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Cysteine S-conjugate β -lyases

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Abstract

Cysteine *S*-conjugate β -lyases are pyridoxal 5'-phosphate (PLP)-containing enzymes that catalyze the conversion of cysteine *S*-conjugates [RSCH₂CH(NH₃⁺)CO₂⁻] and selenium *Se*-conjugates [RSeCH₂CH(NH₃⁺)CO₂⁻] that contain a leaving group in the β position to pyruvate, ammonium and a sulfur-containing fragment (RSH) or selenium-containing fragment (RSeH), respectively. At least ten PLP enzymes catalyze β -elimination reactions with such cysteine *S*-conjugates. All are enzymes involved in amino acid metabolism that do not normally catalyze a β -lyase reaction, but catalyze a non-physiological β -lyase side reaction that depends on the electron-withdrawing properties of the -SR or -SeR moiety. In the case of the cysteine *S*-conjugates, if the eliminated RSH is stable the compound may be *S*-thiomethylated and excreted (thiomethyl shunt) or *S*-glucuronidated and harmlessly excreted [the possibility that RSeH compounds may be similarly metabolized has not been extensively studied]. If, however, RSH is chemically reactive the cysteine *S*-conjugate may be toxic as a result of the β -lyase reaction. The cysteine *S*-conjugate β -lyase pathway is of particular interest to toxicologists because it is involved in the bioactivation (toxicification) of halogenated alkenes and certain drugs.

Keywords

Ammonium; cysteine *S*-conjugates; cysteine *S*-conjugate β -lyases; *S*-(1,2-dichlorovinyl)-L-cysteine; *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; pyruvate

Many potentially toxic electrophiles, whether of endogenous origin (e.g. α,β -unsaturated aldehydes derived from the oxidation of lipids) or of exogenous origin (e.g. some drugs), are detoxified through the mercapturate pathway (Figure 1). In addition, some non-electrophilic toxins may be converted to electrophiles that are then detoxified through the mercapturate pathway. The electrophile is sequentially converted to the glutathione *S*-conjugate, cysteinylglycine *S*-conjugate, cysteinyl *S*-conjugate and *N*-acetyl cysteine *S*-conjugate (mercapturate) by the action of glutathione *S*-transferases, γ -glutamyltransferase (γ -glutamyltranspeptidase), dipeptidases and *N*-acetyl transferases, respectively. The mercapturate is generally more hydrophilic and water soluble than is the starting electrophile and is readily excreted in the urine or bile.

If the cysteine *S*-conjugate contains a good leaving group (nucleofuge) in the β position the metabolism of the electrophile may be diverted irreversibly away from the mercapturate by the action of cysteine *S*-conjugate β -lyases. These PLP-containing enzymes catalyze the conversion of the cysteine *S*-conjugate [RSCH₂CH(NH₃⁺)CO₂⁻] to RSH and aminoacrylate

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[CH₂=C(NH₃⁺)CO₂⁻]. The aminoacrylate non-enzymatically tautomerizes to α-iminopropionate [CH₃C(=NH₂⁺)CO₂⁻], which then hydrolyzes to pyruvate [CH₃C(O)CO₂⁻] and ammonium. The net reaction is shown in Eq. 1.

Inspection of equation 1 shows that the starting electrophile has gained a sulfhydryl (-SH) moiety. If the modified electrophile (RSH) is chemically stable it may be metabolized to a number of products, the most important of which are the thiomethyl- and S-glucuronyl analogues (Figure 1). The thiomethylated and S-glucuronidated species are water soluble and readily excreted. Thus, thiomethylation and S-glucuronidation are detoxification events.

If, however, RSH is chemically reactive, the combined action of the mercapturate pathway and cysteine S-conjugate β-lyase(s) may result in bioactivation. For example, cysteine S-conjugates derived from a large number of halogenated alkenes have been shown to undergo cysteine S-conjugate β-lyase reactions that generate reactive sulfur-containing fragments. As outlined in the Commentary, as a result of the lyase reaction these cysteine S-conjugates are toxic to various organs/organelles, but especially to kidney mitochondria. Much of the interest in cysteine S-conjugate β-lyases among toxicologists relates to their contribution to the bioactivation of haloalkenes. Some of these halogenated alkenes are produced industrially on a large scale and in some cases are environmental contaminants and are present in several Superfund sites (e.g. trichloroethylene, tetrachloroethylene).

Basic Protocol 1 Spectrophotometric Measurement of Pyruvate as its 2,4-Dinitrophenylhydrazone

This end-point assay is quite sensitive and can be accomplished in less than 20 min. The spectrophotometric procedure described below is designed for 96-well plate analysis in which the final volume of assay mix plus reagents is 0.2 ml and the light path length is 0.5 cm. However, the procedure may be scaled up to a 1-ml final volume for a conventional spectrophotometer that utilizes cuvettes with a light path length of 1 cm. Since most studies on cysteine S-conjugate β-lyases have been conducted with S-(1,2-dichlorovinyl)-L-cysteine [DCVC; HC(Cl)=C(Cl)SCH₂CH(NH₃⁺)CO₂⁻; the cysteine S-conjugate of trichloroethylene] and S-(1,1,2,2-tetrafluoroethyl)-L-cysteine [TFEC; HCF₂CF₂SCH₂CH(NH₃⁺)CO₂⁻; the cysteine S-conjugate of tetrafluoroethylene], these are the β-lyase substrates that are recommended in the current protocol. However, since pyruvate is the end-product in all these lyase reactions the assays can be easily adapted in principle to any cysteine S-conjugate β-lyase substrate, provided that the substrate itself does not absorb strongly at 430 nm (the absorbance maximum of pyruvate 2,4-dinitrophenylhydrazone under alkaline conditions).

Although many PLP-containing enzymes have been shown to catalyze cysteine S-conjugate β-lyase reactions the two most important are glutamine transaminase K (GTK) and mitochondrial aspartate aminotransferase (mitAspAT). For several cysteine S-conjugate β-lyases that are aminotransferases, including GTK and mitAspAT, an α-keto acid co-substrate must be present in reaction mixtures containing DCVC or TFEC (see the Commentary).

Materials

Tissue homogenate – see Support Protocols 1 and 2

Cytosol – see Support Protocols 1 and 2
Mitochondrial extract – see Support Protocols 1 and 2
Purified rat liver GTK or purified recombinant human GTK
Purified recombinant rat liver mitAspAT
1 M Potassium phosphate buffer, pH 7.2
1 M Tris-HCl buffer, pH 8.5
100 mM TFEC in 100 mM potassium phosphate buffer, pH 7.2
50 mM DCVC in 100 mM Tris-HCl buffer, pH 8.0.
1 mM α -Keto- γ -methiolbutyrate
1 mM α -Ketoglutarate
2 mM 2,4-Dinitrophenylhydrazine in 2 M HCl
1 M NaOH
0.5 ml Eppendorf tubes
37°C Heating block
96-Well plate spectrophotometer

Procedure 1 – Cysteine S-conjugate β -lyase activity in tissue samples with TFEC as substrate

1. Combine in a small (0.5 ml) Eppendorf tube:
 - 5 μ l of 1 M potassium phosphate buffer, pH 7.2
 - 5 μ l of 100 mM TFEC
 - 2.5 μ l of 1 mM α -keto- γ -methiolbutyrate
 - 2.5 μ l of 1 mM α -ketoglutarate
 - 30 μ l of water
2. Warm the tube to 37°C for 5 min
3. Add 5 μ l of enzyme source (tissue homogenate, cytosol, mitochondrial extract or purified enzyme) such that the final volume of the reaction mixture is 50 μ l
4. Incubate for 5 minutes at 37°C
5. Stop the reaction by the addition of 10 μ l of 2,4-dinitrophenylhydrazine reagent
6. Incubate for 5 minutes at 37°C to ensure complete conversion of pyruvate to its 2,4-dinitrophenylhydrazone
7. Add 140 μ l of 1 M NaOH to generate a red-brown product
8. Transfer the contents quantitatively to a well in a 96-well plate spectrophotometer
9. Read at 430 nm in a 96-well plate spectrophotometer (light path length = 0.5 cm) against a blank containing complete reaction mixture except that TFEC is replaced with 5 μ l of water (the blank is treated identically with 2,4-dinitrophenylhydrazine reagent and NaOH)

10. To ensure that the formation of pyruvate is linear with time, repeat the assay procedure, but in this case incubate the assay mixture for 10 minutes to determine that the increase in absorption is linear with time
11. Calculation of nmols of pyruvate produced: The extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is $16,000 \text{ M}^{-1} \text{ cm}^{-1}$; from Beer's Law, a final volume of 0.2 ml and a path length of 0.5 cm, the absorbance of 1 nmol of pyruvate 2,4-dinitrophenylhydrazone is 0.040

Notes: Both α -keto- γ -methiolbutyrate and α -ketoglutarate also react with 2,4-dinitrophenylhydrazine and therefore will contribute to the absorbance blank. Under the conditions of the assay, the contribution is about 0.2 absorbance unit. Crude tissue homogenates, cytosol and mitochondrial extracts are likely to contain carbonyl-containing compounds that can also react with 2,4-dinitrophenylhydrazine. These endogenous carbonyl-containing compounds will contribute to the blank, the absorbance of which may increase with time. The contribution of these carbonyl compounds to the blank will obviously depend on the nature of the biological material. This background absorbance, however, is generally less than 0.5 absorbance unit. If necessary, endogenous carbonyl compounds may be removed by dialysis or by means of a Microcon YM-100 spin concentrator (Millipore). Stock solutions of α -keto- γ -methiolbutyrate, α -ketoglutarate and TFEC and DCVC can be stored for months at -20°C . The 2,4-dinitrophenylhydrazine stock solution can be stored at room temperature for at least six months.

Procedure 2 – Cysteine S-conjugate β -lyase activity in tissue samples with DCVC as substrate—As for procedure 1, except that 5 μl of 100 mM TFEC is replaced with 10 μl of 50 mM DCVC, keeping the final assay mixture volume to 50 μl . DCVC is less soluble than TFEC and must be dissolved in a buffer with a relatively high pH as for example Tris pH 8.5.

Procedure 3 – Cysteine S-conjugate β -lyase activity with TFEC in preparations of purified GTK—As for procedure 1 except that α -ketoglutarate is omitted and tissue source is replaced with purified GTK.

Notes: Many cysteine S-conjugate β -lyases are syncatalytically inactivated by end products of the reaction (aminoacrylate and RXH) (see the Commentary). Since GTK, however, is not syncatalytically inactivated by end products the assay is linear for at least 60 minutes, provided the concentration of enzyme is such that the increase in absorbance does not exceed 1.5. However, since small volumes are assayed it is important to minimize evaporation by using a tightly sealed 0.5 ml Eppendorf tube. The pH optimum of both rat and human GTK as an aminotransferase is ~ 8.5 . The same is true for the cysteine S-conjugate β -lyase reactions catalyzed by this enzyme. The rate of the cysteine S-conjugate β -lyase reaction with TFEC (and DCVC) catalyzed by human or rat GTK is approximately doubled when 100 mM phosphate buffer (pH 7.2) is replaced with 100 mM Tris-HCl buffer (pH 8.5).

Procedure 4 – Cysteine S-conjugate β -lyase activity with DCVC in preparations of purified GTK—As for procedure 2 except that α -ketoglutarate is omitted and tissue source is replaced with purified GTK.

Procedure 5 – Cysteine S-conjugate β -lyase activity with TFEC in preparations of purified mitAspAT—As for procedure 1 except that α -keto- γ -methiolbutyrate is omitted and the tissue source is replaced with purified mitAspAT.

Procedure 6 – Cysteine S-conjugate β -lyase activity with DCVC in preparations of purified mitAspAT—As for procedure 2 except that α -keto- γ -methiolbutyrate is omitted and the tissue source is replaced by purified mitAspAT.

Procedure 7 – Activity measurement with TFEC in preparations of cysteine S-conjugate β -lyases other than aminotransferases—As for procedure 1 except that it is not necessary to add α -keto- γ -methiolbutyrate and α -ketoglutarate and the tissue source is replaced with purified enzyme.

Procedure 8 – Activity measurement with DCVC in preparations of cysteine S-conjugate β -lyases other than aminotransferases—As for procedure 2 except that it is not necessary to add α -keto- γ -methiolbutyrate and α -ketoglutarate and the tissue source is replaced with purified enzyme.

Basic Protocol 2 HPLC Measurement of Pyruvate as an Azine Derivative

When the cysteine S-conjugate substrate absorbs at 430 nm the 2,4-dinitrophenylhydrazone procedure cannot be used. In that case an HPLC system may be used in which pyruvate is quantitated as an azine derivative with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH).

Materials

Purified enzyme
1 M Potassium phosphate buffer, pH 7.2
3 M Sodium acetate
0.1% (w/v) MBTH in water (= 4.6 mM solution)
Stock solution of cysteine S-conjugate
37°C Heating block
0.5 ml Eppendorf tubes
50% (v/v) Acetonitrile in water
HPLC apparatus with electrochemical detection

Procedure

1. Combine in a 0.5 ml snap-top Eppendorf tube:
 - 10 μ l of potassium phosphate buffer, pH 7.2
 - 10 μ l of 10 mM α -keto- γ -methiolbutyrate
 - 10 – 50 μ l of stock β -lyase substrate so that the final concentration is 5 – 10 mM
 - 2–10 μ l of purified enzyme
 - Water to a final assay volume of 100 μ l
2. Incubate the reaction mixture for 5 – 10 minutes at 37°C
3. Stop the reaction by addition of 50 μ l of 3 M sodium acetate
4. Add 100 μ l of 0.1% MBTH
5. Incubate at 37°C; pyruvate azine formation is complete in 40 minutes

6. Remove a 25- μ l aliquot
7. Add 80 μ l of 50% acetonitrile in water and centrifuge at 10,000 *g*
8. Remove an aliquot (5 μ l) of the supernatant for HPLC analysis

Chromatographic separation and elution of the MBTH-azine α -keto acid derivatives are carried out with an MD-150 analytical column (3.0 $\times$ 150 mm, 3 micron) from ESA, Inc. (Chelmsford, MA), using a mobile phase buffer (75 mM trisodium citrate, 25 mM ammonium acetate, 26% (v/v) acetonitrile, pH 7.64) at a flow rate of 0.8 ml/min. Derivatives are measured using an ESA model 5600 CoulArray module, equipped with two coulometric array cell modules, each with four working electrodes set at 300, 400, 500, 550, 650, 750, 775 and 825 mV, respectively. Solutions of the MBTH-azine derivatives of α -keto acids contain a mixture of *E*- and *Z*-isomers, that can be separated by HPLC. Under the HPLC conditions outlined above, the *E*- and *Z*-isomers of the MBTH-azine derivatives of pyruvate and α -keto- γ -methiolbutyrate elute at 2.8 and 3.1, and 5.2 and 5.8 min, respectively. Peak areas are analyzed using ESA, Inc, software.

Preparation of Cysteine S-Conjugates

DCVC is made by reacting the sodium salt of cysteine with trichloroethylene in liquid ammonia. Yields are typically 60-70%. For details see McKinney et al. (1959).

TFEC is made by bubbling tetrafluoroethylene through a methanolic solution of cysteine hydrochloride and three molar equivalents of 1 M NaOH until the reaction is complete. The solution is dried *in vacuo*, the residue dissolved in water and TFEC is precipitated at pH 5.0 with acetic acid (Hayden and Stevens, 1990)

Support Protocol 1 Preparation of Cytosol and Mitochondria from Rat Liver

Materials

Mitochondrial isolation buffer (Buffer A)

- 300 mM Sucrose
- 5 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)
- 0.5 mM EDTA
- 0.2 mM EGTA
- 0.5% Fatty acids free bovine serum albumin (BSA)
- pH 7.4 adjusted with Tris-base

Mitochondrial wash buffer (Buffer B)

- 300 mM Sucrose
- 5 mM HEPES-KOH, pH 7.4

Guillotine, six clean centrifuge tubes (50 ml) for the J20 Rotor of a Beckman Centrifuge, scissors, tweezers, glass Petri dish (8 cm), 50-ml glass beaker, 50-ml glass beaker with ~25 ml of ice-cold Buffer A, 10-ml- and 100-ml Potter homogenizers with Teflon pestle, glass funnel with two layers of cheesecloth, glass rod.

Procedure

1. Sacrifice a young adult (4-6 week old) rat by decapitation with a guillotine

2. Quickly remove the liver (~7-8 g) and place it into a beaker containing ice-cold Buffer A (From this point on all procedures are performed on ice)
3. Incubate the liver in ice-cold Buffer A for ~5-6 min
4. Place the liver onto a Petri dish and remove fascia and all extrahepatic tissues
5. Cut the liver into small pieces in a glass beaker containing ~25 ml of Buffer A, and stir the contents with tweezers to remove most of the blood from liver pieces
6. Change the buffer
7. Mince the liver tissue with scissors in Buffer A until free of blood
8. Change buffer
9. Continue mincing the liver tissue with scissors in Buffer A until the liver pieces are ~1 × 1 mm in size
10. Discard the buffer and transfer the liver tissue into a 100-ml Potter homogenizer.
11. Adjust the volume to 50 ml with Buffer A
12. Homogenize until there are no more clumps of tissues
13. Adjust the volume to ~90 ml with Buffer A, and after careful mixing transfer the contents to two centrifuge tubes (2 × 45 ml)
14. Centrifuge at 1,000 g for 10 min
15. Filter the supernatant through two layers of cheesecloth into a second set of two centrifuge tubes
16. Discard the pellets
17. Centrifuge the filtered supernatant solution at 10,000 g for 10 minutes to pellet crude mitochondria
18. Save the supernatant fraction for later preparation of the cytosolic fraction
19. Add 1 ml of Buffer B into each tube and carefully scrape off the pelleted mitochondria with a glass rod avoiding a pellet of erythrocytes that underlies the mitochondria
20. Transfer the contents of both tubes into a 10-ml Potter homogenizer
21. Adjust the volume to 5 ml with Buffer B and homogenize the contents
22. Transfer the homogenate to a preweighed centrifuge tube and adjust the volume to 45 ml
23. Centrifuge at 9,000 g for 10 minutes to obtain semi-pure mitochondria
24. Discard the supernatant
25. Add 2 ml of Buffer B into the tube and carefully remove by agitation the top soft layer that covers the mitochondria
26. Repeat the procedure one more time
27. Weigh the tube and calculate the weight of the mitochondrial pellet
28. Add Buffer B such that the pellet:buffer ratio is 400 mg:600 µl
29. Carefully suspend the pelleted mitochondria in the buffer with a glass rod, avoiding the pellet that underlies the mitochondria at the bottom of the tube

30. Careful to avoid air bubble formation, re-suspend the mitochondria by means of a pipette with a 1-ml tip
31. Transfer the mitochondrial suspension into an Eppendorf tube or a special container and maintain it on ice
32. Centrifuge the previously saved 10,000 g supernatant fraction at 100,000 g for 1 h
33. Discard the pellet and save the resulting supernatant material as the cytosolic fraction
34. Proceed to the protein assay (Support Protocol 3)

Support Protocol 2 Preparation of Cytosol and Mitochondria from Rat Kidney

Materials

Mitochondrial Isolation Buffer

70 mM Sucrose
220 mM D-Mannitol
2 mM HEPES, pH 7.4
0.5 mg/ml BSA
0.1% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)
1 mM EGTA
0.5 mM PMSF (phenylmethanesulfonylfluoride)
Aprotinin, antipain and leupeptin (protease inhibitors; each 5 µg/ml)

Mitochondrial Wash Buffer

70 mM sucrose
220 mM D-mannitol
2 mM HEPES, pH 7.4
0.5 mg/ml BSA (OMIT if subsequent protein or proteomic analyses are to be undertaken)
0.5 mM PMSF
Aprotinin, antipain and leupeptin (each 5 µg/ml)

The pH of the two buffers may be adjusted with KOH to 7.4 if necessary

Procedure

1. All steps are carried out on ice
2. Decapsulate the kidneys and dice the tissue into manageable pieces using a scalpel
3. Homogenize the tissue in Mitochondrial Isolation Buffer (10%; w/v) using 4 strokes of a motor driven Teflon™ pestle (Potter-Elvehjem type) set at approximately 1,500 rpm
4. Dilute the homogenate 1:3 with the same buffer and centrifuge in a swinging bucket rotor at 1,000 g for 10 min

5. Carefully remove the resulting supernatant fraction and discard the pellet
6. Centrifuge the supernatant fraction at 10,000 *g* for 15 min
7. Save the supernatant fraction as cytosol
8. Carefully re-suspend the pellet (crude mitochondria) with Mitochondrial Wash Buffer (50% of the volume used in Step 4) by means of a glass Pasteur pipette avoiding contamination with darker material in the lower band
9. Centrifuge the suspension at 10,000 *g* for 15 min and discard the supernatant fraction
10. Carefully re-suspend the pellet (semi-purified mitochondria) with mitochondrial wash buffer in the same volume as used in Step 8 by means of a glass Pasteur pipette and centrifuge at 10,000 *g* for 15 min (if a “fluffy” layer is observed above the pellet this indicates damaged mitochondria, which must be removed)
11. Re-suspend the resulting pellet (pure mitochondria) in a minimal volume of Mitochondrial Wash Buffer (~25% of Step 4)

The procedure outlined immediately above is based on the widely used method of Schnaitman and Greenawalt (1968) and can also be modified to isolate successfully mitochondria from other sources such as cultured mammalian cells. Other methods can also be successfully applied to the isolation of cysteine *S*-conjugate-treated tissue or cells, but it should be noted that under these circumstances variations in mitochondrial density will be observed owing to the induction of a mitochondrial permeability transition (Ho et al., 2005).

The relative purity of isolated mitochondria can be assessed by measuring the activity of compartment-specific enzymatic activities such as lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cytosol) or succinate cytochrome *c* reductase/adenylate kinase (mitochondria). Procedures are also well established for the sub-fractionation of whole mitochondria and the assessment of sub-mitochondrial compartment purity (Schnaitman and Greenawalt, 1968; Bruschi et al., 1993). In our experience cystathionine γ -lyase is a most convenient marker for liver and kidney cytosol (Krasnikov et al., 2005; see support protocol 4 below).

Support Protocol 3 Protein Determination

Materials

96-Well plate reader spectrophotometer

Total protein Biuret reagent (Sigma) must be kept refrigerated, but is used at ambient temperature during protein measurements

BSA stock solution; 100 mg/ml

Mitochondrial suspension

Procedure—In a 96-well plate devote 24 wells (3 × 8) for the standard curve and 3-4 wells for the sample to be measured

Calibration curve (in triplicate):

A 1-3: Blank (100 μ l H₂O)

B 1-3: 10 mg/ml (99 μ l H₂O + 1 μ l of BSA)

C 1-3: 20 mg/ml (98 μ l H₂O + 2 μ l of BSA)

D 1-3: 30 mg/ml (97 μ l H₂O + 3 μ l of BSA)

E 1-3: 40 mg/ml (96 μ l H₂O + 4 μ l of BSA)

F 1-3: 50 mg/ml (95 μ l H₂O + 5 μ l of BSA)

G 1-3: 60 mg/ml (94 μ l H₂O + 6 μ l of BSA)

H 1-3: 70 mg/ml (93 μ l H₂O + 7 μ l of BSA)

Samples:

A-D 4: 90 μ l H₂O + 10 μ l of mitochondria

Add into each well 200 μ l of Biuret reagent and incubate for 15 min

Place the plate into a plate reader and read absorbance at 540 nm

Construct a standard curve and find the protein concentration of the mitochondrial sample

Support Protocol 4 Use of Cystathionine γ -Lyase as A Cytosolic Marker for Rat Liver Subcellular Fractions

Although lactate dehydrogenase is commonly used as a marker enzyme to ensure that mitochondria are not contaminated with cytosolic elements, a small amount of lactate dehydrogenase is naturally present within rat liver mitochondria. We have found that cystathionine γ -lyase (γ -cystathionase) is an exceptionally good marker for rat liver cytosol (Krasnikov et al., 2005). Moreover, the assay procedure is relatively simple, is more sensitive than the lactate dehydrogenase assay and measures product formation rather than substrate disappearance. One caveat is that whereas the enzyme is also present in rat kidney, it is of very low specific activity in most other tissues.

The biological role of cystathionine γ -lyase is to catalyze a γ -elimination reaction with L-cystathionine generating α -ketobutyrate, ammonium and L-cysteine (the eliminated moiety). However, the enzyme will also catalyze the γ -elimination of water from L-homoserine generating α -ketobutyrate and ammonium. The α -ketobutyrate is then quantitated as its 2,4-dinitrophenylhydrazone. L-Homoserine is the preferred substrate in routine assays because it is much less expensive than is L-cystathionine.

Materials

1 M Potassium phosphate buffer, pH 7.2

100 mM L-Homoserine

Rat liver cytosol

Rat liver mitochondria

2 mM 2,4-Dinitrophenylhydrazine in 2 M HCl

1 M NaOH

0.5 ml Eppendorf tubes

37°C Heating block

96-Well plate spectrophotometer

Procedure

1. Combine directly in a well of a 96-well plate:
5 μ l of 1 M potassium phosphate buffer, pH 7.2
10 μ l of 100 mM L-Homoserine
33 μ l of water
2. Warm the plate to 37°C
3. Add 2 μ l of cytosol or mitochondrial suspension
4. Incubate for 5 minutes at 37°C
5. Add 20 μ l of the 2,4-dinitrophenylhydrazine reagent
6. Incubate for 5 minutes at 37°C
7. Determine the absorption at 430 nm relative to a blank in which the L-homoserine is replaced with 10 μ l of water but is otherwise subjected to the same procedure
8. Calculation of nmols of α -ketobutyrate produced in the γ -lyase reaction: The extinction coefficient of α -ketobutyrate 2,4-dinitrophenylhydrazone under these conditions is 15,000 M⁻¹ cm⁻¹; from Beer's Law, a final volume of 0.2 ml and a path length of 0.5 cm, the absorbance of 1 nmol of α -ketobutyrate 2,4-dinitrophenylhydrazone is 0.0375

Note: The activity of cystathionine γ -lyase is very high in rat liver cytosol so that it may be necessary to dilute the liver cytosol at least ten fold with water or buffer before assay.

Commentary

Background Information

More than 90 years ago it was noted that cattle fed soybean oil that had been improperly defatted with trichloroethylene developed aplastic anemia (Stockman 1916). Strong evidence was later presented that a toxic metabolite of trichloroethylene is DCVC (McKinney et al., 1959). DCVC-induced aplastic anemia appears to be unique to cattle (Lock et al., 1996). However, DCVC is nephrotoxic/nephrocarcinogenic and hepatotoxic/hepatocarcinogenic in all experimental animals including cattle, and may also be neurotoxic under certain circumstances. The glutathione-, cysteinylglycine-, cysteine- and mercapturates of a number of haloalkenes, in addition to those of trichloroethylene are all nephrotoxic/hepatotoxic, and, in many cases, nephrocarcinogenic/hepatocarcinogenic in experimental animals. Toxicity is due at least in part to the action of cysteine *S*-conjugate β -lyases that irreversibly divert the cysteine *S*-conjugate away from mercapturate formation and simultaneously generate a reactive sulfur-containing fragment. Even though the mercapturates derived from the halogenated alkenes are downstream from the cysteine *S*-conjugates, they may still be toxic because they can be hydrolyzed back to the corresponding cysteine *S*-conjugate by aminoacylases (e.g. Uttamsingh et al. 2000). Thus, the mercapturate pathway enzymes together with cysteine *S*-conjugate β -lyases may on occasion contribute to toxification (bioactivation) rather than to detoxification. (For reviews see, for example, Anders, 2004, 2008; Cooper and Pinto, 2006; Dekant et al., 1994.) The reactive sulfur-containing fragments are especially cytotoxic in experimental animals to the proximal renal tubules, in particular to the S3 regions and, to a lesser extent, the S2 regions (e.g. Nash et al., 1984; Jones et al., 1988). Toxicity of DCVC is associated in part with covalent modification of macromolecules, depletion of non-protein thiols (e.g. glutathione),

initiation of lipid peroxidation (Chen et al., 1990, 1994) and inhibition of mitochondrial energy metabolism (Cooper and Pinto, 2006).

Tateishi et al. (1978) were the first authors to use the term “cysteine conjugate β -lyase”. These authors showed that rat liver possesses an activity capable of cleaving the C-S (thioether) linkage in the cysteine *S*-conjugates of 2,4-dinitrobenzene and *p*-bromobenzene. Equal quantities of pyruvate, ammonium and a sulfur-containing fragment (XSH) were generated (Eq. 1). The β -lyase activity was purified about 500-fold, but the enzyme responsible was not identified (Tateishi et al., 1978). Subsequently, Stevens and colleagues identified kynureninase and GTK as major cysteine *S*-conjugate β -lyases of rat liver and kidney cytosol, respectively (Stevens 1985; Stevens et al., 1986). Human GTK exhibits the highest specific activity as a cysteine *S*-conjugate β -lyase with DCVC and TFEC as substrates thus far recorded (Cooper et al., 2008). Because of high inherent specific activity as a cysteine *S*-conjugate β -lyase and because early investigators used this name in preference to GTK, the GTK gene in the human genome is listed as *CCBL1* (cysteine conjugate β -lyase 1). However, this nomenclature is a misnomer because at least ten PLP-dependent enzymes (mostly aminotransferases) in addition to GTK are now known to catalyze cysteine *S*-conjugate β -lyase reactions with DCVC and/or TFEC albeit with lower inherent specific activities (reviewed by Cooper and Pinto, 2006). Moreover, in none of these cases is a β -lyase reaction physiologically relevant. In addition to GTK, a particularly important cysteine *S*-conjugate β -lyase is mitAspAT. The specific activity of rat mitAspAT with DCVC and TFEC as substrates is lower than that exhibited by rat and human GTK. However, mitAspAT is exceptionally abundant in almost all tissues including liver, kidney and brain (Parli et al., 1987). This enzyme is a major contributor to the bioactivation of TFEC and DCVC in mitochondria (Cooper and Pinto, 2006). For example, evidence suggests that the nephrotoxicity of cisplatin is due at least in part to formation of its corresponding cysteine *S*-conjugate, which is then a cysteine *S*-conjugate β -lyase substrate of mitAspAT (Zhang et al., 2006).

Critical Parameters and Trouble Shooting

In principle, cysteine *S*-conjugate β -lyase activity may be measured by quantitation of RSH (when it is chemically stable). For example, when *S*-(2-benzothiazolyl)-L-cysteine is used as a substrate, the eliminated RSH fragment (2-mercaptobenzothiazole) can be quantitated by its intense absorbance at 321 nm after quenching the reaction with trichloroacetic acid (Dohn and Anders, 1982). However, *S*-(2-benzothiazolyl)-L-cysteine is a poor substrate of rat kidney GTK compared to TFEC and DCVC. Moreover, this assay cannot be used for analysis of lyase reactions with other cysteine *S*-conjugates. Occasionally ammonia has been measured as an index of β -lyase activity, but this is usually reserved for studies of the stoichiometry of products generated.

Most assays of cysteine *S*-conjugate β -lyases rely on the measurement of pyruvate. This has the advantage that the assay can be used with most cysteine *S*-conjugates. Pyruvate can be measured by HPLC of the fluorescent derivative formed with *o*-phenylenediamine (Stijnes et al., 1992). We also describe here an HPLC-CoulArray method for the detection of pyruvate and other α -keto acids as their azine derivatives with MBTH. The procedure relies on the conversion of pyruvate under mild conditions to an azine in the presence of MBTH (Tanaka et al., 2007). The procedure described here is for the analysis of pyruvate formed in a cysteine *S*-conjugate β -lyase reaction catalyzed by GTK, but could in principle be used for the analysis of pyruvate formed by any combination of lyase substrate and purified cysteine *S*-conjugate. The method detects eluted α -keto acid azines by a coulometric procedure. However, other workers have quantitated α -keto acid azines in HPLC eluates by monitoring absorption profiles at 350 nm (Tanaka et al., 2007). For most assays of cysteine *S*-conjugate β -lyase activity we have relied on measurement of pyruvate as its 2,4-

dinitrophenylhydrazones. This assay is sensitive and, unlike the HPLC procedures, is amenable to the simultaneous measurement of activity in many samples. There are, however, a number of caveats, as described below to be considered, when devising a suitable cysteine *S*-conjugate β -lyase assay.

Assay procedures are complicated by the fact that there are many variables to consider. Despite the fact that the Enzyme Commission lists a single cysteine *S*-conjugate β -lyase (E.C.4.4.1.13) in the C-S lyase family of enzymes, there are, in fact, as emphasized herein, many mammalian PLP-containing enzymes that can catalyze cysteine *S*-conjugate β -lyase reactions to varying degrees. Moreover, cysteine *S*-conjugate β -lyases are not a discreet family of enzymes with a well-defined biochemical role that is related to the β -lyase activity. Instead, they are PLP enzymes (mostly aminotransferases) that are ordinarily involved in well-defined amino acid metabolic pathways, ***but as a result of the electron-withdrawing properties of the –SR group catalyze a β -elimination reaction as a side reaction.***

Because there are multiple cysteine *S*-conjugate β -lyases, the pH optimum of each enzyme under study and the substrate specificity must be carefully defined. Moreover, the initial product of the reaction (aminoacrylate) is reactive and may covalently add to a susceptible residue on the enzyme and/or to the coenzyme thereby syncatalytically inactivating the enzyme. In addition, if the eliminated fragment (RSH) is chemically reactive the fragment (or a breakdown product thereof) may also covalently add to the enzyme thereby contributing to the syncatalytic inactivation. For example, mitAspAT catalyzes a β -lyase reaction with β -chloro-L-alanine and is inactivated by the generated aminoacrylate on average after about 3,850 turnovers (Cooper et al., 2002). The enzyme also catalyzes a β -elimination reaction with the nephrotoxin TFEC. In this case the enzyme is inactivated on average about once in every 2,800 turnover events. The differences in inactivation efficiencies may be ascribed to the fact when β -chloro-L-alanine and TFEC are β -lyase substrates, only one reactive species (aminoacrylate) is generated in the former case whereas two reactive species (aminoacrylate and $\text{HCF}_2\text{CF}_2\text{SH}$) are formed in the latter case. In addition to TFEC, mitAspAT catalyzes β -elimination reactions with DCVC and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine [CTFC; the nephrotoxic cysteine *S*-conjugate of 2-dichloro-1,1,2-ethylene]. DCVC and CTFC syncatalytically inactivate mitAspAT at about one inactivation event for every 2,700 turnovers. Other PLP-containing enzymes (e.g. cytosolic and mitochondrial isozymes of human branched-chain aminotransferase) that catalyze cysteine *S*-conjugate β -lyase reactions with TFEC are even more susceptible to inactivation than mitAspAT, with turnover to inactivation ratios of ≤ 300 (Cooper et al., 2003). On the other hand, GTK, which is a major cysteine *S*-conjugate β -lyase of rat kidney cytosol, is remarkably resistant to inactivation by aminoacrylate or by eliminated sulfur-containing fragments, possibly because the active site possesses several hydrophobic moieties that would not be expected to react with aminoacrylate or the reactive sulfur-containing fragment generated by the β -lyase reaction.

As noted above, most of the mammalian cysteine *S*-conjugate β -lyases discovered thus far are aminotransferases. When aminotransferases are present in the biological sample to be assayed for β -lyase activity, there is the possibility that a half-transamination reaction (Eq. 2) will compete with the β -lyase reaction (Eq. 1). A half transamination reaction will convert the PLP form of the enzyme (PLP-ENZ) to the pyridoxamine 5'-phosphate (PMP) form (PMP-ENZ) plus α -keto analogue of the cysteine *S*-conjugate. The PMP form of the enzyme cannot support another round of β -elimination and the reaction will stop or slow down considerably. In order for the β -elimination reaction to proceed efficiently in the face of a competing half transamination an α -keto acid substrate $[\text{R}'\text{C}(\text{O})\text{CO}_2^-]$ must be present in the assay mixture to ensure that a complete transamination cycle occurs (Eqs. 2 plus 3) and the enzyme is maintained in the PLP form.

As a consequence of these considerations, optimal assay conditions will vary substantially depending on whether the source of the cysteine *S*-conjugate β -lyase is a tissue homogenate, cytosol, mitochondria or purified enzyme. To measure cysteine *S*-conjugate β -lyase activity in tissue homogenate, cytosol or mitochondria, it is recommended that small amounts of α -ketoglutarate plus α -keto- γ -methiolbutyrate be included in the reaction mixture to maximize the cysteine *S*-conjugate β -lyase activity associated with glutamate-dependent and glutamine-dependent aminotransferases, respectively. For measurement of the cysteine *S*-conjugate β -lyase activity of purified GTK, α -keto- γ -methiolbutyrate should be present in the reaction mixture. For measurement of the cysteine *S*-conjugate β -lyase activity of purified mitAspAT, α -ketoglutarate should be present in the assay mixture. For purified enzymes that catalyze cysteine *S*-conjugate β -lyase reactions, but are not aminotransferases (e.g. kynureninase, cystathionine γ -lyase), addition of α -keto acid to the reaction mixture is not necessary.

Finally, because the assays described herein are end-point assays, single readings at relatively long incubation times may give artificially low specific activities as a result of syncatalytic inactivation. Thus, short incubation times are recommended. Measurements at 5 and 10 minutes should be made, if possible, to ensure linearity.

NOTE: All protocols involving the killing of rats for the preparation of cytosol and mitochondria from various tissues must first be reviewed and approved by an Institutional Care and Use Committee and must conform to governmental regulations regarding the care and use of laboratory animals.

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Abbreviations used

CTFC	<i>S</i> -(2-chloro-1,1,2-trifluoroethyl)-L-cysteine
DCVC	<i>S</i> -(1,2-dichlorovinyl)-L-cysteine
GTK	glutamine transaminase K
HPLC	high performance liquid chromatography
MBTH	3-methyl-2-benzothiazolinone hydrazone
mitAspAT	mitochondrial aspartate aminotransferase
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
TFEC	<i>S</i> -(1,1,2,2-tetrafluoroethyl)-L-cysteine

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Figure 1.

The mercapturate pathway and associated side reactions. If the mercapturate precursor contains an electrophilic center it may react directly with GSH (reaction 1). Alternatively, the precursor may be converted to a compound with an electrophilic center (reaction 2) prior to reaction with GSH. Reactions 1 through 5 represent the mercapturate pathway. Reactions 7 through 9 are alternative reactions for elimination of the cysteine *S*-conjugate. Reactions 7 plus 8 denote the thiomethyl shunt. Enzymes: **1)** glutathione *S*-transferases; **2)** oxidases that generate an electrophilic center for attack by GSH (in some cases oxidation may be non-enzymatic); **3)** ectoenzyme γ -glutamyltransferase (GGT); **4)** ectoenzymes aminopeptidase N/cysteinylglycine dipeptidase and possibly cytosolic leucyl aminopeptidase; **5)** *N*-acetyltransferases; **6)** aminoacylases; **7)** cysteine *S*-conjugate β -lyases; **8)** thiomethyltransferase; **9)** UDP-glucuronosyltransferases. *In vivo* the hydrolysis reaction of GGT predominates over the formation of γ -glutamyl amino acids. Additional possible metabolic transformations (not shown) include a) further oxidation of the thiomethyl compound to sulfoxide, sulfone or CO₂ and sulfate, which are excreted, b) conversion of some cysteine *S*-conjugates to the corresponding α -keto acid, α -hydroxy acid and oxidatively decarboxylated product, and c) conversion of the cysteine *S*-conjugate to the corresponding sulfoxide. Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; A, amino acid, dipeptide or GSH acceptor for the γ -glutamyltransferase reaction; γ -GLU-A, γ -glutamyl amino acid (or γ -glutamyl dipeptide; γ -glutamylglutathione). (Cooper, A. J. L.; Hanigan, M. H. In: *Comprehensive Toxicology 2nd Edition: Volume 4. Biotransformations*, McQueen, C. A. (Series Ed), Guengerich, F. P. (Volume Ed), Elsevier Press, Oxford, 2009; with permission of Elsevier]