0026-895X/95/050855-06$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 48:855–860 (1995).

MOL PHARM

MOLECULAR PHARMACOLOGY

Glutamine Transaminase K Is Not a Major Cysteine *S*-Conjugate *β*-Lyase of Rat Kidney Mitochondria: Evidence that a High–Molecular Weight Enzyme Fulfills This Role

DICKY G. ABRAHAM,[1] RANDI J. THOMAS, and ARTHUR J. L. COOPER

Departments of Biochemistry (D.G.A., R.J.T., A.J.L.C.) and Neurology and Neuroscience (A.J.L.C.), Cornell University Medical College, New York, New York 10021

Received April 11, 1995; Accepted August 22, 1995

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

SUMMARY

Glutamine transaminase K (homodimer; M_r of monomer ~45,000) is a major cysteine *S*-conjugate *β*-lyase of rat kidney cytosol. Several cysteine *S*-conjugates are known to cause kidney damage. Mitochondria are especially sensitive, and glutamine transaminase K activity is present in the mitochondrial fraction of rat kidneys. Therefore, it is possible that the mitochondrial form of glutamine transaminase K is a cysteine *S*-conjugate *β*-lyase of the rat kidney and that this activity contributes to the mitochondrial damage. However, the literature contains conflicting data on this point. We obtained highly purified mitochondrial glutamine transaminase K and showed that it possesses little cysteine *S*-conjugate *β*-lyase activity with *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine as substrates. Recently, a high-molecular-weight cysteine *S*-conjugate *β*-lyase (M_r ~330,000) was shown to be present in the cytosol of rat kidney homogenates and partially purified. We present evidence that suggests that a similar high-molecular-weight enzyme is present in rat kidney mitochondria and that this protein may be a major cysteine *S*-conjugate *β*-lyase of these organelles.

Many halogenated xenobiotics are detoxified through the mercapturate pathway. For example, trichloroethylene and dichloroacetylene are converted to the corresponding glutathione conjugate (DCVG) *in vivo*. DCVG is converted subsequently to the corresponding cysteine conjugate (DCVC), which is converted in turn to the *N*-acetyl derivative (mercapturate) and excreted. Alternatively, DCVC may participate in a bioactivation reaction in which it is converted to pyruvate, ammonia, and a reactive fragment by the action of cysteine *S*-conjugate *β*-lyases (see Refs 1–3 for reviews). Trichloroethylene, DCVG, and DCVC are nephrotoxic. In addition, dichloroacetylene is neurotoxic in experimental animals (4) and in humans (5), presumably via conversion to DCVC. The reactive fragment formed from DCVC is a thioacetylating agent and binds to macromolecules (6, 7). The sulfhydryl-containing fragment apparently destroys renal epithelial cells, in part by a combination of covalent binding to macromolecules, depletion of nonprotein thiols, and lipid peroxidation (7, 8). Damage to the kidney by cysteine conjugates is confined largely to the S3 region of the nephron (e.g., Ref. 9). Kidney mitochondria are especially vulnerable to DCVC poisoning (8).

Cysteine *S*-conjugate *β*-lyase of rat liver was first described by Tateishi *et al.* in 1978 (10). Subsequently, Stevens reported that a major cysteine *S*-conjugate *β*-lyase of rat liver is identical to kynureninase (11). Shortly thereafter, Stevens *et al.* showed that a major cysteine *S*-conjugate *β*-lyase of rat kidney is identical to cytGTK (12), an enzyme discovered and characterized in our laboratory (13, 14). Apparently, DCVC binds to the active site of cytGTK, where it can undergo either (a) *β*-elimination to yield pyruvate, ammonia, and a fragment containing a reactive sulfhydryl or (b) transamination to "keto" DCVC. *β*-Elimination results in regeneration of active cofactor (i.e., PLP) at the active site. Transamination, however, results in formation of PMP at the active site. The PMP form of the enzyme cannot productively bind DCVC. The addition of an *α*-keto acid (e.g., phenylpyruvate, *α*KMB) to the reaction mixture stimulates conversion of the PMP form of the enzyme to the PLP form, allowing lyase activity to be maintained (3, 12). The transaminase activity of cytGTK is not stimulated by the addition of PLP, presumably because

This work was supported by National Institutes of Health Grant DK16739.
[1] Current address: Merck Research Laboratories, WP 26–265, West Point, PA 19486.

ABBREVIATIONS: *α*KMB, *α*-keto-*γ*-methiolbutyrate; ammediol, 2-amino-2-methyl-1,3-propandiol; BTC, benzothiazolyl-L-cysteine; Coomassie blue, Coomassie brilliant blue G250; cyt, cytosolic; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DCVG, *S*-(1,2-dichlorovinyl)glutathione; DTT, dithiothreitol; K, kidney type; GTK, glutamine transaminase K; lyase, cysteine *S*-conjugate *β*-lyase; mit, mitochondrial; ND-PAGE, nondenaturing polyacrylamide gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; PLP; pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

aspet

MOL PHARM

the cofactor is tightly bound (12, 13). However, later work showed that the lyase activity of cytGTK is stimulated by addition of PLP. Presumably, the affinity of the enzyme for PLP is much greater than that for PMP and PMP is displaced by PLP (15). Several groups have confirmed that highly purified cytGTK (M_r of the holoenzyme ~90,000; homodimer) of rat (15, 16), bovine (17), and human (17) kidney possesses considerable β-lyase activity toward DCVC, TFEC, and other cysteine *S*-conjugates. In addition to cytGTK, a larger cysteine, *S*-conjugate β-lyase (M_r of holoenzyme ~330,000), that possesses considerable activity toward DCVC (and TFEC) has recently been discovered and partially purified from the cytosolic fraction of rat kidney (15). This activity is well represented in both the cytosolic and mitochondrial fractions of rat kidney (15).

GTK activity is present in the mitochondrial fraction of rat kidney homogenates (13, 14, 18–21). Studies in which the rat kidney mitochondria were fractionated into outer membrane, inner membrane, matrix, and intermembrane space showed that the bulk of the mitGTK activity is in the matrix (18, 19, 21). It follows that because cytGTK has appreciable cysteine *S*-conjugate β-lyase activity, the mitochondrial form might also have such activity. If this were true, then the finding would implicate mitGTK in the process whereby DCVC induces damage to kidney mitochondria. Stevens *et al.* (21) reported that the matrix of rat kidney mitochondria contains DCVC lyase activity. The authors also reported that partially purified mitGTK has DCVC lyase activity (21). However, Lash *et al.* (18) reported that although the bulk of GTK activity in rat kidney mitochondria is in the matrix, most of the DCVC and BTC lyase activities are in the outer membrane. To understand the mechanisms by which cysteine conjugates damage kidney mitochondria, it will be necessary to resolve these contradictory reports. Accordingly, in a partial attempt to resolve the problem, we purified rat kidney mitGTK to the highest specific activity attained thus far and determined its DCVC lyase activity. In the present report, we show that highly purified mitGTK has relatively little DCVC lyase activity.

Experimental Procedures

Materials. All compounds were of the highest quality available. Ammediol, L-phenylalanine, PLP, DTT, sodium αKMB, Coomassie blue, phenazine methosulfate, nitroblue tetrazolium, and *Bacillus subtilis* L-alanine dehydrogenase (20 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). TFEC and DCVC were generous gifts from Dr. James L. Stevens (W. Alton Jones Cell Science Center, Lake Placid, NY). Frozen rat (Wistar) kidneys were obtained from Pel-Freez (Brown Deer, WI). Protein determinations were carried out essentially according to the method of Lowry *et al.* (22) using bovine serum albumin standard.

Enzymes and enzyme assays. GTK activity was routinely assayed by measuring phenylpyruvate formed in reaction mixtures (0.1 ml) containing 100 mM ammediol buffer, pH 9.0, 20 mM phenylalanine, 5 mM αKMB, and 0.1 mM PLP. [Transamination between phenylalanine and αKMB is catalyzed by GTK (23).] After incubation at 37° for 15–60 min, 0.9 ml of 3.3 M KOH is added, and the absorption at 322 nm is read ($\epsilon_{(phenylpyruvate\ enol)}$ = 24,000). In some experiments, the transamination reaction was carried out in the presence of 100 mM potassium phosphate buffer, pH 7.2, or Tris·HCl, pH 8.5. One unit of GTK activity is defined as the amount that catalyzes the formation of 1 μmol of phenylpyruvate/min at 37°. In other experiments, transamination in the reverse direction was measured. The reaction mixture contains 5 mM L-methionine, 0.1 mM phenylpyruvate [K_m ≤20 μM (14)], 100 mM ammediol, and enzyme in a final volume of 0.5 ml. At time zero, 0.2 ml of the reaction mixture is treated immediately with 0.8 ml of 3.3 M KOH, and the absorbance due to phenylpyruvate enol is read at 322 nm. The remainder of the assay mixture (0.3 ml) is incubated at 37° for 1 hr. A portion (0.2 ml) is then treated with 0.8 ml of KOH, and the absorbance at 322 nm is again determined. From the decrease in absorbance between 0 and 60 min, the disappearance of phenylpyruvate is calculated. In another experiment, 5 mM methionine was replaced with 5 mM DCVC or with 5 mM TFEC, and the disappearance of phenylpyruvate was measured. Cysteine *S*-conjugate β-lyase activity was measured according to the method of Abraham *et al.* (15). Typically, the reaction mixture contains 4 mM DCVC, 5 mM DTT, 5 mM αKMB, 0.1 mM PLP, 100 mM buffer (potassium phosphate, pH 7.2, or ammediol, pH 9.0), and enzyme in a final volume of 0.1 ml. After incubation at 37°, pyruvate formation is quantified with alanine dehydrogenase as described (15). To estimate the contribution of the high-molecular-weight enzyme to total lyase activity in whole kidney homogenate, 3000 g supernatant, and mitochondrial supernatant, 1-ml aliquots were added to small columns of hydroxylapatite. The low-molecular-weight lyase (mostly cytGTK) was eluted with 20 ml of 50 mM potassium phosphate buffer, pH 7.4, whereas the high-molecular-weight lyase was eluted with 3 ml of 1 M potassium phosphate, pH 7.4 (15).

Rat kidney cytGTK was purified as described (24). The preparation was homogeneous as judged by the appearance of a single band on SDS-PAGE stained with Coomassie blue or with silver (24). The specific activity of the purified cytGTK was 8.9 μmol/min/mg of protein (transaminase assay, pH 9.0; 37°) and 0.8 μmol/min/mg protein (lyase assay with DCVC, pH 9.0; 37°). Rat kidney mitGTK was purified according to the procedure of Cooper (23) except that

TABLE 1
Purification of mit GTK

Enzyme was purified from rat kidney as described by Cooper (23) except that the heat step was omitted. Values in parentheses are the units of lyase activity in the presence of 4 mM DCVC, 5 mM αKMB, 0.1 mM PLP, and 100 mM ammediol buffer, pH 9.0, 37°. Purification and yield are with respect to the transaminase activity in the mitochondrial homogenate.

Step	Volume	Protein	Activity	Specific activity	Yield	Purification
	ml	*mg*	*U*[d]	*units/mg*	*%*	
Whole homogenate	220	6435	103 (16)[a]	0.016		
3000 g supernatant	157	3376	77 (11.8)[a]	0.023		
Mitochondrial homogenate	30	660	10.5 (6.1)[a]	0.016	100	1
DE-52	60	54	5.3 (ND)[b]	0.098	50	6.5
Hydroxylapatite	45	11	4.1 (ND)[b]	0.37	40	26
Sephadex G-150[c]	2	0.4	1.9 (0.03)	4.75	18	313

[a] The whole homogenate, 3000 g supernatant, and mitochondrial homogenate contained 8.4, 6.3, and 5.5 units, pH 9.0, of high-molecular-weight lyase, respectively.
[b] Not done.
[c] The preparation is devoid of high-molecular-weight lyase.
[d] *U* = *μmol/min/mg of protein* in the standard transaminase assay (20 mM phenylalanine, 5 mM αKMB, 0.1 mM PLP, and 100 mM ammediol buffer, pH 9.0; 37°).

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

MOL PHARM

the heat step was omitted (Table 1). The specific activity of the purified mitochondrial enzyme (transaminase assay, pH 9.0; 37°) was 4.75 U/mg of protein.

PAGE. ND-PAGE was carried out as described previously (15). Activity staining for DCVC lyase and for GTK (transamination between phenylalanine and αKMB) on the gel slabs was carried out essentially according to the method of Abraham and Cooper (24). Protein bands on ND-PAGE and SDS-PAGE were visualized by staining with Coomassie blue (25).

Other procedures. Frozen kidneys were homogenized and fractionated into mitochondria and cytosol as described (26). Analysis of the two fractions with glutamate dehydrogenase (mitochondrial marker) and lactate dehydrogenase (cytosolic marker) showed that despite the freeze-thawing of the kidneys, the cytosolic fraction contained <10% contamination with mitochondrial matrix proteins and that the mitochondrial fractions contained <15% contamination with cytosolic proteins. In some cases, ammonia liberated in the lyase reaction was measured by a modification (24) of the indophenol method of Chaykin (27). Data are expressed as mean ± standard error. Statistical comparisons were carried out using the Mann-Whitney *U* test.

Results

Purification of mitGTK. The three-step procedure (Table 1) results in a preparation of rat kidney mitGTK with a specific activity of 4.75 μmol/min/mg of protein (transaminase assay, pH 9.0). The yield (18%) is somewhat less than that reported previously (31%) (23). However, the specific activity of the present preparation is ≥80% than that reported previously (21, 23). A previously reported purification procedure resulted in a preparation containing two proteins; the relative concentration of mitGTK to the other mitochondrial protein was estimated to be 1:4 (23). Analysis on ND-PAGE (Coomassie blue staining) revealed that the present preparation of mitGTK also contains the contaminating mitochondrial protein (M_r ~72,000), but the ratio of mitGTK to the other protein is estimated to be ~1:1 based on relative intensity of staining. The present preparation of mitGTK is unlikely to be contaminated with cytGTK. The two enzymes have different retention characteristics on DE 52 and hydroxylapatite (21, 23). Moreover, the purified mitochondrial enzyme is poorly recognized by rabbit antibodies to purified rat kidney cytGTK (data not shown). In summary, the present purification procedure, which is identical to that of Cooper (23) except that the heat step is omitted, results in a somewhat lower yield of enzyme but with a preparation of higher specific activity.

Transaminase activity of purified mitGTK. The dependence of phenylalanine-αKMB transaminase activity on pH and the effect of DTT and PLP were determined for the purified mitGTK and cytGTK preparations (Table 2). Neither mitGTK nor cytGTK require a reducing agent for full activity. Therefore, a reducing agent was not included in the standard transaminase assay mixture. The transaminase activity of purified cytGTK is not stimulated by the addition of PLP to the reaction mixture at any of the pH values investigated (Table 2). On the other hand, the transaminase activity of purified mitGTK is slightly, but significantly, stimulated at pH 9.0 by the addition of PLP but not at pH 8.5 or 7.2. The PLP effect on the activity of mitGTK at pH 9.0 was not noted with preparations of enzyme with a lower specific activity (23). It is possible that the PLP is less tightly bound at pH 9.0 or that the PMP formed in the presence of

TABLE 2

The effect of pH, DTT, and PLP on the transaminase activity of purified cytGTK and mitGTK

Assay mixtures (0.1 ml) contained 20 mM phenylalanine, 5 mM αKMB, 100 mM buffer (ammediol, pH 9.0, Tris · HCl, pH 8.5, or potassium phosphate, pH 7.2) and enzyme (~1 munits). After incubation at 37° for 10–60 min, phenylpyruvate was determined as described in the text. The values are given as average ± standard error of at least three determinations.

Conditions	Activity	
	cytGTK	mitGTK
	μmol/min/mg	
pH 9.0 (no addition)	8.9 ± 0.6	3.3 ± 0.2
pH 9.0 (+5 mM DTT)	9.1 ± 0.3	3.8 ± 0.3
pH 9.0 (+0.1 mM PLP)	8.7 ± 0.2	4.7 ± 0.1[a]
pH 9.0 (+5 mM DTT + 0.1 mM PLP)	9.2 ± 0.3	4.2 ± 0.2[a]
pH 8.5 (no addition)	8.1 ± 0.4	2.9 ± 0.3
pH 8.5 (+5 mM DTT)	7.9 ± 0.5	3.3 ± 0.1
pH 8.5 (+0.1 mM PLP)	7.8 ± 0.3	3.0 ± 0.2
pH 8.5 (+5 mM DTT + 0.1 mM PLP)	8.2 ± 0.2	3.5 ± 0.1
pH 7.2 (no addition)	5.3 ± 0.3	1.3 ± 0.1
pH 7.2 (+5 mM DTT)	5.1 ± 0.4	1.3 ± 0.1
pH 7.2 (+0.1 mM PLP)	5.4 ± 0.3	1.5 ± 0.3
pH 7.2 (+5 mM DTT + 0.1 mM PLP)	5.5 ± 0.2	1.5 ± 0.2

[a] Significantly different from activity in the absence of PLP and DTT (i.e., no addition) with $p < 0.05$.

amino acid substrate dissociates more readily. The pH optimum of mitGTK (≥9.0), like that of cytGTK, is high. However, the mitochondrial enzyme is proportionately less active at lower pH values than is the cytosolic enzyme. Thus, the ratio of activity at pH 7.2 to that at pH 9.0 is ~0.6 for the cytosolic enzyme but only ~0.3 for the mitochondrial enzyme (Table 2). The rate of mitGTK-catalyzed transamination (0.1 mM phenylpyruvate as amine acceptor) with 5 mM methionine, 5 mM DCVC, and 5 mM TFEC is 3.0, 0.45, and 0.5 μmol/min/mg of protein, respectively. Stevens *et al.* (21) previously noted that mitGTK catalyzes transamination between DCVC and αKMB.

Cysteine conjugate β-lyase activity of purified mitGTK. Table 1 shows that under the conditions used in the present work, the mitochondrial fraction contains ~35% of the total DCVC lyase activity of rat kidney homogenates. The specific activity of DCVC lyase in the mitochondria (~9 munits/mg of mitochondrial protein; Table 1) is similar to that previously reported by Stevens *et al.* (~8 munits/mg of matrix protein). During purification of mitGTK, most of the lyase activity in the mitochondrial fraction was lost (Table 1). Therefore, our highly purified preparation of mitGTK contains only weak lyase activity (Table 3). In contrast, highly purified cytGTK has considerable cysteine *S*-conjugate β-lyase activity (Table 3). TFEC is a better substrate for the lyase reaction catalyzed by cytGTK than is DCVC (7, 15). This selectivity also appears to be the case with the mitochondrial enzyme (Table 3). However, even with TFEC as substrate, purified mitGTK catalyzes a relatively slow β-lyase reaction compared with that catalyzed by purified cytGTK. The inherent cysteine *S*-conjugate β-lyase activity of mitGTK appears to be <5% that of cytGTK.

Activity staining. The occurrence of DCVC lyase and GTK (phenylalanine-αKMB transamination) activities in rat kidney cytosol and in rat kidney mitochondria was demonstrated by activity staining on ND-PAGE (Fig. 1). The intensity of the band corresponding to purified mitGTK (4.2 munits of transaminase activity at pH 7.2) when stained for

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

TABLE 3

Cysteine *S*-conjugate *β*-lyase activities of purified mitGTK and cytGTK

The reaction mixture contained 4–5 mM cysteine S-conjugate (DCVC or TFEC), 100 mM buffer (ammediol, pH 9.0, or potassium phosphate, pH 7.2), 5 mM αKMB, and enzyme (~10 munits, transaminase assay at pH 9.0). After incubation at 37° for 10–60 min, pyruvate was determined with alanine dehydrogenase. Values are given as mean ± standard error of at least three determinations.

Addition	Activity			
	DCVC (pH 7.2)	TFEC (pH 7.2)	DCVC (pH 9.0)	TFEC (pH 9.0)
	μmol/min/mg			
mitGTK[a]				
No addition	N.D.[b]	0.05 ± 0.00	N.D.	0.07 ± 0.01
+0.1 mM PLP	N.D.	0.10 ± 0.02	0.07 ± 0.02	0.13 ± 0.02
+5 mM DTT	N.D.	0.08 ± 0.01	N.D.	0.06 ± 0.01
+5 mM DTT + 0.1 mM PLP	N.D.	0.08 ± 0.02	0.07 ± 0.02	0.10 ± 0.01
cytGTK[c,d]				
No addition	0.50 ± 0.01	2.84 ± 0.11	0.69 ± 0.06	3.21 ± 0.45
+0.1 mM PLP	1.14 ± 0.18	4.22 ± 0.11	1.31 ± 0.06	4.50 ± 0.29

[a] The specific activity of mitGTK (transaminase assay) = 4.8 units/mg, pH 9.0. The rate of ammonia produced in the lyase reaction was ~0.05–0.1 μmol/min/mg of protein (data not shown).
[b] N.D., not detectable (i.e., ≤0.03 μmol/min/mg).
[c] The specific activity of cytGTK (transaminase assay) = 8.9 units/mg, pH 9.0.
[d] Data from Ref. 15.

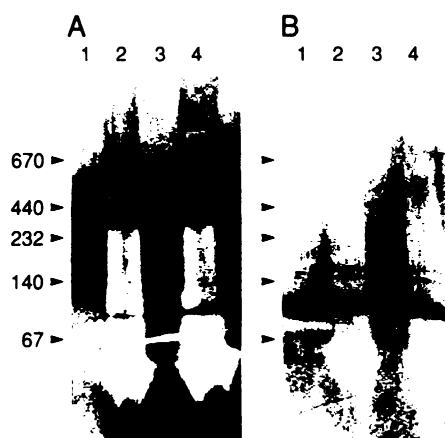

Fig. 1. Activity staining of rat kidney cytosol, kidney mitochondria, purified mitGTK, and purified cytGTK subjected to ND-PAGE. Staining was carried out in 100 mM potassium phosphate buffer, pH 7.2, as described previously (15). A, DCVC lyase activity. B, GTK (phenylalanine-αKMB transamination) activity. *Lane 1*, 3 μg of purified mitGTK (10 munits of transaminase activity at pH 9.0; 4.2 munits at pH 7.2). *Lane 2*, kidney mitochondrial homogenate containing 150 μg of protein (2.4 munits of transaminase activity at pH 9.0; 1 munit at pH 7.2). *Lane 3*, 0.1 μg of purified cytGTK (1.0 munit of transaminase activity at pH 9.0; 0.6 munit at pH 7.2). *Lane 4*, kidney cytosol containing 150 μg of protein (1.7 munits of transaminase activity at pH 9.0; 1 munit at pH 7.2). The activities of high- and low-molecular-weight lyases in the mitochondrial fraction applied to *lane 2* were 1.0 and ~0.03 munit, pH 7.2, respectively. The activities of high- and low-molecular-weight lyases in the cytosolic fraction applied to *lane 4* were 0.24 and 0.22 munit, pH 7.2, respectively. Note that the staining for the lyase activity is more efficient than that for the transaminase activity, presumably because the staining procedure for the transaminase requires a coupling enzyme, whereas that for the lyase does not (24). *Arrows*, positions of molecular-weight markers detected with Coomassie blue on the gel in an adjacent lane: thyroglobulin monomer (M_r 670,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and albumin (67,000). Both gels were exposed to staining solution for ~1 hr and then rinsed and photographed. The high-molecular-weight lyase has very weak GTK-type activity (24) that is not apparent in B. However, on longer incubation times or application of more enzyme to the gel, a faint band of GTK-type activity becomes apparent at M_r ~330,000.

DCVC lyase activity is very low (A, *lane 1*; M_r ~90,000). The intensity is even less than that obtained with 0.6 munit (transaminase assay, pH 7.2) of purified cytGTK (A, *lane 3*). Comparison of *lane 2A* (mitochondrial homogenate containing 1 munit of GTK-type activity, pH 7.2) with *lane 4A* (cytosolic fraction containing 1 munit of GTK-type activity, pH 7.2) shows that although the DCVC lyase activity of the high-molecular-weight lyase (band at M_r ~330,000) is high in both the mitochondrial and cytosolic fractions of rat kidney, the low-molecular-weight lyase activity (band at M_r ~90,000) is proportionately more pronounced in the cytosolic fraction than in the mitochondrial fraction. On the other hand, when the mitochondrial fraction (B, *lane 2*) and cytosolic fraction (B, *lane 4*) were stained for transaminase activity, the bands corresponding to GTK activity were of comparable intensity. Although activity staining provides only a semiquantitative analysis, the procedure provides additional evidence that purified rat kidney cytGTK has considerably greater inherent DCVC-lyase activity than does mitGTK and that this difference can be detected in the various kidney fractions. Moreover, the present findings reinforce the previous suggestion (15) that the high-molecular-weight species is a prominent DCVC lyase of rat kidney mitochondria.

Discussion

Cysteine *S*-conjugate *β*-lyase activity of purified mitGTK. Some previous evidence suggests that the mitochondrial and cytosolic forms of GTK are distinct enzymes. Thus, as mentioned above, the mitochondrial enzyme is bound more tightly to DE 52 (23) and hydroxylapatite (21) than is the cytosolic enzyme. The purified mitochondrial enzyme is relatively more active with glyoxylate, pyruvate, and α-ketobutyrate than is the cytosolic enzyme (14). Finally, antibodies raised in rabbits to purified rat kidney cytGTK react poorly with GTK in preparations of rat brain and kidney mitochondria (29).[2] Results of the present study have also revealed additional differences between the two isoforms. Thus, the cytosolic enzyme is considerably more active as a

[2] Stevens *et al.* showed, however, that antibodies raised in goats to purified cytGTK react with purified mitGTK (21) and recognize a protein in mitochondrial homogenates with a molecular weight similar to that of cytGTK (20). We do not know the reason for the apparent discrepancy between the results obtained by Stevens *et al.* and those obtained by us. It is possible, however, that the polyclonal antibodies raised in goats recognize a greater range of epitopes than do the polyclonal antibodies raised in rabbits.

aspet

DCVC lyase and as a TFEC lyase than is the mitochondrial enzyme (Table 3).

Previously, Stevens *et al.* (21) purified mitGTK from rat kidney by a more detailed procedure than that used in the present study. Both the yield and the specific activity (~5% and 2.1 μmol/min/mg) of mitGTK obtained by these authors were lower than those obtained in the present study (~18% and 4.75 μmol/min/mg) (Table 1). The ratio of phenylalanine-αKMB transaminase activity to αKMB-stimulated DCVC lyase activity reported by Stevens *et al.* was ~9 for the mitochondrial homogenate and 5.3 for the purified mitGTK preparation. However, under the conditions of the present assays, the ratio was ~1.7 for the mitochondrial homogenate and >60 for the purified mitGTK preparation (Table 1). Previous work from our laboratory supports the idea that mitGTK has inherently less DCVC lyase activity than does cytGTK. Aliquots of rat kidney cytosol (containing ~1.7 transaminase munits of cytGTK, pH 9.0) and rat kidney mitochondria (containing ~2.4 transaminase munits of mitGTK, pH 9.0) were subjected in separate experiments to activity staining on ND-PAGE with a mixture containing 1 mM DCVC (or 1 mM TFEC), 0.6 mM αKMB, 100 mM potassium phosphate buffer, pH 7.2, and nitroblue tetrazolium/phenazine methosulfate. The intensity of the formazan band resulting from reduction of the dye by the eliminated -SH fragment was greater in the lane containing the cytosolic fraction than in the lane containing the mitochondrial fraction despite the presence of comparable GTK transaminase activity in the two lanes (15). In the present study, we carried out a similar DCVC-lyase activity staining experiment, except that (a) additional lanes containing purified mitGTK and purified cytGTK (Fig. 1A, *lanes 1 and 3*, respectively) were included in the gel, and (b) a control gel stained for GTK activity (Fig. 1B) was also included. Inclusion of the gel shown in Fig. 1B corrects for any possible denaturation on the gel that might lead to erroneous conclusions about the low lyase activity of mitGTK. The figure shows that the intensity of the transaminase band at the position of GTK (M_r ~90,000) in the mitochondrial fraction (Fig. 1B, *lane 2*) is comparable to that in the cytosolic fraction (Fig. 1B, *lane 4*). This is in accordance with the fact that equal quantities of GTK (i.e., ~1 munit at pH 7.2) were applied to the gel in both cases. However, the intensity of the lyase band at the position of GTK in the mitochondrial fraction (Fig. 1A, *lane 2*) is considerably less than that of the cytosolic fraction (Fig. 1B, *lane 4*). In other words, because Fig. 1A and Fig. 1B were subject to identical electrophoresis conditions, the low level of DCVC lyase in the mitochondrial fraction migrating with an R_F identical to that of mitGTK (Fig. 1A, *lane 2*) is not likely to be due to a denaturing artifact.

In the case of the purified enzymes, as expected the intensity of the band due to the transaminase activity of mitGTK (*lane 1B*, 4.2 munits at pH 7.2) is greater than that due to cytGTK (*lane 3B*, 0.6 munits at pH 7.2). However, the lyase activity of purified mitGTK (*lane 1A*) is less than that of purified cytGTK (*lane 3A*) despite the application of ~7-fold as many units of GTK activity in *lane 1A* compared with *lane 3A*.

In summary, the data in Fig. 1 show that compared with the cytosolic fraction, rat kidney mitochondria contain relatively little DCVC lyase activity in the protein fraction migrating with an apparent molecular weight of ~90,000. The data also provide additional evidence to that presented in Table 3 that mitGTK possesses little DCVC lyase activity.

Regarding the importance of the high-molecular weight enzyme as a cysteine *S*-conjugate β-lyase in rat kidney mitochondria. Stevens *et al.* (21) noted that cysteine *S*-conjugate β-lyase activity of rat kidney mitochondria (DCVC as substrate) is stimulated by both αKMB and α-ketoglutarate and that the addition of the two α-keto acids to the assay mixture does not result in an additive effect. Thus, activities in the absence of added α-keto acid, plus 0.5 mM α-ketoglutarate, plus 0.5 mM αKMB, and plus 0.5 mM α-ketoglutarate and 0.5 mM αKMB were reported to be 1.3, 5.0, 7.1, and 8.2 nmol/min/mg of protein, respectively. α-Ketoglutarate is a poor substrate of the transaminase reaction catalyzed by cytGTK and mitGTK (14). Thus, one would predict that the α-ketoglutarate-stimulated DCVC lyase activity in the rat kidney mitochondria is due to an enzyme distinct from mitGTK. It then follows that the activity of α-ketoglutarate-stimulated lyase (≠mitGTK enzyme) should be additive with that of αKMB-stimulated mitGTK (if the latter truly does possess α-keto acid-dependent DCVC lyase activity). Because the effect of α-ketoglutarate and αKMB is not additive, the data argue that rat kidney mitochondria contain a major DCVC lyase that is stimulated by a broader range of α-keto acids than are substrates for cytGTK and mitGTK. When we analyzed partially purified high-molecular-weight lyase isolated from rat kidney cytosol for the dependence of pyruvate formation from DCVC on added α-keto acid, we found that this activity is stimulated by both α-ketoglutarate and αKMB (24). Moreover, the α-ketoglutarate (and αKMB) stimulation of the high-molecular-weight DCVC lyase and (TFEC lyase) could be detected by activity staining of both crude rat kidney cytosol and mitochondria subjected to ND-PAGE (24). In the present work, we found that ~38% of the DCVC lyase activity in rat kidney homogenates is present in the mitochondrial fraction and that within the mitochondrial fraction ~90% is accounted for by the high-molecular-weight lyase (Table 1). We believe our previous findings (24) and those of the the present findings are consistent with the idea that rat kidney mitochondria contain appreciable levels of a major cysteine *S*-conjugate β-lyase that has a high-molecular-weight and that has a broad specificity for both cysteine *S*-conjugates and activation by α-keto acids. This hypothesis is also in accordance with the previous findings of Stevens *et al.* (21). Finally, we suggested that because the high-molecular-weight rat kidney lyase is present in mitochondria and is activated by near-physiological levels of α-ketoglutarate, this enzyme may well be involved in the activation of cysteine *S*-conjugates in rat kidney mitochondria *in vivo* (15).

Lash *et al.* (18) reported that approximately two thirds of the lyase activity with BTC in rat kidneys is in the cytosolic fraction and one third is in the mitochondrial fraction. Rat kidney mitochondria catalyze a lyase reaction with BTC as effectively as with DCVC (21). However, Stevens *et al.* reported that purified rat kidney mitGTK and cytGTK have little BTC lyase activity (21). Yamauchi *et al.* (16) also reported that highly purified rat kidney cytGTK/DCVC lyase has no discernible BTC lyase activity. [Curiously, preparations of cysteine *S*-conjugate β-lyase/cytGTK purified from bovine and human kidneys appear to possess lyase activity toward BTC (17). Evidently, the substrate specificity of GTK

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

must vary considerably among different species]. BTC has not yet been tested as a substrate for the rat kidney high-molecular-weight lyase, but given the broad specificity of this enzyme (*c.f.*, Ref. 15) the possibility must be considered that this enzyme is responsible for the lyase activity with BTC in both the cytosolic and mitochondrial fractions of rat kidney.

Inhibition of PLP-containing enzymes by DCVC. Stevens *et al.* (29) recently showed that several proteins in kidney mitochondria are thioacetylated after rats are exposed to TFEC. One protein that was tagged by this procedure is mitochondrial aspartate aminotransferase (a matrix enzyme) (29). We found that purified pig heart cytosolic aspartate aminotransferase catalyzes a weak β-elimination reaction with DCVC and TFEC. At the same time, however, the enzyme is slowly inactivated. Cytosolic and mitochondrial aspartate aminotransferases in rat kidney homogenates are likewise inactivated in the presence of DCVC (or TFEC). Purified pig heart alanine aminotransferase is also inactivated by DCVC.[3] Thus, DCVC may induce a metabolic derangement by disrupting key PLP enzymes in addition to depleting thiols and initiating lipid peroxidation. For example, inactivation of cytosolic and mitochondrial aspartate aminotransferases is expected to lead to inhibition of the malate-aspartate shuttle and ultimately to energy failure.

Conclusion. Ample evidence from several laboratories indicates that a major cysteine *S*-conjugate β-lyase of rat kidney cytosol is identical with cytGTK. Rat kidney mitochondria contain GTK activity, and two previous reports suggest that this activity is located primarily in the matrix of these organelles. The present results suggest that mitGTK, unlike cytGTK, has only limited DCVC lyase activity. Furthermore, the results suggest that a major cysteine *S*-conjugate β-lyase activity in the mitochondria is due to a recently described high-molecular-weight enzyme. Detailed fractionation and substrate specificity studies of this high-molecular-weight lyase may help resolve conflicting reports in the literature regarding the location of DCVC and BTC lyases within the mitochondria. Finally, the present results suggest two possible mechanisms by which DCVC and other reactive cysteine *S*-conjugates are toxic. First, the high-molecular-weight lyase may contribute to nondiscriminate release of reactive fragments that attack key macromolecules and low-molecular-weight substances (e.g., thiols) necessary for mitochondrial function. Second, on contact with DCVC, metabolically important PLP-containing enzymes may catalyze their self-destruction.

References

1. Steven, J. L., and D. P. Jones. The mercapturic pathway: Biosynthesis, intermediary metabolism and physiological disposition, in *Glutathione: Chemical, Biochemical and Medical Aspects* (D. Dolphin, R. Poulsen, and O. Avramovic, eds.). John Wiley and Sons, New York, 45–84 (1989).
2. Elfarra, A. A. Aliphatic halogenated hydrocarbons, in *Toxicology of the Kidney* (J. B. Hook, and S. Goldstein, eds.). Raven Press, New York, 387–414 (1993).
3. Cooper, A. J. L. Enzymology of cysteine *S*-conjugate β-lyases. *Adv. Pharmacol.* **27**:71–113 (1994).
4. Reichert, D., G. Liebalt, and F. D. Henschler. Neurotoxic effects of dichloroacetylene. *Arch. Toxicol.* **37**:23–38 (1976).
5. Buxton, P. H., and M. Hayward. Polyneuritis cranialis associated with industrial trichloroacetylene poisoning. *J. Neurol. Neurosurg. Psychiatry* **30**:511–518 (1967).
6. Dekant, W., K. Berthold, S. Vamvakas, D. Henschler, and M. W. Anders. Thioacetylating intermediates as metabolites of *S*-(1, 2-dichlorovinyl)-L-cysteine and *S*-(1, 2, 2-trichlorovinyl)-L-cysteine formed by cysteine conjugate β-lyase. *Chem. Res. Toxicol.* **1**:175–178 (1988).
7. Hayden, P. J., and J. L. Stevens. Cysteine conjugate toxicity, metabolism, and binding to macromolecules in isolated rat kidney mitochondria. *Mol. Pharmacol.* **37**:468–476.
8. Chen, Q., T. W. Jones, P. C. Brown, and J. L. Stevens. The mechanism of cysteine conjugate cytotoxicity in renal epithelial cells: Covalent binding leads to thiol depletion and lipid peroxidation. *J. Biol. Chem.* **265**:21603–21611 (1990).
9. Nash, J. A., L. J. King, E. A. Lock, and T. Green. The metabolism and disposition of hexachloro-1:3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol. Appl. Pharmacol.* **73**:124–137 (1984).
10. Tateishi, M., S. Susuki, and S. Shimuzu. Cysteine conjugate β-lyase in rat liver: A novel enzyme catalyzing formation of thiol-containing metabolites of drugs. *J. Biol. Chem.* **253**:8854–8859 (1978).
11. Stevens, J. L. Isolation and characterization of a rat liver enzyme with both cysteine conjugate β-lyase and kynureninase activity. *J. Biol. Chem.* **260**:7945–7950 (1985).
12. Stevens, J. L., J. D. Robbins, and R. A. Byrd. A purified cysteine conjugate β-lyase from rat kidney cytosol: Requirement for an α-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K. *J. Biol. Chem.* **261**:15529–15537 (1986).
13. Cooper, A. J. L., and A. Meister. Isolation and purification of a new glutamine transaminase from rat kidney. *J. Biol. Chem.* **249**:2554–2561 (1974).
14. Cooper, A. J. L., and A. Meister. Comparative studies of glutamine transaminases from rat tissues. *Comp. Biochem. Physiol. B. Comp. Biochem.* **69B**:137–145 (1981).
15. Abraham, D. G., P. P. Patel, and A. J. L. Cooper. Isolation from rat kidney of a cytosolic high molecular weight cysteine-*S*-conjugate β-lyase with activity toward leukotriene E_4. *J. Biol. Chem.* **270**:180–188 (1995).
16. Yamauchi, A., G. J. Stijntjes, J. N. M. Commandeur, and N. P. E. Vermeulen. Purification of glutamine transaminase K/cysteine conjugate β-lyase from rat renal cytosol based on hydrophobic interaction HPLC and gel permeation FPLC. *Protein Expr. Purif.* **4**:552–562 (1993).
17. Lash, L. L., R. M. Nelson, R. A. Van Dyke, and M. W. Anders. Purification and characterization of human kidney cytosolic cysteine conjugate β-lyase activity. *Drug Metab. Dis.* **18**:50–54 (1990).
18. Lash, L. A., A. A. Elfarra, and M. W. Anders. Renal cysteine conjugate β-lyase: Bioactivation of nephrotoxic cysteine *S*-conjugates in mitochondrial outer membrane. *J. Biol. Chem.* **261**:5930–5935 (1986).
19. Elfarra, A. A., L. L. Lash, and M. W. Anders. α-Ketoacids stimulate rat renal cysteine conjugate β-lyase activity and potentiate the cytotoxicity of *S*-(1,2-dichlorovinyl)-L-cysteine. *Mol. Pharmacol.* **31**:208–212 (1987).
20. Jones, T. W., C. Qin, V. H. Schaeffer, and J. L. Stevens. Immunohistochemical localization of glutamine transaminase K, a rat kidney cysteine conjugate β-lyase, and the relationship to the segment specificity of cysteine conjugate nephrotoxicity. *Mol. Pharmacol.* **34**:621–627 (1988).
21. Stevens, J. L., N. Ayoubi, and J. D. Robbins. The role of mitochondrial matrix enzymes in the metabolism and toxicity of cysteine conjugates. *J. Biol. Chem.* **263**:3395–3401 (1988).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Cooper, A. J. L. Purification of soluble and mitochondrial glutamine transaminase K from rat kidney: Use of a sensitive assay involving transamination between phenylalanine and α-keto-γ-methiolbutyrate. *Anal. Biochem.* **89**:451–469 (1978).
24. Abraham, D. G., and A. J. L. Cooper. Glutamine transaminase K and cysteine *S*-conjugate β-lyase activity stains. *Anal. Biochem.* **197**:421–427 (1991).
25. Blakesly, R. W., and J. A. Boezi. A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G250. *Anal. Biochem.* **82**:580–582 (1977).
26. Curthoys, N. P., T. Kuhlenschimdt, and S. S. Godfrey. Regulation of renal ammoniagenesis. *Arch. Biochem. Biophys.* **174**:82–89 (1976).
27. Chaykin, S. Assay of nicotinamide deaminase: Determination of ammonia by the indophenol method. *Anal. Biochem.* **31**:375–382 (1964).
28. Cooper, A. J. L., D. G. Abraham, A. S. Gelbard, J. C. K. Lai, and C. K. Petito. High activities of glutamine transaminase K (dichlorovinylcysteine β-lyase) and ω-amidase in the choroid plexus of rat brain. *J. Neurochem.* **61**:1731–1741 (1993).
29. Bruschi, S. A., K. A. West, J. W. Crabb, R. S. Gupta, and J. L. Stevens. Mitochondrial HSP60 (P1 protein) and a HSP70-like protein (Mortalin) are major targets for modification during *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine-induced nephrotoxicity. *J. Biol. Chem.* **268**:23157–23161 (1993).

[3] Y. Kato and A. J. L. Cooper, unpublished results.

Send reprint requests to: Dr. Arthur J. L. Cooper, Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, NY 10021.

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 2, 2012

aspet