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Cysteine S-Conjugate N-Acetyltransferase from Rat Kidney Microsomes

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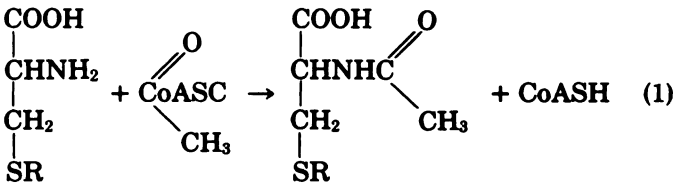
SUMMARY

An acetyltransferase from rat kidney microsomes that catalyzes the *N*-acetylation of thioethers of L-cysteine has been solubilized, stabilized, and separated from hydrolytic enzymes active against both the acetylated product, a mercapturic acid, and acetyl coenzyme A. Efficiency of catalysis varies with the lipophilicity of the substituent at sulfur in the order, ethyl < propyl < benzyl < butyl, as predicted by the Hansch π constants. Although L-tryptophan is acetylated at a very low rate, acetylation is not detectable for L-cysteine, L-methionine, L-serine, L-leucine, L-phenylalanine, or L-glutamic acid. The properties and substrate specificity reported here, along with previous studies on enzyme distribution, suggest that cysteine *S*-conjugate *N*-acetyltransferase is responsible for the final step in mercapturic acid biosynthesis.

INTRODUCTION

The formation of mercapturic acids, the *S*-conjugates of *N*-acetylcysteine, represents a major route for detoxication of a wide variety of reactive electrophiles (1–3). The process of mercapturate formation requires four distinct steps: (a) thioether formation with glutathione, either spontaneously or catalyzed by the glutathione transferases (3, 4); (b) transfer of the γ-glutamyl group of the GSH moiety as catalyzed by the membrane-bound γ-glutamyl transpeptidase (3, 5); (c) cleavage of glycine, for which several dipeptidases and aminopeptidase M are candidates (6, 7); and the final step, (d) *N*-acetylation of the remaining thioether of cysteine (Reaction 1).



(1)

Detailed studies on purified preparations of each of the participating enzymes have been reported (4–6) with the exception of the acetylation step. Although *N*-acetyltransferases with broad specificity are known to occur in the cytosol (8), the enzyme active with thioethers of cysteine has been reported as membrane-bound in kidney and liver (9, 10). Difficulties in solubilizing and stabilizing the membrane-bound enzyme required that it be studied at the level of tissue slices or microsomes (10).

In this report, we present a method for extraction of cysteine *S*-conjugate *N*-acetyltransferase from rat kidney microsomes, with subsequent minor purification and more satisfactory stabilization of the enzyme. The resulting *N*-acetyltransferase is free of hydrolytic activity toward CoASAc and *N*-acetyl-*S*-benzyl-L-cysteine, thereby allowing a more precise assessment of specificity.

MATERIALS AND METHODS

Kidneys from male Sprague-Dawley rats of 175–200 g (ARS Sprague-Dawley, Madison, Wisc.) were stored at −70° prior to use. [1-^{14}C]CoASAc (51–59 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.) and used without further purification. Hydroxylapatite-spheroidal was obtained from Gallard-Schlessinger (Carle Place, N. Y.). The non-ionic detergent DGDC[2] was synthesized by the method of Hjelmeland and Klee.[3] *S*-1-propyl-L-cysteine, *S*-1-butyl-L-cysteine, *S*-benzyl-D-cysteine, and *S*-2,4-dinitrophenyl-L-cysteine were synthesized by the method previously described for *S*-1-propyl-L-cysteine (11). *O*-Benzyl-L- and D-serine were obtained from Vega-Fox Biochemicals (Tucson, Ariz.). All other compounds tested for substrate activity were obtained from Sigma Chemical Company (St. Louis, Mo.).

Assay of Cysteine S-Conjugate N-Acetyltransferase

Enzyme activity was measured with 1 mM *S*-benzyl-L-cysteine, 0.8 mM [^{14}C]CoASAc (0.25 μCi/μmole), and enzyme in a final volume of 250 μl of 0.1 M potassium phosphate, pH 7.0 at 37°. After preincubation of the mixture at 37° for 2 min, the enzyme was added and

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[2] The abbreviation used is: DGDC, *N,N*-bis(3-D-gluconamidopropyl)-3α,12α-dihydroxy-5β-cholan-24-amide.

[3] L. M. Hjelmeland and W. Klee, in preparation.

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incubation was continued for 4 min. Reaction was terminated with 0.75 ml of 1.33 M acetic acid and followed immediately by 2 ml of cyclohexanone. The mixture was subjected to Vortex mixing for 10 sec, and the phases were separated by centrifugation for 3 min at 1500 rpm. To an aliquot of 1 ml of the cyclohexanone layer, 10 ml of Hydrofluor (National Diagnostics, Somerville, N. J.) were added and radioactivity was determined with a Beckman LS-8100 scintillation spectrometer. Values from control incubations, conducted in the absence of S-benzyl-L-cysteine, were subtracted from values obtained with the complete assay mixture; for the standard assay, conditions were considered as acceptable when control values were less than 5% of experimental values. Under the conditions of the standard assay, product formation was linear for at least 8 min with 0.5 mg or less of rat kidney microsomes. For the soluble enzyme, activity was a linear function of protein concentration when less than 60 nmoles of product were formed.

Protein concentration was determined by the method of Bradford (12).

Measurement of Cyclohexanone Extraction Efficiency

In order to obtain comparable values for V_m, it was necessary to ascertain the efficiency of extraction for the *N*-acetyl derivative of *S*-benzyl-L-cysteine, and for the derivatives of such other compounds that were evaluated as substrates. After the standard incubation mixture had been extracted, an aliquot of the cyclohexanone layer was itself extracted with fresh 1.0 M acetic acid, and a portion of the cyclohexanone layer was taken for determination of radioactivity present. The radioactivity present in the second extract was compared with that in an aliquot of the first extraction to determine the distribution of product between the organic and aqueous phases. The partitioning of *N*-acetyl-L-cysteine between cyclohexanone and 1.0 M acetic acid was measured by reaction with 5-5′-dithiobis-2-nitrobenzoic acid (13). Aside from its lower volatility, the use of cyclohexanone instead of ethyl acetate (10) as extraction solvent allowed quantitative transfer of the product in the standard assay: 25 nmoles of *N*-acetyl-*S*-benzyl-L-cysteine were extracted by cyclohexanone with an efficiency of 101 ± 1.5% (n = 4).

Preparation of Cysteine S-Conjugate N-Acetyltransferase

Step 1: microsomes. Rat kidneys (77 g) were thawed and homogenized in 315 ml of 0.25 M sucrose containing 1 mM dithiothreitol. Homogenization was accomplished with three pulses of 10 sec each of a Waring Blendor followed by four passes with a Teflon-glass homogenizer. Homogenization and all subsequent purification steps were carried out at 4°. Microsomes were obtained by differential centrifugation using a standard procedure (14). The microsomal fraction was suspended in a solution of 0.25 M sucrose, 1 mM dithiothreitol, and 0.2 M potassium chloride, and centrifuged at 100,000 × g for 80 min. The pellet was suspended in Buffer A to a protein concentration of between 10 and 15 mg/ml.

Step 2: solubilization. The microsomal suspension was treated with an equal volume of a mixture that contained 0.25 M sucrose, 20 mM potassium phosphate (pH 7.0), 1 mM dithiothreitol, 0.2 M KCl, and the DGDC detergent (20 mg/ml); the ratio of DGDC to microsomal protein was approximately 2:1 (w/w).[4] The mixture was stirred for 10 min, homogenized in a Teflon-glass tissue grinder, and subjected to centrifugation at 100,000 × g for 80 min.

Step 3: concentration. The supernatant liquid was added to 50% (w/w) polyethylene glycol 6000 to yield a final concentration of 20% (w/v) of the polymer. The mixture was stirred for 20 min and the precipitate was collected by centrifugation at 18,000 × g for 25 min. The pellet was resuspended in Buffer A [0.01 M Tris-HCl (pH 7.8 at 25°), 30% (v/v) glycerol, 1 mM dithiothreitol, and DGDC (5 mg/ml)] to a volume approximately equal to that of the supernatant liquid after extraction.

Step 4: DEAE-cellulose. A column (4 × 22 cm) of DEAE-cellulose (DE-52, Whatman) was equilibrated with Buffer A. The enzyme preparation was applied to the column at a flow rate of 1.2 ml/min, collecting fractions of 15 ml. After sample application, the column was washed with 200 ml of Buffer A and eluted with a linear gradient formed between 700 ml of Buffer A and 700 ml of Buffer A supplemented to 0.3 M KCl. Fractions 51 through 65, containing *N*-acetyltransferase activity (Fig. 1A), were combined and concentrated to approximately 5 mg of protein per milliliter in an Amicon apparatus using a PM10 membrane. Salt was removed by precipitation of the enzyme with 20% (w/v) polyethylene glycol 6000. After centrifugation at 25,000 × g for 30 min, the pellet was suspended in 43 ml of Buffer B [10 mM potassium phosphate (pH 7.0 at 25°), 30% (v/v) glycerol, 1 mM dithiothreitol, and DGDC (5 mg/ml)].

Step 5: hydroxylapatite chromatography. A column of spheroidal hydroxylapatite (2.5 × 28.5 cm) was equilibrated with Buffer B and was charged with the product of Step 4 at a rate of 1.2 ml/min. The column was washed with 280 ml of Buffer B and eluted with a linear gradient formed between 225 ml of Buffer B and 225 ml of Buffer B containing 0.3 M potassium phosphate (pH 7.0 at 25°). After this gradient, 250 ml of Buffer B containing 0.3 M potassium phosphate were applied; fractions of 10 ml were collected (Fig. 1B). Fractions 67 through 85 containing *N*-acetyltransferase activity, were combined, supplemented with sodium azide to 3 mM, and concentrated with an Amicon PM10 membrane.

Gel Filtration Chromatography

The partially purified *N*-acetyltransferase was subjected to gel filtration with Sepharose 6B (2.0 cm × 37.5 cm) which was equilibrated with Buffer C [50 mM potassium phosphate (pH 7.0), 30% (v/v) glycerol, 1 mM dithiothreitol, DGDC (5 mg/ml), and 3 mM sodium azide]. The enzyme solution, 4 mg of protein in 0.5 ml, was

[4] Use of DGDC represents a marked improvement over other nonionic detergents (Triton X-100, octyl-β-D-glucopyranoside, Zwittergent 3-14, Tween 20, Lubrol 12A9, polyoxyethylene-10 lauryl ether) that solubilized less than 25% of the enzyme from rat kidney microsomes. A zwitterionic cholic acid derivative (15), 3-[(3-cholamidopropyl)-dimethyl-ammonia]-1-propanesulfonate, resulted in a good yield of the soluble transferase, although difficulties were encountered with chromatography on DEAE-cellulose in the presence of this detergent.

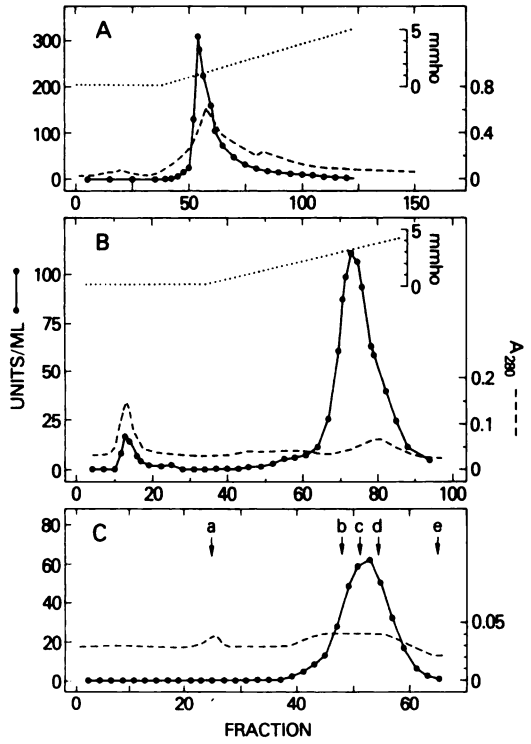

FIG. 1. *Elution patterns from (A) DEAE-cellulose, (B) Hydroxylapatite, and (C) Sepharose 6B*

Enzyme activity is indicated by *solid lines*, A_{280} by *dashed lines*, and the conductivity by *dotted lines*. In C, the *arrows* indicate peaks of elution for (*a*) Blue Dextran 2000, (*b*) bovine serum albumin, (*c*) ovalbumin, (*d*) chymotrypsinogen, and (*e*) [^{14}C]valine.

applied to the column and eluted with the same buffer. Molecular weight standards, including bovine serum albumin (68,000), hen egg albumin (45,000), chymotrypsinogen (25,000), Blue Dextran 2000 and [^{14}C]valine, were used in the same buffer system (Fig. 1C).

Assay of Deacetylation Activities and of the Reverse Reaction

N-[1-^{14}C]Acetyl-*S*-benzyl-L-cysteine was synthesized by incubation of *S*-benzyl-L-cysteine in a standard assay mixture with [^{14}C]CoASAc and the purified (Step 5) cysteine *S*-conjugate *N*-acetyltransferase. The product was extracted into chloroform and, after evaporation of the chloroform, was dissolved in 0.1 M potassium phosphate (pH 7.0). When 35 μM *N*-[1-^{14}C]-acetyl-*S*-benzyl-L-cysteine was incubated with 0.6 mg of the partially purified enzyme (Step 5) at pH 7.0 and 37° for 10 min, in either the presence or absence of 1 mM coenzyme A, no change was seen in cyclohexanone-extractable radioactivity. When [1-^{14}C]CoASAc was incubated with purified enzyme in a standard assay without acetyl acceptor, no hydrolysis of the acetyl donor was detected.

RESULTS

Purification. It is clear from the results of purification as summarized in Table 1 that this membrane protein has not been purified significantly with respect to high specific activity. What has been accomplished is that two major interfering activities, i.e., a deacetylase acting on *N*-acetyl-*S*-benzyl-L-cysteine and a CoASAc hydrolase,

TABLE 1

Purification of cysteine S-conjugate N-acetyltransferase

Step	Volume	Total units	Total protein	Specific activity
	ml	*nmoles/min*	*mg*	*units/mg*
1. Microsomes	86.5	126,000	969	130
2. Extract	167	131,000	935	140
3. Precipitation	159	92,000	715	129
4. DEAE-cellulose	43	21,000	90	232
5. Hydroxylapatite	10	11,000	28	394

both present at early stages of purification, were eliminated at Step 5.

The primary problem in the purification of cysteine *S*-conjugate *N*-acetyltransferase was the lability of the enzyme. Upon extraction, 20% of *N*-acetyltransferase activity was lost at 4° overnight. Despite improvement in stability resulting from the presence of 30% (v/v) glycerol and 1 mM dithiothreitol, it was necessary to carry out the procedure rapidly to the stage of Step 5. The purified enzyme, at a protein concentration of 5 mg/ml in 150 mM potassium phosphate (pH 7.0), 1 mM dithiothreitol, 3 mM sodium azide, and DGDC (5 mg/ml), lost only 30% of its activity upon storage at 4° for 3 months. Nevertheless, subsequent attempts at purification met with large losses and minimal increase in specific activity.[5]

Based on the use of globular proteins of known size that were assumed to have similar partial specific volumes, the M_r of cysteine *S*-conjugate *N*-acetyltransferase was estimated as 34,000 by gel filtration with Sepharose 6B (Fig. 1C). The optimal pH for the acetylation of *S*-benzyl-L-cysteine with the partially purified preparation was at about pH 6.8–7.0 (Fig. 2). In contrast to a previous report in which microsomes from rat liver were used (10), the pH optimum of the soluble enzyme was the same in both phosphate and Tris buffers.

Substrates. The purified *N*-acetyltransferase catalyzed the acetylation of low polarity, *S*-substituted derivatives of L-cysteine but not of D-cysteine (Table 2). The D and L isomers of cysteine were not substrates, nor were L-serine, L-methionine, L-leucine, L-phenylalanine, and L-glutamate; only L-tryptophan served as an acetyl acceptor, albeit at a low rate. The apparent K_m for acetylation of cysteine *S*-conjugates (Table 2) decreased with increasing lipophilicity of the substituent, i.e., K_m for ethyl > propyl > benzyl > butyl. The correlation ($r = 0.99$) of the Hansch π constants, as a measure of lipophilicity, with V_m/K_m as a measure of catalytic efficiency, is shown in Fig. 3.

Substrates in which oxygen was substituted for sulfur, as in *O*-benzyl-L-serine, were acetylated but with a much greater apparent K_m than for *S*-benzyl-L-cysteine. Substrates effective with cytosolic *N*-acetyltransferase, e.g., aniline and p-aminobenzoate, were not acetylated in the standard assay system.

With *N*-[^{14}C]acetyl-*S*-benzyl-L-cysteine and CoASH,

[5] Chromatography on Phenyl Sepharose (Pharmacia) as well as on the *S*-cysteine and *S*-coenzyme A derivatives of Affigel 501 (Bio-Rad) was attempted with poor recovery. Gel filtration on Sepharose 6B resulted in a 20% loss of enzyme activity with only a 1.5-fold increase in specific activity.

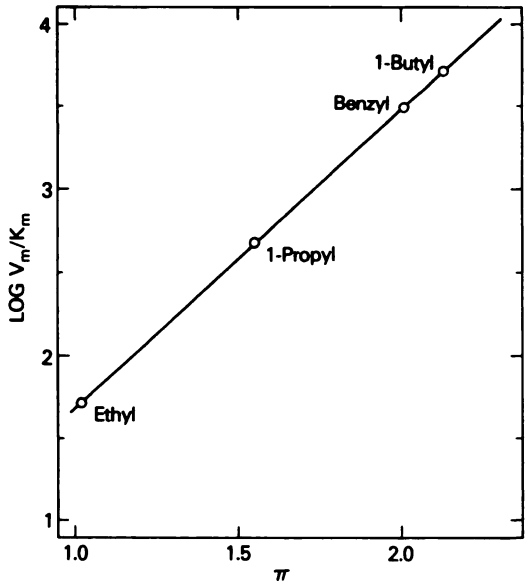

FIG. 2. *pH optimum*

All reaction rates were determined under standard assay conditions with 50 mM sodium acetate (▲), 100 mM potassium phosphate (○), or 100 mM Tris-HCl (■).

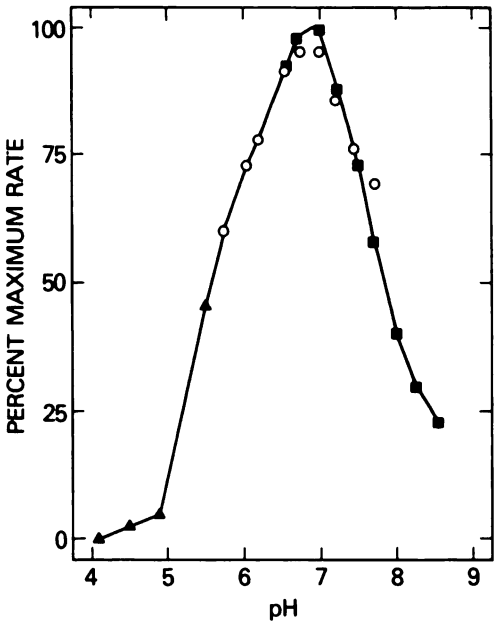

FIG. 3. *Correlation of catalytic efficiency of cysteine S-conjugate N-acetyltransferase with substituent lipophilicity*

Log V_m/K_m is correlated with Hansch π constants (16) for the indicated substituents.

Reaction 1 was not experimentally reversible. For Reaction 1, in an otherwise standard assay system, an apparent K_m of 26 μM (V_m = 420 nmoles/min^{-1} mg^{-1} protein) was calculated for CoASAc.

TABLE 2

Substrate specificity of cysteine S-conjugate N-acetyltransferase

Compound	Relative[a] rate	Apparent K_m[b]	Apparent V_m[b]	Extraction[c] efficiency
		μM	*nmoles/min/mg protein*	%
L-Cysteine	<0.1[d]			75
S-Carboxymethyl-L-cysteine	<0.1			
S-Methyl-L-cysteine	0.7			100
S-Ethyl-L-cysteine	11	7100	360	92
S-1-Propyl-L-cysteine	46	670	330	95
S-1-Butyl-L-cysteine	69	63	320	98
S-Benzyl-L-cysteine	100	140	440	101
S-Benzyl-D-cysteine	1.1			
S-Benzyl-L-cysteine methyl ester	7	4200	120	98
O-Benzyl-L-serine	16	2600	210	101
O-Benzyl-D-serine	<0.1			
S-2,4-Dinitrophenyl-L-cysteine	110	250	670	99
L-Tryptophan	0.3			104

[a] Under standard assay conditions at an acetyl acceptor concentration of 1 mM.

[b] Apparent K_m and V_m with 0.8 mM CoASAc under standard assay conditions.

[c] Percentage of acetylated product extracted by cyclohexanone.

[d] In all cases, <0.1 indicates that the rate was below the limit of detection of the assay.

DISCUSSION

Cysteine *S*-conjugate *N*-acetyltransferase has been solubilized from rat kidney microsomes and stabilized to a degree allowing characterization of its substrate spectrum. The low level of purification that has been achieved nevertheless removed interfering hydrolytic enzymes that included proteases, presumably the major cause of instability in crude enzyme solutions, and those enzymes catalyzing deacylation of both mercapturic acids and CoASAc.

The enzyme appears to be broadly effective with thioethers of L-cysteine and its analogues, e.g., *O*-benzyl-L-serine (Table 2). Efficiency of catalysis is a function of the lipophilicity of the specific substituent at sulfur (Fig. 3). Although absolute specificity for cysteine derivatives has not been observed with these preparations, it is clear that the enzyme is inactive with normal substrates for arylamine *N*-acetyltransferases (8).

The specificity for lipophilic *S*-conjugates of L-cysteine correlates with reports of metabolism studies with intact rats (17, 18). Although the *in vivo* investigation was carried out with a different strain of rat, and must necessarily take into account such factors as reabsorption, binding to serum proteins, and fat deposition, the trend of increasing acetylation of cysteine thioethers with increasingly lipophilic substituents was entirely similar (17, 18).

The distribution of cysteine *S*-conjugate *N*-acetyltransferase also deserves comment. Although present in rat liver, specific activity in rat kidney was almost twice as great (10). The enzyme was found in the heavy microsomal fraction of rat kidney along with the γ-glutamyltranspeptidase and aminopeptidase activities (19) necessary for conversion of glutathione thioethers to cysteine thioethers. All three enzymes have been histologically localized in the outer stripe of the kidney medulla

(19), although the peptidases are thought to be at the brush-border membranes whereas the acetyltransferase is probably associated with the endoplasmic reticulum (19).

On the basis of the substrate specificity of the solubilized enzyme as well as the rates of mercapturate excretion and tissue distribution, the cysteine *S*-conjugate *N*-acetyltransferase from kidney appears to be a good candidate for the enzyme acting in the last step of mercapturate formation.

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