Purification and characterization of cysteine-S-conjugate *N*-acetyltransferase from pig kidney

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Microsomal cysteine-S-conjugate *N*-acetyltransferase catalyses the *N*-acetylation of various S-substituted cysteines in liver and kidney. We describe here the purification and more detailed characterization of this enzyme catalysing the final reaction of mercapturic acid biosynthesis, and thus playing a crucial role in the detoxicating metabolism of many xenobiotics. The solubilization of cysteine-S-conjugate *N*-acetyltransferase by deoxy-BIGCHAP [*N*,*N*′-bis-(3-D-gluconamidopropyl)deoxycholamide] was the prerequisite for partial purification by means of anion-exchange chromatography. The molecular mass of the enzyme was determined by gel filtration. A polyclonal antiserum was raised against the excised protein band from SDS/PAGE and purified antibodies were used for the complete purification of native cysteine-S-conjugate *N*-acetyltransferase by immunoaffinity chromatography. A dimeric form of the enzyme was sometimes detected on SDS/PAGE, depending on the degree of purification. For further characterization of cysteine-S-conjugate *N*-acetyltransferase, the stability of catalytic activity, the pH optimum and K_m values were determined. The inhibitory effects of various agents were tested, revealing a substantial, yet not complete, loss of cysteine-S-conjugate *N*-acetyltransferase activity after treatment with cysteine proteinase inhibitors or probenecid under various conditions.

INTRODUCTION

Cysteine-S-conjugate *N*-acetyltransferase (EC 2.3.1.80) is a membrane-bound enzyme that catalyses the acetylation of various thioethers, and hence the final reaction of mercapturic acid biosynthesis. As several cysteine-S-conjugates are highly mutagenic and/or toxic and appear in the urine only after acetylation, this process of mercapturate formation represents a major route for detoxification of a wide variety of reactive electrophiles [1–3]. At least in some species the enzyme may also be responsible for intracellular acetylation of leukotriene E_4 leading to the formation of *N*-acetyl-leukotriene E_4 [4–6].

In the rat, cysteine-S-conjugate *N*-acetyltransferase is located mainly in the liver and kidney, with its active site on the cytoplasmic side of the endoplasmic reticulum [7,8]. Cysteine-S-conjugate *N*-acetyltransferase exhibits a very similar distribution profile in the kidney to the other enzymes of the mercapturic acid pathway (i.e. γ-glutamyl transpeptidase and several dipeptidases), with the maximum enzymic activity localized in the inner cortex [9]. However, in spite of the important function of cysteine-S-conjugate *N*-acetyltransferase in detoxification, only limited information is available about this enzyme, which is not identical with other soluble or membrane-bound acetyltransferases specific for glutamate, aspartate, or for various amines [7]. It catalyses the acetylation of hydrophobic, S-substituted derivatives of L-cysteine with acetyl-CoA as a co-substrate. Assays have been established on the basis of the acetylation of different model compounds [7,10], the most recent method being non-radioactive and confirming enzymic activity in pig kidney [11]. Some data concerning the catalytic properties of cysteine-S-conjugate *N*-acetyltransferase were published more than a decade ago [7,8,10]. However, except for a method for the extraction of the protein and minor enrichment of enzymic activity [10], all attempts to purify the enzyme further have been unsuccessful.

In contrast to γ-glutamyl transpeptidase and dipeptidases, cysteine-S-conjugate *N*-acetyltransferase is not involved in any metabolic pathways other than the mercapturic acid pathway and possibly the formation of *N*-acetyl-leukotriene E_4. Therefore the enzyme serves as a good representative of these detoxification pathways. In pig brain, cysteine-S-conjugate *N*-acetyltransferase is detected in microcapillaries and hence at the site of the blood–brain barrier (A. Aigner and S. Wolf, unpublished work), indicating a possible role of the mercapturic acid pathway in the enzymic blood–brain barrier. This may be further elucidated by immunohistochemical studies using a polyclonal antiserum against the purified cysteine-S-conjugate *N*-acetyltransferase.

The aim of the present study was to purify and characterize the physical and kinetic properties of cysteine-S-conjugate *N*-acetyltransferase. We have determined the molecular mass of the enzyme by gel filtration, and describe the partial purification of the membrane protein from pig kidney by column chromatography. The enzyme was subjected to SDS/PAGE and polyclonal antibodies were raised against the excised protein band of cysteine-S-conjugate *N*-acetyltransferase. We describe the purification of the enzyme to apparent homogeneity by immunoaffinity chromatography. Finally, we present data concerning the catalytic properties of pig cysteine-S-conjugate *N*-acetyltransferase.

EXPERIMENTAL

Materials

Chemicals were of analytical grade and were obtained from Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Materials for column chromatography were purchased from Whatman (U.K.), Pharmacia Fine Chemicals (Uppsala, Sweden) and Sigma. Nitro-

Abbreviations used: deoxy-BIGCHAP, *N*,*N*′-bis-(3-D-gluconamidopropyl)deoxycholamide; DTT, dithiothreitol; PEG, poly(ethylene glycol).

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cellulose paper was obtained from Schleicher & Schuell (Dassel, Germany). Equipment for FPLC was from Pharmacia, and for HPLC from Beckman (München, Germany). Biochemicals were purchased from Boehringer, Fluka or Sigma.

Deoxy-BIGCHAP [*N*,*N*′-bis-(3-D-gluconamidopropyl)deoxycholamide] was synthesized according to [12]. *S*-4-Nitrobenzyl-L-cysteine was synthesized as described in [13]. Antiserum against the 34 kDa protein band of cysteine-S-conjugate *N*-acetyltransferase was prepared by EUROGENTEC (Seraing, Belgium).

Preparation of microsomes

Microsomal fractions were prepared from pig kidney cortex by differential centrifugation as described in [11]. Pig kidneys were obtained from a regional slaughterhouse. Homogenization and all subsequent purification steps were carried out at 4 °C unless otherwise noted.

Solubilization

Proteins were extracted from microsomal fractions as described in [10], with modifications. A 16.8 g portion of microsomes was suspended in 240 ml of 250 mM sucrose containing 1 mM dithiothreitol (DTT) and 200 mM KCl, then 240 ml of the same buffer supplemented with 20 mM potassium phosphate (pH 7.0) and 20 mg/ml deoxy-BIGCHAP was added. The mixture was stirred for 15 min prior to homogenization (Dounce homogenizer; tight-fitting pestle) and subjected to centrifugation for 80 min at 100000 ***g*** ($r_{av.}$ 11.82 cm). For sedimentation of the solubilized proteins, 320 ml of 50 % (w/v) polyethylene glycol (PEG) 6000 was added slowly to the supernatant to yield a final concentration of 20 % (w/v) of the polymer. The mixture was stirred for 30 min, and the precipitate ('PEG precipitate') was collected by centrifugation for 30 min at 18000 ***g*** ($r_{av.}$ 11.9 cm) and stored at −20 °C.

For solubilization experiments, 10 mg of microsomes was suspended in 5 ml of 250 mM sucrose containing 1 mM DTT. Detergent (CHAPS, deoxycholate, deoxy-BIGCHAP, n-octyl glucoside, SDS, Triton X-100 or Tween 20) was added to a concentration of 2.5× critical micelle concentration prior to homogenization in a Dounce homogenizer (tight-fitting pestle).

Ion-exchange chromatography

A 4 g portion of PEG precipitate from the above step was resuspended in 100 ml of TDG buffer, pH 7.8 [10 mM Tris/HCl, 1 mM DTT, 30 % (v/v) glycerol], and applied on to a column (20 cm × 4 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with TDG buffer, pH 7.8 (200 ml), and eluted with 300 ml of 0–1 M KCl prepared in the same buffer, at a flow rate of 120 ml/h. Subsequently the column was eluted with 200 ml of TDG buffer (pH 7.8) containing 1 M KCl and 5 mg/ml deoxy-BIGCHAP. Fractions from this second elution step containing cysteine-S-conjugate *N*-acetyltransferase activity were pooled, and were then concentrated and desalted using an Amicon ultrafiltration cell with a PM-10 membrane.

FPLC Mono Q chromatography and SDS/PAGE

TDG buffer (pH 7.3) containing 5 mg/ml deoxy-BIGCHAP was used for Mono Q column chromatography. Elution was performed with a non-linear gradient (0-0.3 M KCl) as indicated in Figure 1 (lower panel) at a flow rate of 60 ml/h. Fractions 11–21 were pooled, concentrated and rediluted in 500 μl of buffer containing 250 mM Tris/HCl, 2 M glycine and 1 % SDS for SDS/PAGE. Discontinuous SDS/PAGE was performed according to Laemmli [14] on 12.5 % acrylamide gels. Samples were heated for 5 min at 100 °C before application. The gels were stained with Coomassie Brillant Blue or by a silver staining method as described in [15].

Gel filtration

For gel filtration, 350 μl of the concentrated and desalted fractions from anion-exchange chromatography on DEAE-cellulose were further concentrated and rediluted in 100 μl of TD buffer, pH 7.8 (10 mM Tris/HCl, 1 mM DTT), containing 5 mg/ml deoxy-BIGCHAP to remove glycerol. The sample was applied on to an FPLC Superose 6 column equilibrated with TD buffer (pH 7.8) containing 5 mg/ml deoxy-BIGCHAP and eluted with the same buffer. For better separation performance, glycerol was omitted from this step. For calibration, molecular mass standards, including cytochrome *c* (12.5 kDa), myoglobin (17 kDa), ovalbumin (44 kDa) and BSA (66 kDa), were used in the same buffer system.

Preparation of antiserum

The 34 kDa protein band, obtained after SDS/PAGE of fractions from FPLC Mono Q chromatography, was excised from the gel and used as an antigen. Rabbit antibodies were generated by EUROGENTEC, according to a standard protocol comprising subcutaneous injection with about 100 μg of protein emulsified in an equal volume of Freund's adjuvant. After three booster injections with the same amount of antigen, the rabbit was bled to obtain antiserum. Isolation of IgGs from the antiserum was performed according to [16].

Immunoblotting

The proteins from SDS/PAGE were transferred to sheets of nitrocellulose paper by semi-dry blotting (40 min) as described in [17] at a constant voltage of 25 V. The membrane was pretreated with 5 % (w/v) skimmed milk powder in TBST buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.05 % Tween-20] for 60 min at room temperature, washed and then treated with anti-(cysteine-S-conjugate *N*-acetyltransferase) serum (diluted 1:1000) at 4 °C overnight. This was followed by extensive washing with TBST buffer. Antigenic proteins were visualized using alkaline phosphatase-conjugated goat anti-rabbit antibodies with bromochloroindolyl phosphate/Nitro Blue Tetrazolium as substrate [20].

Amino acid analysis

After SDS/PAGE, Coomassie Blue-stained protein bands were excised and proteins were electroeluted for 2 h at 200 V using a Mini-Protean II apparatus from Bio-Rad. The buffer used was 100 mM Tris/HCl (pH 8.3), 100 mM boric acid, 2.5 mM EDTA and 0.1 % SDS.

Total hydrolysis of proteins with HCl and determination of amino acid concentrations was performed as described in [18,19], including post-column derivatization with ninhydrin.

Immunoaffinity chromatography

A (Protein A bead)–antibody affinity column was prepared as described in [20] by direct coupling of the antibodies with dimethylpimelimidate. For antigen binding, 50 mg of PEG precipitate was dissolved in 3 ml of TG buffer [10 mM Tris/HCl (pH 7.8), 30 % (w/v) glycerol] containing 5 mg/ml deoxy-BIGCHAP and applied on to the column, passing through it three times. After washing the column with 20 ml of TG buffer, pre-

elution was performed with 5 ml of 10 mM sodium phosphate (pH 6.8). Antigen was eluted by applying 5 ml of 100 mM glycine/HCl (pH 3.0) containing 5 mg/ml deoxy-BIGCHAP. Fractions of 500 μl were collected, and each fraction was supplemented with 50 μl of 1 M potassium phosphate (pH 7.0). Finally, fractions were treated with 20 mg of Protein A–Sepharose for removal of small amounts of IgGs.

Assays

Protein concentration was determined by amino acid analysis (see above) or as described in [21]. In column effluents, protein elution was monitored by measurement of A_{280}.

Cysteine-S-conjugate *N*-acetyltransferase activity was determined as described in [11]. According to [22], 1 unit of activity is the amount of enzyme required for formation of 1 nmol of product per min under assay conditions.

The K_m values of different protein samples were determined with substrate concentrations ranging from 0 to 2000 μM (*S*-4-nitrobenzyl-L-cysteine) or from 0 to 4000 μM (acetyl-CoA). The effects of inhibitors on enzyme activity were determined by addition of the substance (1.7 mM *p*-chloromercuribenzoate, 2.1 mM *N*-ethylmaleimide, 4.4 mM PMSF, 2.0 mM probenecid or 5.0 mM probenecid) to different samples and incubation of the mixture at 37 °C for 1 h prior to measurement of enzyme activity. For determination of enzyme activities at different pH values, the incubation mixtures contained 150 mM Bistris/HCl with a pH range 5.4–7.0 or Tris/HCl with a pH range 7.0–9.5 instead of 150 mM potassium phosphate (pH 7.0), and were incubated for 10 min.

RESULTS

Solubilization of cysteine-S-conjugate *N*-acetyltransferase.

Purification of the enzyme very much depends on solubilization from microsomal preparations without substantial loss of activity. In order to determine optimal conditions for solubilization of pig cysteine-S-conjugate *N*-acetyltransferase, microsomal pellets were treated with different detergents as described in the Experimental section. Satisfactory results during purification of pig cysteine-S-conjugate *N*-acetyltransferase were obtained only with buffers containing deoxy-BIGCHAP. Consequently, despite a 45 % loss of enzyme activity upon treatment with this detergent, deoxy-BIGCHAP was used for preparative solubilization and for the subsequent chromatography steps.

Gel filtration

Based on the use of globular proteins of known size, the molecular mass of cysteine-S-conjugate *N*-acetyltransferase was about 32 kDa as estimated by gel filtration on a Superose 6 column using FPLC. For calibration of the column and for determination of the molecular mass of the enzyme, the same buffer system was used, and the relationship between log (molecular mass) and volume was linear for all molecular mass standards. Thus it was ensured that aberrant results due to detergent binding to proteins are highly unlikely when using deoxy-BIGCHAP. However, chromatography on Superose 6 led to a marked decrease of enzyme activity and only negligible purification of cysteine-S-conjugate *N*-acetyltransferase.

Chromatographic purification

Cysteine-S-conjugate *N*-acetyltransferase was partially purified as described in the Experimental section, and details are listed in Table 1. The protocol comprised preparation of microsomes, solubilization with deoxy-BIGCHAP, anion-exchange chromatography on DEAE-cellulose with two elution steps (Figure 1, upper panel) and anion-exchange chromatography on a FPLC Mono Q column using a non-linear KCl gradient (Figure 1, lower panel).

Table 1 Protocol of partial purification of cysteine-S-conjugate *N*-acetyltransferase from 479 g of pig renal cortex

Purification step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Microsomes	–	15398	1294	11.9	100	1.0
PEG precipitate	–	9568	1044	9.2	62	0.8
DEAE-cellulose	242	2245	169	13.3	14.5	1.1
Mono Q	798	642	8	80.4	4	6.8

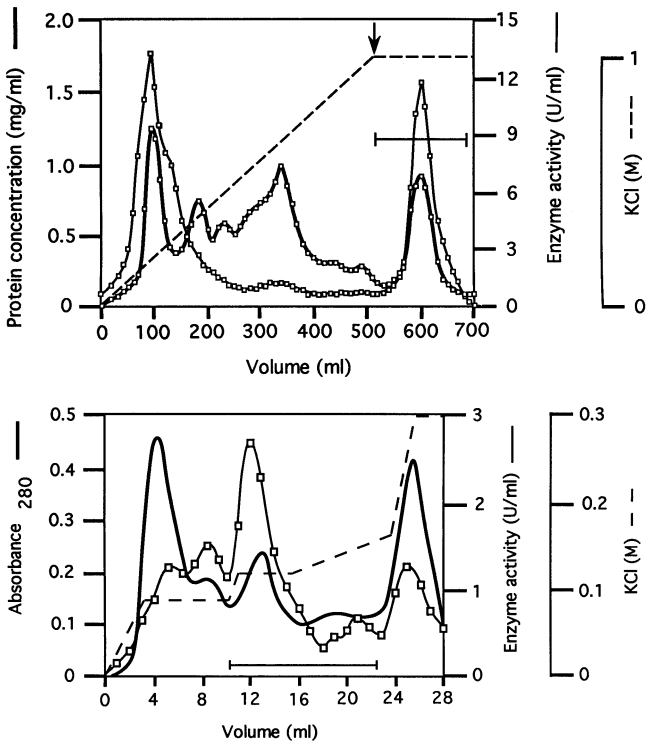

Figure 1 Partial purification of deoxy-BIGCHAP-solubilized cysteine-S-conjugate *N*-acetyltransferase

Elution profiles are shown of enzyme activity (thin lines) and of protein (thick lines) as measured at 280 nm. The broken lines show the KCl gradient. Fractions pooled for further purification steps are indicated. Upper panel: anion-exchange chromatography on DEAE-cellulose. The fraction size was 10 ml. The arrow indicates the beginning of the second elution step with deoxy-BIGCHAP-supplemented buffer. Lower panel: anion-exchange chromatography on a Mono Q column. The fraction size was 1 ml.

The specific activity increased from 11.9 units/mg in the microsomal fraction to 80.4 units/mg in pooled fractions 11–21 from the Mono Q column, corresponding to a 7-fold enrichment during partial purification.

During anion-exchange chromatography on DEAE-cellulose, only the active fractions of the second elution step were collected for further purification. Omitting detergent as described in the Experimental section during application of the sample on to the column and subsequent elution with 0–1 M KCl led to visible

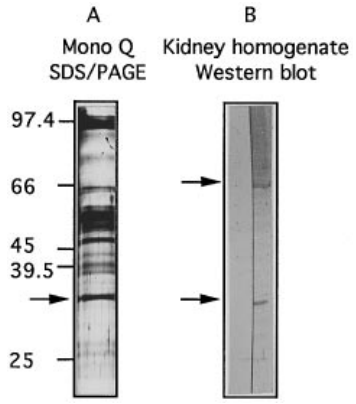

Figure 2 Protein bands of 34 kDa and 68 kDa on SDS/PAGE of cysteine-S-conjugate *N*-acetyltransferase

(**A**) Silver-stained SDS/12.5%-polyacrylamide gel of the pooled fractions from the Mono Q column. The 34 kDa protein band (arrow) was excised from the gel and used as an antigen. Numbers on the left indicate molecular mass in kDa. (**B**) Western blot. Kidney homogenate was separated and immunoblotted with purified IgG from the polyclonal antiserum (right lane) or preimmune serum (left lane). The two bands (arrowed) correspond to the monomer (34 kDa) and the dimeric form (68 kDa) of cysteine-S-conjugate *N*-acetyltransferase.

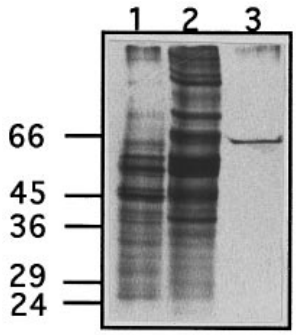

Figure 3 Purification of cysteine-S-conjugate *N*-acetyltransferase

Silver-stained SDS/12.5%-polyacrylamide gel from total kidney homogenate (lane 1) and dissolved PEG precipitate (lane 2), the latter being used for immunoaffinity chromatography. Lane 3 shows cysteine-S-conjugate *N*-acetyltransferase purified by immunoaffinity chromatography with subsequent removal of traces of co-eluting IgGs, as described in the Experimental section. Numbers on the left indicate molecular mass in kDa.

partial precipitation of proteins. Nevertheless, large amounts of proteins were eluted as the KCl concentration was increased, whereas most of the enzymic activity remained on the column and was obtained in fractions 57–65, corresponding to the second elution step. Compared with the presence of deoxy-BIGCHAP in all buffers, this procedure was advantageous with DEAE-cellulose because of better purification performance and the need for smaller amounts of the costly detergent.

The best result was obtained with the Mono Q column (6-fold increase in specific activity). Only fractions with high specific activity were pooled.

Losses of activity during purification were partly due to the decrease in cysteine-S-conjugate *N*-acetyltransferase activity caused by deoxy-BIGCHAP, but were mainly a result of the removal of fractions with lower specific activity not being used in subsequent steps.

SDS/PAGE of fractions from FPLC Mono Q chromatography revealed two bands at 34 kDa and 68 kDa, corresponding to cysteine-S-conjugate *N*-acetyltransferase and its dimeric form respectively (see below). The other bands at about 100 kDa and above 45 kDa represented contaminating proteins, the latter often being more intense than is shown in Figure 2(A).

Immunological identification and complete purification of cysteine-S-conjugate *N*-acetyltransferase

In order to obtain homogeneous and enzymically active cysteine-S-conjugate *N*-acetyltransferase by immunoaffinity chromatography, rabbit antibodies were generated against the excised 34 kDa protein band. Anti-(34 kDa protein) serum was prepared as described in the Experimental section. Purification of IgG from 25 ml of serum yielded 75 mg of IgG. Western blotting with kidney homogenate revealed two protein bands at 34 kDa and 68 kDa. No signals were visible with preimmune serum (Figure 2B). To ensure that equal amounts of protein were present on the nitrocellulose paper, a single lane was bisected for comparison of immune serum and preimmune serum.

For immunoaffinity chromatography, the matrix was prepared as described in the Experimental section. On applying the dissolved PEG precipitate to the column, activity was specifically bound by the matrix and, essentially, was not removed during pre-elution. For specific elution, glycine buffer (100 mM glycine/HCl buffer, pH 3.0) was selected. Addition of 5 mg/ml deoxy-BIGCHAP ensured higher yields of eluted protein but was not essential. Several other standard elution buffers tested were not satisfactory because of complete enzyme inactivation (5 M LiCl, 3.5 M $MgCl_2$, 1% or 5% SDS, 3 M trifluoroacetic acid, 3 M trichloroacetic acid) or small amounts of enzyme eluted (2 M or 8 M urea). Treatment with glycine buffer or with 100 mM sodium phosphate buffer (pH 11.5) resulted in an irreversible decrease in enzyme activity (21% or 26% of the initial level respectively after 10 min). Nevertheless, the selected glycine buffer was advantageous due to high elution efficiency. To decrease inactivation, fractions were adjusted to pH 7.0 with 1 M potassium phosphate immediately after elution. With glycine buffer for elution, the affinity matrix could be used at least three times without substantial loss of binding capacity.

Traces of co-eluting IgGs were removed by treatment with Protein A–Sepharose. Despite elution with glycine buffer (pH 3.0), the fractions containing cysteine-S-conjugate *N*-acetyltransferase still possessed enzyme activity. For example, after applying a 1 ml sample (24.5 units/ml) on to the column, an activity of 1.5 units/ml (5.8 units/mg) was determined for fraction 2, which contained most of the eluting cysteine-S-conjugate *N*-acetyltransferase.

On SDS/PAGE analysis of this fraction the 34 kDa band was not detectable (Figure 3). Even when antibodies were purified by immunoadsorption on to the blotted 34 kDa protein band prior to coupling to Protein A–Sepharose, the 68 kDa band was obtained exclusively on SDS/PAGE after immunoaffinity chromatography. Amino acid analysis of the excised and electro-eluted bands from SDS/PAGE after Mono Q chromatography revealed identical amino acid compositions of the 34 kDa and the 68 kDa proteins (Table 2).

The observations that the 68 kDa band is recognized by the anti-(34 kDa protein) serum, that it displays an identical amino acid composition to the 34 kDa protein and that the corresponding fraction possesses cysteine-S-conjugate *N*-acetyltransferase activity indicate the occurrence of a dimeric form of cysteine-S-conjugate *N*-acetyltransferase. As the dimeric form was not found on gel filtration, the 68 kDa band seems to be formed during SDS/PAGE, presumably upon treatment with

Table 2 Amino acid analysis of the 34 kDa and 68 kDa forms of cysteine-S-conjugate *N*-acetyltransferase

The corresponding protein bands of SDS/PAGE were excised and electroeluted prior to acid total hydrolysis, as described in the Experimental section. n.d., not determined.

	Content	
Amino acid	34 kDa form	68 kDa form
Asp	9.3	7.4
Ser	4.5	4.5
Thr	8.9	8.1
Glu	8.9	8.0
Pro	4.2	5.9
Gly	24.4	23.1
Ala	6.8	7.7
Cys	n.d.	n.d.
Val	5.4	4.8
Met	n.d.	n.d.
Ile	3.7	4.1
Leu	7.5	8.1
Tyr	2.7	2.9
Phe	3.4	3.8
His	n.d.	n.d.
Trp	n.d.	n.d.
Lys	6.0	6.2
Arg	4.1	5.5

Table 3 K_m values of cysteine-S-conjugate *N*-acetyltransferase for acetyl-CoA and for the model substrate *S*-4-nitrobenzyl-L-cysteine

Assays were carried out as described in the Experimental section. Results are means ± S.D. of 2 determinations.

	K_m (μM)	
Sample	*S*-4-Nitrobenzyl-L-cysteine	Acetyl-CoA
Kidney total homogenate	28.76 ± 0.17	153.5 ± 0.7
Microsomes	77.19 ± 0.26	308.0 ± 22.2
Immunoaffinity chromatography	176.40 ± 0.48	273.3 ± 41.7

SDS and despite the presence of 2-mercaptoethanol in the sample buffer. Clearly the occurrence of the 68 kDa band depends on the grade of purification of cysteine-S-conjugate *N*-acetyltransferase, with fractions containing the completely purified enzyme displaying exclusively the dimeric form on SDS/PAGE.

Catalytic properties

Kidney homogenate, the microsomal fraction and detergent-solubilized cysteine-S-conjugate *N*-acetyltransferase purified to homogeneity by immunoaffinity chromatography were used for determination of K_m values for both *S*-4-nitrobenzyl-L-cysteine and acetyl-CoA. Table 3 shows the K_m values, which essentially increase with the degree of purification of the enzyme.

As indicated in Figure 4, the pH optimum of enzyme activity is about pH 6.5.

Whereas cysteine-S-conjugate *N*-acetyltransferase activity was stable for several months at −20 °C, considerable inactivation of the homogeneous enzyme stored at 4 or 25 °C in glycine/deoxy-BIGCHAP buffer adjusted to pH 7.0 was observed (52% and 81% respectively). With a crude kidney homogenate in the same buffer, the loss of activity was even greater (66% and 97% respectively), presumably due to the action of proteinases.

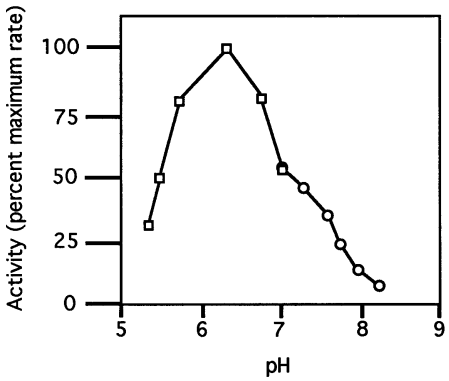

Figure 4 pH-dependence of cysteine-S-conjugate *N*-acetyltransferase activity

Enzyme activities were determined as described in the Experimental section with 150 mM Bistris/HCl (□) or 150 mM Tris/HCl (○), and are given as a percentage of the maximal activity.

The inhibitory effects of various agents were tested as described in the Experimental section. Enzyme activity was markedly decreased by the cysteine proteinase inhibitors *p*-chloromercuribenzoate and *N*-ethylmaleimide (85% and 72% inhibition respectively). However, higher concentrations of inhibitor, addition of either *S*-4-nitrobenzyl-L-cysteine or acetyl-CoA to the enzyme/inhibitor mixture in the preincubation step or longer incubation of the mixture at 37, 25 or 4 °C prior to measurement of enzyme activity did not result in complete inactivation (results not shown). The decrease in enzyme activity was similar for samples of homogeneous and partially purified cysteine-S-conjugate *N*-acetyltransferase, and was independent of the initial activity. In contrast PMSF, an inhibitor of serine proteinases, had no effect on enzyme activity.

The effect of probenecid, which is known as an inhibitor of peritubular transport processes, was also investigated. Enzyme activity was decreased to a degree dependent on the probenecid concentration (40% inhibition with 2 mM and 61% inhibition with 5 mM probenecid). However, the decrease was less than those caused by the inhibitors of cysteine proteinases.

DISCUSSION

We have described the solubilization and purification of cysteine-S-conjugate *N*-acetyltransferase from kidney microsomes. We found that only one detergent, deoxy-BIGCHAP, was capable of satisfactorily solubilizing the pig enzyme for subsequent purification. Treatment with other detergents resulted in a nearly complete loss of enzyme activity or in a poor separation performance during purification steps (results not shown). These findings are in agreement with data from attempts to solubilize the enzyme from rat kidney [10]. However, even deoxy-BIGCHAP caused a 45% decrease in enzyme activity.

On the basis of the solubilization of the enzyme with deoxy-BIGCHAP and a non-radioactive assay for enzymic activity described recently [11], our aim was the purification of pig cysteine-S-conjugate *N*-acetyltransferase. Anion-exchange chromatography resulted in partial purification, and gel-filtration chromatography allowed the identification of the corresponding 34 kDa protein band in SDS/polyacrylamide gels. However, all attempts to completely purify the enzyme by means of these chromatographic methods were unsuccessful. Whereas gel filtra-

tion was performed to determine the molecular mass of the native enzyme, it was not chosen as a further step in the purification procedure due to large losses in enzyme activity and poor purification results.

Immunoblotting with an antiserum raised against the excised 34 kDa protein band suggested the existence of a dimeric form of cysteine-S-conjugate *N*-acetyltransferase with a molecular mass of 68 kDa in SDS/polyacrylamide gels. Acid hydrolysis of the electroeluted proteins at 34 kDa and 68 kDa and comparison of the results of amino acid analysis confirmed this finding. However, the values for glycine seem to be comparatively high, which may be due to contaminating substances co-eluted from the gel matrix. We are currently attempting to determine the DNA sequence of cysteine-S-conjugate *N*-acetyltransfease by screening an expression library.

By means of immunoaffinity chromatography, we purified cysteine-S-conjugate *N*-acetyltransferase to homogeneity. Although the pH of the elution buffer resulted in a marked decrease, the eluting enzyme still exhibited cysteine-S-conjugate *N*-acetyltransferase activity. Interestingly, after complete purification we found exclusively the 68 kDa band on SDS/PAGE. However, the 68 kDa band seems to be formed during SDS/PAGE, as on gel filtration no enzymically active dimeric form was present. There is good evidence that the occurrence of the dimeric form (possibly upon treatment with SDS) increases with the grade of purification of cysteine-S-conjugate *N*-acetyltransferase.

For further characterization of pig cysteine-S-conjugate *N*-acetyltransferase, we determined the catalytic properties of the homogeneous enzyme. The pH optimum of the enzymic activity (6.5) was similar to the value for the enzyme from rat kidney [10]. On the other hand, determination of K_m values produced somewhat different results. Whereas the K_m for our model compound 4-nitro-*S*-benzyl-L-cysteine was comparable with previous results for *S*-benzyl-L-cysteine [7,10], the K_m for acetyl-CoA was considerably higher. In our experiments, with progressive purity of the enzyme the K_m values increased substantially for both substrates. Whether the basis for this change is solubilization or some additional modification remains to be elucidated.

Enzyme activity was markedly inhibited by *p*-chloromercuribenzoate or *N*-ethylmaleimide, but not by PMSF. However, as cysteine-S-conjugate *N*-acetyltransferase retained at least 15% of its activity after treatment with even the potent and irreversible thiol reagent chloromercuribenzoate, the mechanism of this inhibition remains to be elucidated. To exclude product formation due to the activity of other (unknown) enzyme(s), we confirmed our findings by using homogeneous cysteine-S-conjugate *N*-acetyltransferase. Inhibition was dependent neither on the incubation time prior to measurement of enzyme activity nor, over a wide range, on the concentration of cysteine proteinase inhibitor. Likewise, the decrease in cysteine-S-conjugate *N*-acetyltransferase activity after addition of *p*-chloromercuricenzoate or *N*-ethylmaleimide was not a result of the reaction of the inhibitor with a substrate of the assay decreasing its initial concentration. Finally, the addition of either *S*-4-nitrobenzyl-L-cysteine or acetyl-CoA to the enzyme/inhibitor mixture in the preincubation step did not alter the effects of the cysteine proteinase inhibitors. This excludes the possibility of the enzyme being more liable to inhibition after conformational changes induced by one substrate.

Another inhibitor was probenecid, a well known inhibitor of the peritubular transport of organic anions. In several experiments it has been shown that mercapturic acids representing the final *N*-acetylated metabolites of cysteine-S-conjugates are excreted into urine or bile via a probenecid-sensitive peritubular transport system [23,24]. However, our findings for pig cysteine-S-conjugate *N*-acetyltransferase agree with previous results that probenecid, in addition to its inhibitory effect on mercapturate transport, inhibits *N*-acetylation itself.

For our experiments we chose pig kidney rather than liver due to the more than 30-fold higher specific activity of cysteine-S-conjugate *N*-acetyltransferase in this organ (A. Aigner and S. Wolf, unpublished work). Now, with the enzyme having been purified to homogeneity, it will be possible to investigate whether cysteine-S-conjugate *N*-acetyltransferase is also capable of acetylating leukotriene E_4 in different organs. This will allow a greater understanding of the final step of metabolism and inactivation of leukotrienes. Furthermore, by immunohistochemical studies with the polyclonal antiserum raised against cysteine-S-conjugate *N*-acetyltransferase, the precise cellular localization of the enzyme, and hence of the mercapturic acid pathway, can be shown. We propose that, among other findings, this will elucidate a possible function for this detoxification pathway at the blood–brain barrier.

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