicate the presence of an endogenous inhibitor, as suggested by Ward et al. [8], in the U937 cells. To determine further the roles of NAT1 and NAT2 in pABG acetylation, a number of arylamines were examined as potential inhibitors (Table 1). The NAT1-selective substrates *p*-aminobenzoic acid and *p*-aminosalicylic acid almost completely inhibited acetylation by both the U937 cells and the rNAT1. By

contrast, the NAT2-selective substrate sulphamethazine showed little or no inhibition. For the remaining arylamines, a range of activities was seen. The correlation between inhibitor activity with the U937 cells and that with the rNAT1 was highly significant ($r^2 = 0.98$, $P < 0.01$), suggesting that NAT1 was responsible for pABG acetylation by the U937 cells. In agreement with this, Coroneos et al. [14] have shown constitutive expression of NAT1 in U937 cells with little or no expression of NAT2.

The possibility that NAT1 has a function in addition to xenobiotic metabolism has been suggested previously [8]. However, it has been difficult to isolate or identify candidate endogenous substrates for the arylamine acetyltransferases partly because, with the exception of pABG, endogenous aromatic amines are uncommon in mammalian cells. Most endogenous substrates that undergo acetylation do so via specific aliphatic acetylases [14a,15]. The present study has identified for the first time a potential endogenous substrate for human NAT1.

During the second and third trimester, pregnant women excrete each day the equivalent of an additional 200–300 μg of folic acid, mainly as N-Ac-pABG [10]. pABG can compete with folic acid for folate-binding protein [16] and it appears to be important in the binding of folate to 5,10-methylenetetrahydrofolic dehydrogenase [11]. Thus, it may act as an antagonist to some folate-dependent enzymes. pABG does not readily cross biological membranes [17]. Acetylation, therefore, may assist in the removal of the catabolite from the cell since, based on C_{18} HPLC retention times, N-Ac-pABG is considerably more lipophilic than pABG. However, the activity of NAT1 varies considerably between individuals [18] and there is growing evidence to suggest a genetic polymorphism at the NAT1 gene locus [18,19]. In individuals with low or absent NAT1, pABG may accumulate in rapidly dividing cells leading to a 'folate-deficiency'-like syndrome. Currently, we are investigating this hypothesis in various human cell lines.

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