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Biochemical Pharmacology 62 (2001) 1345–1353

Biochemical Pharmacology

Co-purification of mitochondrial HSP70 and mature protein disulfide isomerase with a functional rat kidney high-M_r cysteine *S*-conjugate β-lyase

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Received 12 March 2001; accepted 17 May 2001

Abstract

S-(1,1,2,2-Tetrafluoroethyl)-L-cysteine (TFEC, the cysteine *S*-conjugate of tetrafluoroethylene) is an example of a nephrotoxic, halogenated cysteine *S*-conjugate. Toxicity results in part from the cysteine *S*-conjugate β-lyase(s)-catalyzed conversion of TFEC to a thioacylating fragment with the associated production of pyruvate and ammonia. In the present study, we have demonstrated that rat kidney homogenates contain at least three enzyme fractions that are capable of catalyzing a cysteine *S*-conjugate β-lyase reaction with TFEC. One of these fractions contains a high-M_r lyase. At least two proteins co-purify with this high-M_r complex. N-Terminal analysis (15 cycles) revealed that the smaller species was mature protein disulfide isomerase (M_r ~54,200) from which the 24 amino acid endoplasmic reticulum signal peptide had been removed. Internal amino acid sequencing (15 cycles) revealed that the larger species was mitochondrial HSP70 (mtHSP70; M_r ~75,000). The present findings offer an explanation for the previous observation that mtHSP70 in kidney mitochondria is heavily thioacylated when rats are injected with TFEC (Bruschi *et al.*, J Biol Chem 1993;268:23157–61).

Keywords: Cysteine *S*-conjugates; Halogenated xenobiotics; mtHSP70; Protein disulfide isomerase; *S*-(1; 1; 2; 2-Tetrafluoroethyl)-L-cysteine

1. Introduction

Many electrophiles are detoxified in part by the mercapturate pathway (electrophile → glutathione *S*-conjugate → L-cysteinylglycine *S*-conjugate → L-cysteine *S*-conjugate → *N*-acetyl L-cysteine *S*-conjugate (mercapturate) → excretion) [reviewed in Refs. 1–5]. Alternatively, however, L-cysteine *S*-conjugates that contain a good leaving group may be converted to aminoacrylate and a thiol-containing fragment (RSH) by cysteine *S*-conjugate β-lyases. Aminoacrylate is unstable and is hydrolyzed non-enzymatically to pyruvate and ammonia [net reaction:

$$RSCH_2CH(CO_2^-)NH_3^+ + H_2O \rightarrow CH_3C(O)CO_2^- + NH_4^+ + RSH].$$

Generally, if the RSH is stable, the cysteine *S*-conjugate (e.g. BTC) is not particularly toxic [5]. However, several halogenated cysteine *S*-conjugates, such as DCVC, *S*-(1,2,2-trichlorovinyl)-L-cysteine, CTFC, and TFEC are toxic because the eliminated fragments are reactive thioacylating agents [3,5]. Although the kidneys (especially the S_2 and S_3 segments of the proximal tubules) are the major target of toxic cysteine *S*-conjugates, damage to liver, brain, and other organs may also occur [3–7].

Several PLP-containing enzymes, such as kynureninase

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Abbreviations: AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; BTC, benzothiazolyl-L-cysteine; CTFC, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; cyt, cytosolic; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DTT, dithiothreitol; GTK, glutamine transaminase K; mt, mitochondrial; NBT, nitroblue tetrazolium; PDI, protein disulfide isomerase; PLP, pyridoxal 5′-phosphate; PMS, phenazine methosulfate; PVDF, polyvinylidene difluoride; T_3, 3,3′,5-triiodo-L-thyronine; TFA, trifluoroacetic acid; and TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

0006-2952/01/$ – see front matter
PII: PII S0006-2952(01)00802-4

[8], cytGTK [9], and kynurenine-pyruvate aminotransferase, possess strong cysteine *S*-conjugate β-lyase activity [10,11]. (cytGTK and kynurenine-pyruvate aminotransferase may be products of alternative splicing [12][2]). In addition, alanine aminotransferase and cytAspAT have weak cysteine *S*-conjugate β-lyase activity [13–15]. Kynureninase, cytGTK, kynurenine-pyruvate aminotransferase, alanine aminotransferase, and cytAspAT have M_r values of ~90,000–100,000, and are collectively referred to here as low-M_r cysteine *S*-conjugate β-lyases.

Mitochondria are especially vulnerable to toxic cysteine *S*-conjugates such as DCVC and TFEC [e.g. Refs. 16 and 17]. When TFEC was injected into rats, several kidney mitochondrial proteins (most notably mtHSP70) were tagged by a thioacylating fragment [18,19]. A less heavily tagged protein was mtAspAT [18]. The mitochondrial α-ketoglutarate dehydrogenase complex is inactivated in the kidneys of rats injected with TFEC. Thioacylation of both the E2o (E2k) and E3o (E3k) subunits was observed [19]. Mitochondria are targeted in PC12 cells exposed to TFEC with concomitant inactivation of the α-ketoglutarate dehydrogenase complex, mtAspAT, and the regulatory machinery of the pyruvate dehydrogenase complex [20]. The findings [19,20] support the earlier work of Stonard and Parker [21] who suggested that a metabolite of DCVC inhibits mitochondrial α-ketoglutarate and pyruvate oxidation. The toxicity of several cysteine *S*-conjugates toward human Chang liver cells follows the rank order of these compounds as substrates of cysteine *S*-conjugate β-lyase(s) in the mitochondrial fraction [22]. Finally, apoptosis follows mitochondrial damage in LLC-PK1 cells exposed to 0.5 mM DCVC [23].

Several factors could contribute to the selective vulnerability of the S_2/S_3 segments of the kidney proximal tubules such as site-selective uptake and/or rapid rates of mitochondrial transport of cysteine *S*-conjugates (or precursors). Nevertheless, a possibility for the selective vulnerability is the presence of highly active cysteine *S*-conjugate β-lyases in the kidney tubules. We previously discovered and partially purified a high-M_r cysteine *S*-conjugate β-lyase in rat kidney homogenates [24]. The enzyme appears to be especially abundant in the S_2 and S_3 regions of rat kidney proximal tubules [25]. In the present work, we show that mtHSP70 and PDI in rat kidney homogenates co-purify with this high-M_r cysteine *S*-conjugate β-lyase.

[2]Rat kidney cytGTK is a homodimer. The sequence data of Abraham and Cooper [12] show the true molecular mass of the monomer to be 48.5 kDa. However, because of an unfortunate typographical error, it was reported to be 45.8 kDa. Thus, the mass of the cytGTK monomer (48.5 kDa) sequenced by Abraham and Cooper is slightly larger than the monomer of the closely related cysteine *S*-conjugate β-lyase/kynurenine pyruvate aminotransferase (47.8 kDa) reported by Perry *et al.* [10] and Mosca *et al.* [11].

2. Materials and methods

2.1. Materials

Ammediol (2-amino-2-methyl-1,3-propanediol), Tris, sodium pyruvate, aminooxyacetate, gabaculine, hydroxylamine–HCl (neutralized with NaOH before use), T_3, 3,3′,5,5′-tetraiodo-L-thyroxine, PLP, 2,4-dinitrophenylhydrazine, DTT, 2-mercaptoethanol, bovine serum albumin, Amido Black, Coomassie Brilliant Blue R, PMS, NBT, methanol, acetic acid, bromophenol blue, glycerol, Sephacryl S-200, and Phenyl-Sepharose CL-4B were obtained from the Sigma Chemical Co. SDS was obtained from Boehringer-Mannheim. Hydroxyapatite was purchased from Bio-Rad. DE-52 was obtained from Whatman. TFEC was synthesized as previously described [26]. DCVC and CTFC were gifts from Dr. James Stevens (University of Vermont). Stock solutions of 20 mM BTC (acetate salt; synthesized as described below) in 100 mM ammediol–HCl buffer (pH 9.0), 40 mM TFEC in 100 mM Tris–HCl buffer (pH 8.5), 40 mM CTFC in 100 mM Tris–HCl buffer (pH 8.5), and 20 mM DCVC in 100 mM Tris–HCl buffer (pH 8.5) were stored at –20°.

2.2. Synthesis of BTC

BTC was synthesized by a new procedure. Sodium hydroxide (1.83 g; 45 mmol) was dissolved in 50 mL of methanol. L-Cysteine (2.63 g; 15 mmol) was added, and the suspension was stirred under argon for 10 min. 2-Chlorobenzothiazole (2.53 g; 1.95 mL; 15 mmol) (Aldrich Chemical) was added drop-wise from a syringe, and the suspension was refluxed under argon for 10 min. The reaction was then stirred at room temperature overnight. Water (200 mL) was added, and the methanol was removed by rotary evaporation. At this point, solid sodium hydroxide was added until the entire white residue was dissolved, yielding a yellow solution. The solution was extracted with ether (3 × 50 mL). The aqueous layer was transferred to a 250-mL flask and acidified with glacial acetic acid to approximately 6.0. The resultant copious white precipitate was cooled on ice overnight, vacuum filtered, and washed with 150 mL of ice-cold ammonium acetate solution (100 mM; pH 6.7). The white solid was dried thoroughly under vacuum to yield 3.07 g of white solid (82% crude yield). This solid was recrystallized from isopropanol:water:acetic acid (7:3:1, by vol.) after hot filtration.

The resultant white crystals of BTC acetate were air dried, and then washed with ice-cold ether. The material was judged to be pure by the criterion of HPLC analysis [C-18 column (4.6 × 250 mm), Altech] on a Hewlett-Packard 1090 II/L system with eluant monitored at 280 nm by a diode array detector. Conditions for chromatography were as follows: solvent A = water containing 0.6% (v/v) TFA; solvent B = acetonitrile containing 0.6% (v/v) TFA. The column was equilibrated with 30% B/70% A, and

eluted with this mixture for 5 min after injection of the sample. A gradient was then run to 90% B/10% A over the next 15 min. Finally, the column was eluted with 90% B/10% A for 5 min. All elutions were carried out at ambient (room) temperature at a flow rate of 1.0 mL/min. The retention times of the starting material (2-chlorobenzothiazole) and product were 16.5 and 6.9 min, respectively. Proton NMR spectroscopy (Varian VXR 300) yielded results identical to those reported previously [27]. Finally, LC-MS (VG Quattro II LC/ES-MS/MS, Micromass Ltd.) showed the expected pseudo-molecular ion $m/z = 255$.

2.3. Enzyme assays

Cysteine *S*-conjugate β-lyase was assayed using two alternative substrates. Assay mixture 1 contained 10 mM BTC, 50 μM PLP, and 100 mM ammediol–HCl buffer (pH 9.0) plus enzyme; final volume, 20 μL. Assay mixture 2 contained 20 mM TFEC, 50 μM PLP, and 100 mM Tris–HCl buffer (pH 8.5) plus enzyme; final volume, 20 μL. After incubation at 37° in a covered 96-well plate for 10 min, the reaction was terminated by the addition of 20 μL of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After 10 min at room temperature, 180 μL of 1 M NaOH was added, and the absorbance at 430 nm was determined in a SpectraMax 96-well plate spectrophotometer (Molecular Devices). The blank contained the complete reaction mixture to which was added enzyme after the addition of 2,4-dinitrophenylhydrazine reagent. The molar extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is ~15,000. TFEC, but not BTC, undergoes some non-enzymatic conversion to pyruvate in the presence of 50 μM PLP (~2 nmol/hr/20 μL of assay mixture).

GTK was assayed by the method of Cooper [28] as modified for multi-well plate analysis. The reaction mixture (50 μL) contained 5 mM α-keto-γ-methiolbutyrate, 20 mM L-phenylalanine, and 100 mM ammediol–HCl (pH 9.0). After incubation at 37°, the reaction was terminated by the addition of 150 μL of 1 M KOH, and the absorbance was read at 322 nm at 25°. The molar extinction coefficient of phenylpyruvate (enol) under these conditions is 16,000.

For all of the enzymes studied here, one unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product/min at 37°. Protein was measured by the procedure of Lowry *et al.* [29] adapted to a microplate reader. Bovine serum albumin was used as a standard.

2.4. Electrophoresis

Non-denaturing (native) PAGE was carried out using precast gels (4% stacking gel, 7.5% separating gel) (Bio-Rad). Conditions: upper and lower buffers, 25 mM Tris–192 mM glycine; 150 V; 22°. A 10-μL aliquot of the native protein solution was applied to each lane. After 2 hr of electrophoresis, the gel was subjected to activity staining (see below) or stained with 0.1% (w/v) Coomassie Brilliant Blue R in 45% (v/v) methanol/10% (v/v) acetic acid. Denaturing PAGE was identical to non-denaturing PAGE except that (a) the buffers contained 0.1% (w/v) SDS, and (b) electrophoresis was for 1 hr. Prior to loading on the gel, the protein samples were boiled in sample buffer [12 mM Tris–HCl (pH 6.8), 5% glycerol, 0.4% (w/v) SDS, 3 mM 2-mercaptoethanol, 0.02% (w/v) bromophenol blue], and 10–15 μL of the denatured protein solution was applied to each lane. Protein bands in the denaturing gel were either transferred to a PVDF membrane (see below) or visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R in 45% (v/v) acetic acid.

2.5. N-terminal and internal protein sequencing

Following SDS–PAGE, proteins in the gel were transferred to a PVDF membrane (Bio-Rad) using degassed 10 mM cyclohexylaminopropane sulfonic acid (ICN Biochemicals) in 10% (v/v) methanol (pH 11.0) as the transfer buffer (600 V, 2.5 hr). Proteins on the PVDF membrane were visualized by staining for 5 min with 0.1% (w/v) Amido Black in 40% (v/v) methanol/10% (v/v) acetic acid. Stained bands were cut from the membrane and were subjected to either N-terminal analysis or internal sequence analysis. Microsequencing was carried out at the Protein/DNA Technology Center of Rockefeller University according to the method of Fernandez *et al.* [30,31].

2.6. Activity staining

The procedure used was a modification of that described previously from our laboratory [24]. After non-denaturing PAGE, the gel was immersed in a solution containing 2 mM cysteine *S*-conjugate, 0.05 mM PLP, 1 mM NBT, 0.1 mM PMS, and 100 mM potassium phosphate buffer (pH 7.4). The eliminated sulfhydryl-containing fragment reduces the NBT/PMS solution to form an insoluble blue formazan band corresponding to enzyme activity. After incubation in the dark at 22° for 2 hr, the gel was rinsed thoroughly with water and photographed.

3. Results

3.1. Partial purification of three fractions containing cysteine S-conjugate β-lyase activity from rat kidney homogenates

All steps were carried out at 0–4°. Frozen rat kidneys (32 g) from young, male Fischer 344 rats were thawed and homogenized in 300 mL of 5 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTT and 1 mL of protease inhibitor "cocktail" (Sigma) using a Waring blender. The homogenate was centrifuged at 10,000 *g* for 30 min, and the precipitate was discarded. Solid ammonium sulfate was added to the supernatant to achieve 40% saturation with

Table 1
Purification of cysteine *S*-conjugate β-lyases from rat kidney

Step	Protein (mg)	Volume (mL)	TFEC lyase (U)	BTC lyase (U)	GTK (U)
Homogenate	4820	300	80	27	164
Ammonium sulfate cut (40–80%)	1670	120	53	16	82
DE-52	818	290	40	15	64
Phenyl Sepharose	405	56	35	10	50
Hydroxyapatite (fraction eluted with 50 mM potassium phosphate, pH 7.4; Fraction A)	80	330	30	4.2	30
Hydroxyapatite (fraction eluted with 250 mM potassium phosphate, pH 7.4; fraction B)	33	50	1.0	2.0	0.17
Fraction A					
Sephacryl S-200 (portion eluted in the low-M_r range; Fraction 1)	26	1	12.4	0.4	18
Sephacryl S-200 (portion eluted in the high-M_r range; Fraction 2)	18	1	5.6	1.2	5
Fraction B					
Sephacryl S-200 (portion eluted in the high-M_r range; Fraction 3)	10	1	1.0 (1.0)[a]	1.2	0.12

[a] The value in parentheses refers to cysteine-*S*-conjugate β-lyase activity with DCVC.

gentle stirring for 5 min. After centrifugation at 10,000 *g* for 10 min, the precipitate was discarded. Additional solid ammonium sulfate was added to bring the concentration to 80% saturation. After centrifugation at 10,000 *g* for 10 min, the supernatant was discarded. The precipitate was dissolved in the minimal amount of distilled water and dialyzed extensively against 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTT (buffer A). An inactive precipitate in the dialysate was removed by centrifugation at 10,000 *g* for 10 min. The dialysate was then applied to a DE-52 column (3 × 12 cm) equilibrated with buffer A. Cysteine *S*-conjugate β-lyase activities (with both TFEC and BTC) were eluted with a 1-L gradient of 1–200 mM NaCl in buffer A. These activities also co-eluted with GTK (Table 1).

The active fractions were collected, and solid ammonium sulfate was added to a final concentration of 1.5 M. Precipitated protein was removed by centrifugation at 10,000 *g* for 10 min, and the supernatant was applied to a Phenyl-Sepharose CL-4B column (1.5 × 12 cm) equilibrated with 1.5 M ammonium sulfate in buffer A. The column was eluted with 100 mL of 1.5 M ammonium sulfate in buffer A, followed by 400 mL of a linear gradient of 1.5 to 0.0 M ammonium sulfate in buffer A. GTK, TFEC lyase, and BTC lyase activities all eluted toward the end of the 1.5 M ammonium sulfate wash and the beginning of the gradient. Activity staining of this fraction with TFEC showed two bands with apparent M_r values of ≥ 200,000 [high-M_r cysteine *S*-conjugate β-lyase(s)] and ~95,000, respectively [low-M_r cysteine *S*-conjugate β-lyases] (data not shown).

The active fractions from the Phenyl-Sepharose column were pooled, dialyzed extensively against buffer A, and added to a hydroxyapatite column (1.5 × 7 cm) equilibrated with buffer A. The column was eluted with 500 mL of 50 mM potassium phosphate buffer (pH 7.4) (Fraction A). GTK, most of the TFEC lyase, and a portion of the BTC lyase activity were eluted with this buffer. The hydroxyapatite column was then eluted with 150 mL of 100 mM potassium phosphate buffer (pH 7.4), followed by 200 mL of 250 mM potassium phosphate buffer (pH 7.4) (Fraction B). The 250 mM potassium phosphate fraction contained some BTC lyase and TFEC lyase activities, but very little GTK activity. Activity staining with TFEC showed the presence of a high-M_r cysteine *S*-conjugate β-lyase in this fraction (data not shown).

Fraction A was dialyzed extensively against 2 mM potassium phosphate buffer (pH 7.4) and concentrated by addition to a small DE-52 column (0.8 × 2.0 cm). Elution was with 0.2 mL of 1 M potassium phosphate buffer (pH 7.4) followed by distilled water. The yellow eluate (~1 mL) was added to a Sephacryl S-200 column (3 × 22 cm) equilibrated with buffer A and eluted with buffer A. The fraction eluting in the low-M_r range (i.e. after the void volume) of the Sephacryl S-200 column, containing TFEC lyase and GTK, was pooled and concentrated on a small DE-52 column (as described above) to ~1 mL (Fraction 1). The fractions containing TFEC lyase and BTC lyase in the void volume of the Sephacryl S-200 column (apparent $M_r \geq$ 200,000) were collected and concentrated on a small DE-52 column to ~1 mL (Fraction 2). Fraction B was dialyzed against 2 mM potassium phosphate buffer (pH 7.4) and concentrated by addition to a small DE-52 column to ~1 mL. The yellow eluate (~1 mL) was added to a Sephacryl S-200 column (3 × 22 cm) equilibrated with buffer A and eluted with buffer A. BTC lyase and TFEC lyase activities eluting in the void volume (apparent $M_r \geq$ 200,000) were collected and concentrated on a small DE-52 column to ~1 mL (Fraction 3). No detectable cysteine *S*-conjugate β-lyase activity eluted in the low M_r range.

3.2. Inhibitor studies

The BTC lyase reaction in Fraction 3 was not inhibited by either 1 mM T_3 or 1 mM 3,3′,5,5′-tetraiodo-L-thyroxine. The BTC lyase activity of Fraction 3 was not inhibited by 10 mM gabaculine, but was inhibited strongly by 10 mM aminooxyacetate (~70% inhibition) and by 10 mM hydroxylamine (>95% inhibition).

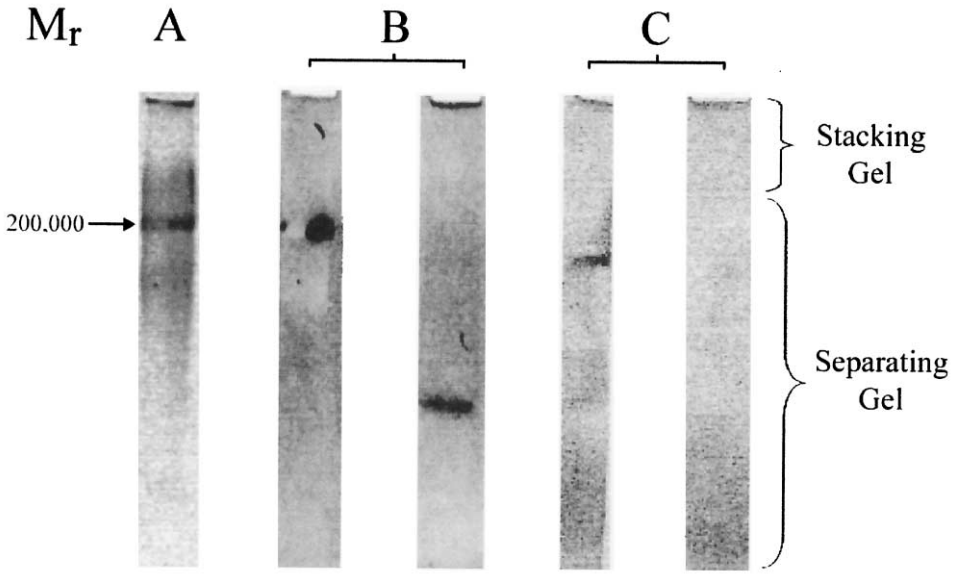

Fig. 1. Native PAGE of Fractions 1 and 3. (A) Coomassie Brilliant Blue staining of Fraction 3 (100 μg). (B, right lane) Activity staining of Fraction 1 (260 μg) with TFEC. (B, left lane) Activity staining of Fraction 3 (100 μg) with TFEC. (C, right lane) activity staining of Fraction 1 (260 μg) with BTC. (C, left lane) Activity staining of Fraction 3 (100 μg) with BTC. Note that the lanes in A and B are cut from a single slab, whereas the lanes in C are cut from a different slab subjected to non-denaturing PAGE at a different time. The arrow represents the position of a myosin standard (M_r 200,000) subjected to electrophoresis in the same slab gel as A and B.

3.3. Activity staining of fractions 1 and 3

Two aliquots of Fraction 3 and one aliquot of Fraction 1 were subjected to non-denaturing PAGE (Fig. 1). Staining of Fraction 3 with Coomassie Blue revealed a prominent band with an apparent M_r similar to that of a 200,000-M_r standard (Fig. 1, lane A). The position of this protein-stained band is identical to that obtained after activity staining with TFEC (Fig. 1, B, left lane). For comparison, activity staining of Fraction 1 with TFEC revealed a band only in the low-M_r region (estimated M_r ~90,000–100,000) (Fig. 1, B, right lane). Staining of Fraction 1 with Coomassie Brilliant Blue R showed three prominent bands in the low-M_r region, only one of which corresponded to the activity stain (data not shown). Activity staining of Fractions 1 and 3 with BTC (right and left lanes of Fig. 1, C, respectively) showed that the high-M_r cysteine *S*-conjugate β-lyase in Fraction 3 can catalyze a lyase reaction with both TFEC and BTC. On the other hand, the low-M_r cysteine *S*-conjugate β-lyase in Fraction 1 can catalyze a cysteine *S*-conjugate β-lyase reaction with TFEC but not with BTC. Activity staining showed that the high-M_r cysteine *S*-conjugate β-lyase (Fraction 3) and the low-M_r cysteine *S*-conjugate β-lyase (Fraction 1) were also active with CTFC and DCVC (data not shown). In summary, the high-M_r cysteine *S*-conjugate β-lyase (Fraction 3) was active with TFEC, DCVC, CTFC, and BTC, whereas the low M_r cysteine *S*-conjugate β-lyase (Fraction 1) was active with TFEC, DCVC, and CTFC, but not with BTC.

3.4. N-Terminal and internal microsequencing of proteins associated with the high-M_r cysteine S-conjugate β-lyase of Fraction 3

Only one major protein band was detected by staining with Coomassie Brilliant Blue R following electrophoresis under non-denaturing conditions (Fig. 1, lane A). However, when a 10-μL aliquot (100 μg) of Fraction 3 was subjected directly to SDS–PAGE, two prominent bands with apparent M_r values of about 50,000 (protein 1) and 70,000 (protein 2) were detected by Coomassie Brilliant Blue staining (data not shown). In a separate experiment, a sample of Fraction 3 (10 μL; 100 μg) was subjected to non-denaturing PAGE, and activity stained. The stained band was cut from the gel, boiled in 100 μL of sample buffer (see "Materials and methods"), and 15 μL of the supernatant was subjected to SDS–PAGE. Two bands with apparent M_r values of about 50,000 and 70,000, respectively, were again detected by staining with Coomassie Brilliant Blue R (data not shown). N-Terminal analysis (15 cycles) of protein 1 (~7.5 μg) revealed the following sequence: XXVLELTDENFESRV. A BLAST search showed a perfect match to mature rat/mouse PDI in which the first 24 amino acid residues at the N terminus of the PDI precursor protein had been removed. N-Terminal analysis of protein 2 (~7.5 μg) was not successful, but internal sequencing (15 cycles) was readily accomplished. The sequence obtained was: RQAVT-NPNNTFYAYK. A BLAST search revealed a perfect match encompassing residues 107–121 of rat mtHSP70.

4. Discussion

The present findings show that rat kidney homogenates contain at least three fractions that can catalyze a cysteine *S*-conjugate β-lyase reaction with TFEC. The low-M_r fraction (M_r ~90,000–100,000; Fraction 1) was highly enriched in GTK. This fraction contained very little BTC lyase activity compared with TFEC lyase activity (Table 1). Moreover, this fraction could be activity stained with TFEC, DCVC, and CTFC, but not with BTC. Our findings are in agreement with previous work showing that highly purified cytGTK catalyzes a β-lyase reaction with TFEC, DCVC, and TFEC, but not with BTC [26,32].

The high-M_r fraction (Fraction 2) contained some contaminating GTK. However, the high ratio of TFEC lyase to GTK and the presence of BTC lyase suggest that Fraction 2 contains cysteine *S*-conjugate β-lyases in addition to cytGTK. Fraction 2 was enriched in alanine-glyoxylate aminotransferase II (M_r ~210,000) activity (data not shown). The possibility that a TFEC/BTC lyase in Fraction 2 is alanine-glyoxylate aminotransferase II is currently under investigation.

The high-M_r fraction ($M_r \geq$ 200,000; Fraction 3) contained the enzyme that we previously detected in rat kidney preparations by activity staining [24,25]. In a previous study, we tentatively suggested that this high-M_r cysteine *S*-conjugate β-lyase contained at least two components with apparent M_r values of about 50,000 and 70,000, respectively [24]. Our current preparation, which is more pure and was obtained by a slightly different procedure, also contains two major proteins with apparent M_r values of about 50,000 (protein 1) and 70,000 (protein 2). Microsequencing showed a perfect match with mature rodent PDI (M_r of precursor protein ~56,600; M_r of mature protein ~54,200; accession number P11598 or S41661) and mtHSP70 (M_r ~75,000), respectively. We have observed proteins 1 and 2 in three separate preparations of the rat kidney high-M_r cysteine *S*-conjugate β-lyase.

Our data show that the mature PDI (M_r ~54,200) in our preparation of purified rat kidney high-M_r cysteine *S*-conjugate β-lyase (Fraction 3) is part of a larger protein complex ($M_r \geq$ 200,000). Precedence exists for PDI in a protein complex. PDI is a component of prolyl hydroxylase (an $\alpha_2\beta_2$ tetramer; M_r ~250,000) and of microsomal triacylglycerol transfer protein (MTP) [33–35]. PDI is also thought to regulate the modulation of rat liver *S*-adenosylmethionine synthetase by glutathione [36] and to participate in peptide binding, cell adhesion, and perhaps chaperoning [37]. In addition, PDI is a membrane-associated thyroid-hormone binding protein that strongly binds T_3 [38]. However, neither T_3 nor 3,3′,5,5′-tetraiodo-L-thyroxine inhibited the BTC lyase activity of Fraction 3.

PDI appears to be particularly sensitive to attack from small, halogenated electrophiles [39–41]. Thus, if PDI is part of a high-M_r cysteine *S*-conjugate β-lyase, it is possible that it will be thioacylated in the presence of TFEC.

In the cell, PDI is thought to be predominantly present in the endoplasmic reticulum. However, recent work has shown that PDI is also present in the mitochondrial outer membrane [42,43]. Therefore, a portion of cellular PDI and mtHSP70 may be present in the same compartment.

Previously, the nucleotide sequence of rat liver PDI was identified with that of iodothyronine 5′-monodeiodinase [44]. However, it is now known that the three major mammalian deiodinases are selenoproteins. Because PDI seems to bind to type I iodothyronine 5′-monodeiodinase, it may play a regulatory role in the deiodination reaction [45]. Perhaps PDI also plays a regulatory role in the rat kidney high-M_r cysteine *S*-conjugate β-lyase complex of Fraction 3. Further work is necessary, however, to determine whether PDI is a true component of this high-M_r cysteine *S*-conjugate β-lyase or whether it co-purifies fortuitously with the complex.

As noted for PDI, mtHSP70 (M_r 75,000) in our preparation of rat kidney high-M_r cysteine *S*-conjugate β-lyase (Fraction 3) is part of a larger protein complex ($M_r \geq$ 200,000). Dekant, Anders and their colleagues have shown that the sulfhydryl-containing fragment [CF_2HCF_2SH] eliminated from TFEC spontaneously loses HF to yield difluorothionoacetyl fluoride [$CF_2HC(=S)F$], which is a powerful thioacylating agent [7 and references quoted therein]. Because of its reactivity, it is likely that the thionoacetyl fluoride after its formation would not diffuse far before reacting with a suitable nucleophile *in vivo*. Thus, the presence of mtHSP70 within (or associated with) a mitochondrial high-M_r cysteine *S*-conjugate β-lyase complex provides a satisfactory explanation for the previous finding [18] that kidney mtHSP70 is adducted after injection of TFEC into rats.

mtHSP70 is known to be present not only in the mitochondria, but also in extramitochondrial compartments with proposed roles in antigen binding and acquisition of cellular immortalization [46–49]. Significant amounts of mtHSP70 have been found in association with plasma membranes, cytoplasmic vesicles, and cytoplasmic granules [e.g. Refs. 50–52]. mtHSP70 mediates protein transport across the inner mitochondrial membrane and protein folding in the matrix [e.g. Ref. 53]. High-M_r cysteine *S*-conjugate β-lyase was found previously to be present in both the cytosolic and mitochondrial fractions of rat kidney homogenates [24]. Whether the same high-M_r cysteine *S*-conjugate β-lyase is present in both compartments remains to be established, but it is possible that mtHSP70 is a component of both complexes.

Binding of fragments derived from toxic cysteine *S*-conjugates to macromolecules is associated with thiol depletion, oxidative stress, lipid peroxidation, and increased cytosolic calcium in kidney epithelial cells [54]. The oxidative stress induced by cysteine *S*-conjugates in LLC-PK1 cells leads to elevated expression of the stress-responsive genes *gadd153* [55] and *hspP70* [56]. It will be interesting to determine whether the lyase protein in the high-M_r cys-

teine *S*-conjugate β-lyase complex is up-regulated in conjunction with HSP70 and whether this leads to increased formation of tagged mtHSP70 in the presence of a suitable cysteine *S*-conjugate.

A membrane-associated form of brain L-glutamate decarboxylase (a PLP-dependent enzyme) co-purifies with the 70-kDa heat shock cognate (HSC70) [57], another member of the HSP70 family [58]. Therefore, precedence exists for a PLP-containing enzyme co-purifying with a member of the HSP70 family. The high-M_r cysteine *S*-conjugate β-lyase can be stimulated by α-keto acids or by PLP [24]. Fraction 3 exhibited a small amount of aminotransferase activity with phenylalanine and α-keto-γ-methiolbutyrate as substrates (Table 1), which we previously showed to co-migrate with the high-M_r lyase [59]. The present work indicates that the activity of the high-M_r cysteine *S*-conjugate β-lyase is strongly inhibited by the general PLP antagonists hydroxylamine and aminooxyacetate, but not by gabaculine (a selective, mechanism-based inhibitor of GABA aminotransferase [60]). Our findings are in accord with the presence of a PLP-containing subunit in the high-M_r cysteine *S*-conjugate β-lyase that is likely to be (or related to) an α-amino acid/α-keto acid aminotransferase. Many aminotransferases have subunits with M_r values close to 50,000 (e.g. cytGTK, mtAspAT, cytAspAT, AlaAT, alanine-glyoxylate aminotransferase II), and many have a blocked N terminus. Therefore, it is possible that the associated PLP-containing subunit co-migrates with the mature PDI (M_r ~54,200) in the SDS gel, but that it is not detected by N terminal analysis because of blockage at the N terminus.

mtAspAT can form an association with cytosolic HSC70 [61]. For example, HSP70 can bind to acid-unfolded apo-mtAspAT, slowing its refolding. It was proposed that the folding might be necessary for efficient uptake of apo-mtAspAT from its site of synthesis into the mitochondria [62]. It is interesting that the productive binding of a protein substrate to HSP70 is coupled to the ATPase activity of the chaperone, which in turn is controlled by several co-chaperones [63]. In the absence of a nucleotide and co-chaperones, the protein substrate may become kinetically trapped [63]. Therefore, it is conceivable that (a) a partially unfolded mtAspAT or another PLP-containing enzyme is "frozen" into an HSP70 complex upon homogenization of the kidney, (b) this partially unfolded PLP-containing enzyme allows the binding of large cysteine *S*-conjugates to the active site, and (c) such binding results in the creation of an active site that can catalyze an effective β-elimination with cysteine *S*-conjugates but slow transamination with phenylalanine. The nature of the PLP enzyme within the complex is currently under investigation.

Acknowledgments

This work was supported by NIH Grants RO1 ES008421 to A.J.L.C. and R29 GM5196 to S.A.B.

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