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Measurement of Cysteine Dioxygenase Activity and Protein Abundance

Martha H. Stipanuk, John E. Dominy Jr., Iori Ueki, and Lawrence L. Hirschberger

Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853

Abstract

Cysteine dioxygenase is an iron (Fe^{2+})-dependent thiol dioxygenase that uses molecular oxygen to oxidize the sulfhydryl group of cysteine to generate 3-sulfinoalanine (commonly called cysteinesulfinic acid). Cysteine dioxygenase activity is routinely assayed by measuring cysteinesulfinate formation from substrate L-cysteine at pH 6.1 in the presence of ferrous ions to saturate the enzyme with metal cofactor, a copper chelator to diminish substrate oxidation, and hydroxylamine to inhibit pyridoxal 5'-phosphate-dependent degradation of product. The amount of cysteine dioxygenase may be measured by immunoblotting. Upon SDS-PAGE, cysteine dioxygenase can be separated into two major bands, with the upper band representing the 23-kDa protein and the lower band representing the mature enzyme that has undergone formation of an internal thioether cross link in the active site. Formation of this cross link is dependent upon the catalytic turnover of substrate and produces an enzyme with a higher catalytic efficiency and catalytic half-life.

Keywords

cysteine dioxygenase; cysteine; cysteinesulfinic acid; taurine; sulfate

Introduction

Cysteine dioxygenase (CDO) is a 23-kDa, iron (Fe^{2+})-dependent thiol dioxygenase that uses molecular oxygen to oxidize the sulfhydryl group of cysteine to generate 3-sulfinoalanine (commonly called cysteinesulfinic acid, or CSA). CDO plays a central role in the regulation of intracellular cysteine concentrations and, hence, the availability of cysteine for glutathione synthesis, as evidenced by the fact that over-expression of CDO in HepG2/C3A cells reduced both cysteine and glutathione levels and potentiated the toxicity of CdCl_2 (Dominy et al., 2007).

Basic Protocol 1 describes a procedure for assaying the activity of CDO in tissue homogenates or extracts. This assay for CDO has been optimized for application to crude

tissue preparations under air-saturated conditions. The addition of NAD to the incubation mixture is optional as it is not required for the enzyme reaction. Two alternative protocols are also described. Alternate Protocol 1 is a simplified assay that is appropriate for measuring the activity of purified CDO. When purified CDO is being assayed, it is not necessary to add hydroxylamine as the enzyme preparation should not contain any enzymes that will degrade the product. For both the basic and first alternative assays, the product (cysteinesulfinic acid) is derivatized with o-phthalaldehyde, and the fluorescent derivatives are separated by HPLC. Alternate Protocol 2 is described for situations in which very high sensitivity is required or for kinetic studies of inhibition by product; this assay involves using radiolabeled substrate. Finally, Basic Protocol 2 describes measurement of CDO amount by SDS-PAGE and immunoblotting.

Basic Protocol 1: Standard Assay for Cysteine Dioxygenase Activity in Tissue Samples or Cell Extracts

This protocol is similar to the one we previously described (Bagley et al., 1995) but includes some additional modifications we have made over the past decade. It is appropriate for assay of CDO in tissue samples. Because some tissues have very low activity, it is wise to do an initial pre-test of the assay using a liver supernatant from a rat or mouse fed a standard or high protein diet.

Materials

2-(*N*-morpholino)ethanesulfonic acid (MES), free acid, monohydrate ($C_6H_{13}NO_4S \cdot H_2O$, FW 213.2)

Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, FW 278.02)

Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$, FW 69.49)

NAD (β -Nicotinamide adenine dinucleotide) ($C_{21}H_{27}N_7O_{14}P_2$, MW 663.43)

L-cysteine, free base ($C_3H_7NO_2S$, MW 121.16)

Bathocuproinedisulfonic acid (BCS), disodium salt ($C_{26}H_{18}N_2Na_2O_6S_2$, FW 564.54)

0.5 M potassium hydroxide for pH adjustment (KOH, FW 56.11; dissolve 2.8 g per 10 ml for 0.5 M solution)

5-Sulfosalicylic acid, dihydrate ($C_7H_6O_6S \cdot 2H_2O$, FW 254.2)

Potassium dihydrogen phosphate (KH_2PO_4 , MW 136.09) and potassium monohydrogen phosphate (K_2HPO_4 , MW 174.18)

Tetrahydrofuran (THF), HPLC grade (C_4H_8O , MW 72.11)

Acetonitrile, HPLC grade (CH_3CN , MW 41.05)

ortho-Phthalaldehyde (OPA) ($C_8H_6O_2$, FW 134.13)

Ethanol (C_2H_5OH , MW 46.07)

Boric acid (H_3BO_3 , MW 61.83)

Potassium hydroxide (KOH, FW 56.11)

2-Mercaptoethanol ($HOCH_2CH_2SH$, 14.3 M pure liquid)

L-Asparagine ($C_4H_8N_2O_3$, MW 132.119)

L-cysteinesulfinic acid ($C_3H_7NO_4S$, MW 153.2)

L-cysteic acid ($C_3H_7NO_5S$, MW 169.2)

Dowex 50 WX8 200-400 mesh, H⁺ form (Supelco, Bellefonte, PA)

Nova-Pak C₁₈, 4.6 × 150-mm column for HPLC (4 μm spherical particles; Waters Corporation)

C₁₈ guard column cartridge for HPLC (5 μm spherical particles; Alltech)

Prepare samples

1. 1a. *For liver*: Prepare 20% to 40% homogenate in 50 mM MES buffer, pH 6.1 (e.g., 2.0 g tissue plus 8.0 ml MES buffer for a 20% homogenate).

Samples can also be homogenized in other buffers, but check to ensure that the final assay mixture has a pH of 6.1.

Measurements of tissue weights and buffer volumes need to be precise if results are to be expressed per g tissue but not if results are to be expressed only as per mg protein, as protein concentrations will be determined later in these samples.

Most of the CDO activity is recovered in the soluble fraction of most tissues, so it is usually acceptable to use supernatant fractions instead of homogenates, if preferred.

It is also acceptable to include detergents or protease or phosphatase inhibitors in the buffer or lysis solutions used for tissue or cell preparation, but use of iron chelators should generally be avoided as CDO requires Fe(II) for activity.

1b. *For isolated hepatocytes or other cells in suspension*: Centrifuge hepatocyte suspension ?? min at ?? × g, ??°C, to pellet cells and then homogenize cells in 50 mM MES buffer, pH 6.1, to prepare an ~0.33% homogenate (e.g., 0.33 g cells plus 0.67 ml MES buffer). Sonicate the homogenate for three 15-sec periods; cool the suspensions on ice between sonication.

1c. *For primary or other attached cell cultures*: Add ~250 μl 50 mM MES, pH 6.1 (or other lysis buffer) per 100-mm dish. Scrape cells from dish and then sonicate the suspension three time for 15 sec on ice to lyse the cells.

2. To obtain supernatants, centrifuge homogenates for 30 minutes at 25,000 × g at 4 °C.

It is also acceptable to assay homogenates directly.

3. To be able to express results on the basis of protein content (particularly recommended for cell preparations), determine the protein content of the homogenates using an assay such as the bicinchoninic acid (BCA; *APPENDIX 3I*) assay with bovine serum albumin as a standard.

Prepare reagents for enzyme assay

1. Prepare the following reagents (sufficient for 180+ assays; see recipes). The final concentrations of assay components, in a final incubation volume of 0.4 ml, will be 62.5 mM MES, 0.3 mM ferrous sulfate, 5 mM hydroxylamine, 5 mM cysteine, and 0.0625 mM bathocuproine disulfonate.

50 mM MES buffer, pH 6

200 mM MES buffer, pH 6.1

*Fe (II), NAD, and hydroxylamine premix:

Cysteine and BCS solution*Sulfosalicylic acid (SSA)****Perform enzyme assay**

Run assay and blanks as enzyme versus time duplicates (replicate contains half as much enzyme incubated for twice the time). For each sample, set up four tubes: two with 0.12 ml sample and two with 0.06 ml sample; one of each will be used as acid-killed, zero-time blanks; the others will be incubated for 5 (0.12 ml) or 10 (0.06 ml) min (See Table 6.15.1).

1. Set up assays in 1.5-ml microcentrifuge tubes that are sitting in ice. To each tube, add:
 - 0.08 ml Fe(II) and hydroxylamine premix (freshly prepared)
 - 0.1 ml 200 mM MES buffer
 - 0.12 ml sample *or* 0.06 ml sample plus 0.06 ml 50 mM MES buffer
2. For blanks, add 0.1 ml of the freshly prepared cysteine plus BCS solution and mix. Then immediately add 0.2 ml of 5% (w/v) SSA to stop the reaction. Return to ice.
3. Using a timer, start reactions at 20- or 30-sec intervals by adding 0.1 ml of the cysteine and BCS solution, mixing, and immediately placing it in an Eppendorf Thermomixer (Brinkmann Instruments) with shaking at 900 rpm at 37 °C.
4. Incubate for 5 (0.12 ml sample) or 10 (0.06 ml sample) minutes.
5. Using a timer, stop reactions at 20- or 30-sec intervals by removing the tube from the mixer, adding 0.2 ml of 5% (w/v) sulfosalicylic acid, and placing the tube on ice.
6. Let tubes with stopped reaction mixtures sit on ice for 15 min. Centrifuge tubes at $\sim 16,000 \times g$ for 15 min to pellet the denatured protein.
7. Remove the acid supernatant and store in labeled tubes at -20°C until ready to measure the product, CSA, by HPLC.

Prepare reagents for HPLC

1. Prepare reagents for HPLC analysis of reaction products in acid supernatant from enzyme assays.
 - Dowex 50 WX8 resin, 200-400 mesh (H⁺) form
 - 0.10 mM L-asparagine
 - 0.2 M borate buffer, pH 10.4
 - OPA derivatization mixture (5 mM o-phthalaldehyde + 27.5 mM 2-mercaptoethanol in ~ 0.2 M borate buffer)
 - 1.0 M potassium phosphate, pH 7.0
 - Buffer A (50 mM potassium phosphate with 3% THF, pH 7)
 - Buffer B (50 mM potassium phosphate with 3% THF and 60% acetonitrile, pH 7)
 - 100 mM L-cysteinesulfinic acid (CSA) standard
 - 100 mM L-cysteic acid (CA) standard

Measure product formation by HPLC

Measure product formation by derivatizing the acid supernatants from the incubation mixtures to form the OPA derivatives of CSA and any CA that might be present as a secondary product by high performance liquid chromatography coupled with fluorescent detection. Use of an internal standard (e.g., asparagine) is recommended.

1. To prepare the sample for HPLC, apply 0.5 ml of the acid supernatant to a 3-cm column of Dowex 50 WX8, 200-400 mesh (H^+ form) in a Pasteur pipet plugged with glass wool. Follow sample loading with two 1-ml aliquots of deionized water. Collect all 2.5 ml of eluate for analysis.

This procedure removes cations in the sample that could interfere with HPLC analysis. If you find that this step is unnecessary, simply use undiluted samples in step 2.

2. Mix 10 μ l of the Dowex eluate (or sample or standard) with 10 μ l of 0.10 mM L-asparagine (an internal standard) and 80 μ l of 200 mM borate buffer, pH 10.4.
3. Precolumn derivatize samples with o-phthalaldehyde (OPA) using an automatic sample injector (e.g., WISP model 717 plus, Waters Corporation). Program the injector to mix 70 μ l of the o-phthalaldehyde (OPA)/2-mercaptoethanol derivatization reagent with 30 μ l of the sample or standard and wait for 1 minute prior to sample injection.
4. Perform HPLC on a system equipped with a 4.6 $\times$ 150-mm Nova-Pack C_{18} column (4 μ m spherical particles; Waters Corporation) equipped with a C_{18} cartridge (5 μ m spherical particles; Alltech), using a gradient elution at a flow rate of 1.0 ml/min and a column temperature of 30.0 $^{\circ}$ C. Begin elution with 5 minutes of 100% of Buffer A (50 mM potassium phosphate with 3% THF, pH 7) followed by a linear gradient to 70% Buffer A:30% of Buffer B (50 mM potassium phosphate with 3% THF and 60% acetonitrile, pH 7) over 7 minutes (i.e., 5 min to 12 min of run time). Maintain the mobile phase at 70% Buffer A:30% Buffer B for a total of 3 min. Then linearly increase Buffer B from 30% to 100% over 3 min (i.e., 15 min to 18 min of run time) and maintain 100% Buffer B for 2 min. [Following elution of each sample, the gradient should be switched back to 100% Buffer A by running a linear gradient over 3 min, and then running 100% Buffer A for 7 minutes to reequilibrate the column with buffer A prior to injection of the next sample.]
5. Detect derivatized amino acids in the column eluate using a fluorescence detector (e.g., Model 2475, Waters Corporation) set with an excitation wavelength of 360 nm and an emission wavelength of 455 nm. Set the excitation wavelength at 200 nm for the first 3.5 min of the run in order to eliminate a large void volume peak due to the SSA. Collect output from the fluorometer by a personal computer equipped with Empower 2 software (Waters Corporation) for integration of chromatographic peaks and interpolation of analyte concentrations.

CSA is the immediate and major product that accumulates. A small amount of CSA may be nonenzymatically further oxidized to CA. CSA elutes at $\sim$ 6.5 min and CA elutes at $\sim$ 3.8 min. Check for the production of CA as a secondary reaction product, especially if samples have been stored for a long period of time between the assay and the HPLC measurement of product. If needed, run a standard curve for both CSA and CA.

6. Determine the average area of the asparagine peak from all samples and standards in the run. Then divide the asparagine area for each sample or standard by this average asparagine peak area to obtain a ratio. Multiply each CSA and CA standard

value (area) by the asparagine (internal standard) ratio for that standard before establishing the standard curve. Then multiply the CSA and/or CA value (area) for each sample by its asparagine (internal standard) ratio. Determine the sample CSA and CA concentrations for each sample by comparison with the standard curves.

Because standards and sample Dowex 50 eluate are treated identically from step 2 onwards, the concentration of CSA or CA read from the standard curve will be equivalent to the concentration of the product in the Dowex 50 eluate.

- Express enzyme activity as nmol cysteinesulfinate·min⁻¹·g wet weight of tissue⁻¹ or as pmol cysteinesulfinate·min⁻¹·mg protein⁻¹. Calculate the amount of CSA (plus CA) produced per tube and then divide by the assay time (min) the amount of protein (mg) or tissue (g) added to the assay tube, as shown below.

Calculate the amount of CSA (plus CA) produced per tube:

$$\frac{\mu\text{mol CSA}}{\text{ml Dowex eluate}} \times \frac{2.5 \text{ ml Dowex eluate total}}{0.5 \text{ ml stopped assay supernatant loaded on Dowex}} \times \frac{0.60 \text{ ml total stopped assay volume}}{\text{assay tube}} = \frac{\mu\text{mol CSA}}{\text{assay tube}}$$

Divide the μmol product produced per assay flask or tube by the amount of protein or tissue in the sample and by the incubation time to calculate CDO activity:

$$\frac{\mu\text{mol CSA}}{\text{assay tube}} \times \frac{\text{assay tube}}{\text{g tissue or mg protein}} \times \frac{1}{\text{assay time (min)}} = \frac{\mu\text{mol CSA}}{\text{min} \times \text{g tissue or mg protein}}$$

Alternate Protocol 1: Simplified Assay for Measurement of Activity of Purified Cysteine Dioxygenase

This simplified assay is appropriate for measuring CDO activity of purified CDO. We have used it with recombinant CDO expressed with or without fusion peptide tags. The assay protocol is similar to the standard assay for measurement of CDO activity in tissue preparation. However, several important modifications have been made. (1) Hydroxylamine can be omitted because PLP-containing enzymes that degrade cysteinesulfinate (i.e., aminotransferases and cysteinesulfinate decarboxylase and glutamate decarboxylase) should not be present. (2) NAD can be omitted because NAD has no effect on activity measurements for purified CDO. (3) The cysteine concentration should be reduced to 3 mM because the purified enzyme is more subject to substrate inhibition at a given concentration of added cysteine. (4) It is not necessary to use the Dowex 50 pre-HPLC treatment as the sample matrix is much simpler.

Materials

2-(*N*-morpholino)ethanesulfonic acid (MES), free acid, monohydrate (C₆H₁₃NO₄S·H₂O, FW 213.2)

Ferrous sulfate heptahydrate (FeSO₄·7H₂O, FW 278.02)

L-cysteine, free base (C₃H₇NO₂S, MW 121.16)

Bathocuproinedisulfonic acid (BCS), disodium salt (C₂₆H₁₈N₂Na₂O₆S₂, FW 564.54)

5-Sulfosalicylic acid, dihydrate (C₇H₆O₆S·2H₂O, FW 254.2)

Prepare samples

- Use bacterial expression system to generate recombinant fusion CDO protein and purify protein.

Possible expression and purification methods have been reported (Dominy et al., 2006b; Simmons et al., 2006b; Dominy et al., 2008).

2. Measure protein content of the purified CDO using a colorimetric protein assay such as the bicinchoninic acid (BSA) assay with bovine serum albumin as a standard (*APPENDIX I*).

Alternatively, if no interfering compounds are present, the protein content can be determined by reading the absorbance at 280 nm and converting this to molar concentration using an extinction coefficient of $25230 \text{ cm}^{-1}\text{M}^{-1}$. Based on the molecular weight of rat/mouse CDO (23,026 Da), a 1 M solution of CDO would contain 23 g CDO protein per ml or 23 mg CDO protein per μl .

3. Dilute purified CDO in 50 mM MES buffer (pH 6.1) just prior to assay to obtain desired concentration.

You will need to use ~ 1 to $5 \mu\text{g}$ of CDO protein per 400- μl assay tube. Thus, you need a CDO solution that contains $\sim 2.2 \times 10^{-6} \text{ M}$ CDO (i.e., $5 \mu\text{g}/100 \mu\text{l}$ or $2.5 \mu\text{g}/50 \mu\text{l}$).

Prepare reagents

1. Prepare reagents (sufficient for 120 microtube assays). The final concentrations of assay components, in a final incubation volume of 0.40 ml, will be 62.5 mM MES, 0.3 mM ferrous sulfate, 3 mM cysteine, and 0.0625 mM bathocuproine disulfonate.
 1. **50 mM MES buffer**, pH 6.1
 2. **200 mM MES buffer**, pH 6.1
 3. ***Fe (II)**: 2.5 mM ferrous sulfate heptahydrate
 4. ***Cysteine and BCS solution**

Perform enzyme assay

Run assay and blanks as “enzyme $\times$ time” duplicates (i.e., the duplicate contains half as much enzyme incubated for twice the time). For each sample, set up in 4 tubes: two with 120 μl sample and two with 60 μl sample; one of each will be used as acid-killed, zero-time blanks; the others will be incubated for 5 (100 μl) or 10 (50 μl) min.

1. Set up assays in microfuge tubes that are sitting on ice. To each flask, add:
 - 50 μl of 2.5 mM Fe(II) (freshly prepared)
 - 100 μl 200 mM MES buffer
 - 100 μl sample **OR** 50 μl sample plus 50 μl 50 mM MES buffer
 - 50 μl 50 mM MES buffer
2. For blanks, add 100 μl of the freshly prepared cysteine plus BCS solution and mix. Then immediately add 200 μl of 5% SSA to stop the reaction. Return to ice.
3. For assay tubes, using a timer, start reactions at 20 or 30 sec intervals by adding 100 μl of the 20 mM cysteine plus 0.25 mM BCS solution, mixing, and immediately placing it in an Eppendorf Thermomixer at 37 °C with shaking at 900 rpm.
4. Incubate for 5 (100 μl sample) or 10 (50 μl sample) minutes. [If there is concern about the conversion of immature CDO to mature thioether-linked CDO over time,

consider using a shorter incubation time and increasing the CDO concentration as needed.]

5. Using a timer, stop reactions at 20 or 30 sec intervals by removing the tube from the Thermomixer, adding 200 μ l 5% SSA, and placing the tube on ice.
6. Let tubes with stopped reaction mixtures sit on ice for 10 minutes. Then centrifuge the tubes at $\sim 16,000 \times g$ for 15 minutes to pellet the denatured protein.
7. Remove the acid supernatant and store in labeled tubes at -20°C until ready to measure the product, CSA, by HPLC.

Measure product formation by HPLC

1. For quantification of CSA from the reactions, mix 10 μ l of the acid supernatant (or standard solution) with 10 μ l of 200 mM asparagine and 380 μ l of 200 mM borate buffer, pH 10.4. Proceed with the HPLC analysis as above.
2. Calculations will be slightly different because, in this case, the CSA concentration determined by comparison with standard concentrations refers to the concentration in the acid supernatant from the assay mixture.

Calculate the amount of CSA (plus CA) produced per tube:

$$\frac{\mu\text{mol CSA}}{\text{ml acid supernatant}} \times \frac{0.60 \text{ ml total stopped assay volume}}{\text{assay tube}} = \frac{\mu\text{mol CSA}}{\text{assay tube}}$$

Divide the μ mol product produced per assay flask or tube by the amount of protein (purified CDO) added to the assay tube and by the incubation time to calculate CDO activity:

$$\frac{\mu\text{mol CSA}}{\text{assay tube}} \times \frac{\text{assay tube}}{\text{mg CDO protein}} \times \frac{1}{\text{assay time (min)}} = \frac{\mu\text{mol CSA}}{\text{min} \times \text{mg CDO protein}}$$

III. Alternate Protocol 2: Radiolabeled Assay for Cysteine Dioxygenase Activity

This modification of the CDO assay is useful for measuring activity in tissue samples or cell extracts that have lower activity than liver. The radioactive assay follows the standard protocol except that (1) the substrate solution is radiolabeled with [^{35}S]-cysteine and (2) an anion-exchange HPLC procedure is used to separate un-derivatized [^{35}S]-cysteinesulfinate with quantification of product by scintillation counting of fractions containing [^{35}S]-cysteinesulfinate.

1. Modification #1: Preparation of ^{35}S -labeled cysteine substrate solution

^{35}S -labeled cysteine and BCS solution—20 mM L-cysteine (free base) plus 0.25 mM bathocuproine disulfonate (BCS). Dissolve 0.0485 g L-cysteine (free base) and 0.2823 g bathocuproinedisulfonic acid (disodium salt) in 20 ml deionized water. Take the volume of cysteine/BCS solution needed for the number of assays to be done (plus a little extra for measurement of specific activity of the final solution) and add 2 to 4 microcuries of [^{35}S]-cysteine** per ml cysteine/BCS solution (i.e., 1 to 2 μCi of [^{35}S]-cysteine per 2-ml final assay mixture for the standard assay, or 0.2 to 0.4 ml per assay mixture for the microassay; or a specific radioactivity of 0.2 to 0.4 $\mu\text{Ci}/\mu\text{mol}$). Take 10 μL aliquots of the substrate solution and measure radioactivity by scintillation counting; divide cpm per 10 μL aliquot by the amount of cysteine in 10 μL (i.e., 0.4 μmol) to calculate specific radioactivity of the

[³⁵S]cysteine substrate solution. [**Check the purity of the [³⁵S]cysteine prior to use, as [³⁵S]cysteine does undergo oxidation to cysteinesulfinic acid and cysteic acid. To remove impurities, the [³⁵S]cysteine can be applied to a 0.6 × 4-cm Dowex 1-X8 column (acetate form) and eluted with ~20 ml deionized water. After purification, lyophilize and store [³⁵S]cysteine at -80 °C until needed.]

2. Modification #2: HPLC and scintillation counting to measure [³⁵S]CSA production

Because OPA derivatization of CSA gives rise to a relatively unstable derivative, resulting in distribution of radioactivity among several peaks, it is preferable to measure [³⁵S]CSA directly using anion-exchange HPLC. The HEMA-IEC BIO Q columns described in this protocol are, unfortunately, no longer available. We have not yet tried to modify this procedure to run on a different anion-exchange column, but describe the method we have previously used as a starting point for setting up HPLC with a different column.

Because there can be some further nonenzymatic oxidation of CSA to CA, particularly during long-term storage of acid supernatant fractions at -20°C, both the CSA and CA peaks should be measured.

Although not described in detail here, if purified CDO is being assayed with the radioactive method, it should be possible to separate radiolabeled substrate from radiolabeled product simply by running the acid supernatant over a Dowex 50 column to trap the [³⁵S]cysteine substrate and eluate the [³⁵S]cysteinesulfinate product. This approach, however, should not be applied to crude preparations as ³⁵S-sulfate would also elute with the ³⁵S-cysteinesulfinate, and ³⁵S-sulfate production may not be completely inhibited by addition of hydroxylamine. Load 2 ml of the acid supernatant on a 0.6 × 5 cm column of Dowex 50-X8 (200-400 mesh, hydrogen form). Elute cysteinesulfinate with 11 ml of water (pH 7). Determine the radioactivity in an aliquot of the eluate by liquid scintillation spectrometry.

- a. To prepare the acid supernatant from the enzyme assay for analysis by HPLC, apply 0.5 ml of sample to a 3 cm column of Dowex 50 (H⁺ form) in a Pasteur pipet plugged with glass wool. Follow this with 2 × 1 ml aliquots of deionized water to eluate anions, including cysteinesulfinic acid and CA. Collect all 2.5 ml of the eluate for analysis. This procedure removes cations, including the [³⁵S]cysteine, in the sample that can interfere with the HPLC analysis.
- b. Carry out anion-exchange HPLC using a HEMA-IEC BIO Q analytical column (150 × 4.6 mm, 10-μm particles, Alltech) and a HEMA-IEC BIO Q DEAE guard cartridge (10 × 4.6 mm, 10-μm particles, Alltech), with the system set up for fraction collection during elution of CSA and CA. The column should be equilibrated with deionized water (eluant A) prior to sample injection and the flow rate should be 1 ml/min. Inject 100 μl of the sample eluate from the Dowex 50W clean-up column. Following sample injection, linearly change the eluant over 5 min from 100% eluant A (water) to 15% eluant B (100 mM borate buffer with 250 mM NaCl, pH 8). Over the following 10 min, change eluant linearly to 33% eluant B. Then, over the next 3 min, linearly increase eluant B to 100%, and then wash the column with 100% eluant B for 4 min. [Finally, bring the column back to initial conditions (100% eluant A) over the next 3 min, and allow the column to equilibrate with 100% eluant A for 10 min before the next sample is injected.] The mean retention times for CSA and CA are ~11.8 and ~14.5 min, respectively.

If molar quantification of CSA or CA are desired, or to identify the location of the product peaks, postcolumn derivatization can be done using a derivatization solution containing 3 mM *o*-phthalaldehyde, 0.4% (v/v) methanol, 0.2% (v/v) 2-mercaptoethanol, and 200 mM borate buffer, pH 10.4, that is pumped at a rate of

0.5 ml/min. This derivatization solution should be prepared fresh and kept in the dark; it should be stable for ~36 hr at room temp or for 3 or 4 days at 4°C when kept in the dark or in an opaque bottle. Fluorescence can be detected using excitation and emission wavelengths of 360 and 455 nm, respectively. L-CSA and L-CA can be used to generate standard curves, which should be linear over the range of zero to 10 nmol per 100-μl injection volume (equivalent to injecting 100 μl of a standards containing zero to 100 μmol/L cysteinesulfinate or CA).

Fractions of eluate should be collected over a time frame that encompasses the elution times for CSA and CA, with fraction size corresponding to 1 min or less of run time. If postcolumn derivatization is done, the presence of the derivatizing solution in the eluate should not affect counting efficiency.

- c. Measure ^{35}S radioactivity in the eluate fractions corresponding to the CSA and CA peaks by liquid scintillation spectrometry. Correct for counting efficiency and radioactive decay. To avoid the need to calculate decay of ^{35}S between running the enzyme assay and analysis of the product, save the substrate aliquots for determination of specific radioactivity and count these along with the HPLC eluate fractions containing the ^{35}S -labeled products in the same batch. Calculate the molar quantity of CSA measured by HPLC by dividing the amount of [^{35}S]CSA (plus CA) produced by the specific radioactivity of the L- ^{35}S]cysteine used as substrate. Calculate the molar quantity of CSA produced in the enzyme assay by correcting this value back to the final assay volume:

$$\frac{\text{total cpm}^{35}\text{S-CSA in HPLC eluate}}{\text{cpm}^{35}\text{S}/\mu\text{mol substrate}} \times \frac{2.5 \text{ ml Dowex eluate total}}{0.10 \text{ ml Dowex eluate injected}} \times \frac{0.60 \text{ ml total stopped assay volume}}{0.50 \text{ ml stopped assay supernatant loaded on Dowex}} = \frac{\mu\text{mol CSA}}{\text{assay tube}}$$

Divide the umol product produced per assay flask or tube by the amount of protein or tissue in the sample and by the incubation time to calculate CDO activity:

$$\frac{\mu\text{mol CSA}}{\text{assay tube}} \times \frac{\text{assay tube}}{\text{g tissue or mg protein}} \times \frac{1}{\text{assay time (min)}} = \frac{\mu\text{mol CSA}}{\text{min} \times \text{g tissue or mg protein}}$$

IV. Basic Protocol 2: Western Blotting to Measure CDO Protein Levels

Western blotting involves the separation of CDO from other proteins in the sample by one-dimensional SDS-PAGE, followed by the detection of immunoreactive protein. The method described here has been successfully used to measure CDO in rat, mouse and human tissue samples.

Reagents and supplies

1. For sample preparation prior to SDS-PAGE:

Lysis buffer with protease inhibitor cocktail: We usually use a TNES (Tris/Nonidet-P40/EDTA/NaCl) buffer as described in reagent preparation with 1× mammalian protease inhibitor cocktail

Loading buffer 6× (See reagent preparation.)

2. For SDS-PAGE

Apparatus for one-dimensional electrophoresis

Electrode buffer for Lammeli method. (See reagent preparation for instructions for preparing a 5× stock of electrode buffer.)

SDS-polyacrylamide gels (12 % or 15%,w/v, acrylamide). [We have obtained the best resolution of the three CDO protein species when we use manually-prepared 14 cm × 16 cm Tris-Glycine SDS-PAGE gels. The gel should be poured so the stacking layer has at least 1.5 cm between the bottom of the well and the top of the resolving portion of the gel. The stacking layer consists of 5.1% (w/v) acrylamide with a final pH of 6.8. The resolving layer consists of 12% (w/v) acrylamide with a final pH of 8.8.]

Protein molecular weight markers (various markers are listed in 10.2A)

3. For electroblotting

Polyvinylidene difluoride (PVDF) membranes such as 0.45 Immobilon-P
PVDF membranes (Millipore, Medford, MA, U.S.A)

Adjustable speed rocking platform

Transfer buffer: We use a buffer containing 25 mM Tris base, 193 mM glycine, and 15% methanol with PVDF membranes (see reagent preparation).

4. For immunoblotting

1× TBST (See reagent preparation.)

Blocking buffer (1× TBST containing 5% w/v non-fat dry milk; see reagent preparation)

Primary antibody (anti-CDO): Anti-rat/mouse CDO from our laboratory is available from Abcam as the IgG fraction of the rabbit anti-CDO immune serum. The antibody was raised against His6-tagged IMAC-purified recombinant rat CDO, and the IgG fraction was purified from rabbit serum using a protein A column (Stipanuk et al., 2004). We have used this antibody to detect rodent and human CDOs. [Note about other antibodies to CDO: Several antibodies raised against linear epitopes of CDO (e.g., residues 167-177) have been used and results have been published for a 68-kDa protein detected by these antibodies (Parsons et al., 1998a, 1998b, 2001a, 2001b; Qusti et al., 2000). There is no evidence for a 68-kDa CDO species of CDO, and because these antibodies to linear epitopes of CDO did not detect any protein migrating with a molecular mass close to the 23 kDa molecular mass of CDO, it would seem that these antibodies are unacceptable for measuring CDO levels. The antibody to purified rat liver CDO that was raised by Hosokawa et al. (1990) yielded similar results as those antibodies we have raised against recombinant rat CDO; to our knowledge, this antibody is no longer available.]

Control protein antibody such as anti-rat actin

Secondary antibody: horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (e.g., Pierce product # 1858415) and chemiluminescent substrate (e.g., Supersignal West Pico or Supersignal West Dura from Thermo Scientific)

X-ray film

5. For quantification

Scanner

Software (e.g., NIH Image 1.63 software)

6. Chemicals for reagent preparation

Tris base ($C_4H_{11}NO_3$, MW 121.14)

Nonidet P40 (octylphenolpoly(ethyleneglycolether))

EDTA, or Ethylenediaminetetraacetic acid (disodium salt, dihydrate, $C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$, FW 372.24)

NaCl, or Sodium chloride (FW 58.44)

HCl, or Hydrochloric acid (concentrated = 12N)

Tween-20 (polyoxyethylene sorbitan monolaurate)

DTT, or DL-dithiothreitol ($HSCH_2CH(OH)CH(OH)CH_2SH$, FW 154.25)

SDS, or sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$, FW 288.38)

Glycerol ($HOCH_2CH(OH)CH_2OH$, FW 92.09)

Bromophenol blue ($C_{19}H_{10}Br_4O_5S$, FW 669.96)

Glycine (NH_2CH_2COOH , FW 75.07)

Methanol (CH_3OH , FW 32.04)

Non-fat dry milk

Prepare samples

1. Homogenize tissue or lyse cells.
 - a. For fresh or frozen tissue samples, homogenize samples in ~4 volumes of ice-cold lysis buffer prepared with addition of a protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A) prior to use. If tissues are being prepared for both enzyme assay and western blotting, 0.05 M 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.0, with protease inhibitors is acceptable for both methods.
 - b. For cultured cells, harvest cells with 200-250 μ l of lysis buffer for a 60 mm culture dish.
2. Centrifuge to obtain supernatant, if desired. Use the homogenate as such or prepare supernatant by centrifuging the homogenate at $\sim 20,000 \times g$ for 30 min at 4 °C. Most, if not all, of the CDO is soluble.
3. Determine the protein levels in the samples (supernatants or homogenates) that are to be loaded using an appropriate method, such as the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Use the protein concentration to calculate dilutions and the volume of sample to be loaded. Typically, we load 30-40 μ g of total protein per lane for cultured cell lysates and 40-50 μ g of total protein per lane for tissue lysates.
4. Prepare samples for loading by diluting samples with 6 $\times$ loading buffer (5 volumes sample:1 volume 6 $\times$ loading buffer to yield a 1 $\times$ final concentration of loading buffer) and heating samples to 95-100 °C for 5 minutes. Cool heated samples to room temperature prior to loading them on the gel.

Prepare reagents

1. **TNES lysis buffer with protease inhibitors:** Prepare buffer to contain 0.5% (v/v) Nonidet P40, 1 mM EDTA, 150 mM sodium chloride, and 1 $\times$ mammalian protease cocktail in 50 mM Tris buffer, pH 7.4. Dissolve 0.6057 g Tris base in 75 ml water

and add 1 N HCl to adjust pH to 7.4. Add 0.03722 g EDTA (disodium salt, dihydrate) and 0.8766 g NaCl; mix to dissolve. Add 0.5 ml Nonidet P40. Check pH and adjust if necessary. Bring final volume up to 100 ml and refrigerate. Just before use, add a mammalian protease inhibitor cocktail to yield a 1× concentration.

2. **Loading buffer, 6×:** The final composition of the 6× loading buffer should be 1.3 % (w/v) SDS, 0.5 M DTT, 0.0012% w/v bromophenol blue and 30% v/v glycerol in 1 M Tris buffer, pH 8.8. Step 1: Prepare 50 mM Tris buffer, pH 6.8. For 100 ml of 50 mM Tris buffer, dissolve 6.05 g Tris base in 70 ml of water, adjust the pH to 6.8 with 1 N HCl, and bring volume up to 100 ml. Step 2: Dissolve 1 g SDS (1% w/v), 0.93 g DTT (0.5 M), and 1.2 mg bromophenol blue (0.0012% w/v) into 7 ml of the 50 mM Tris buffer, pH 6.8; add 3ml glycerol (30% v/v). Store at -70 °C.
3. **Electrode buffer.** The final composition of the 1× solution should be 25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS. To prepare 1 liter of a 5× stock of electrode buffer, dissolve 15 g of Tris base, 72 g of glycine, and 5 g of SDS in 900 ml of water; bring volume up to 1 L with water. Refrigerate. Dilute to 1× just before use. (The pH of this buffer does not need to be adjusted; the 1× solution will have a pH of ~8.3.)
4. **Transfer buffer (for PVDF membranes):** Prepare solution containing 25 mM Tris base, 193 mM glycine and 15 % (v/v) methanol. To prepare 2 liters of transfer buffer, dissolve 6.05 g Tris base and 29 g glycine in 1.5 L of water. Add 300 ml of methanol and adjust final volume to 2 L. [The pH of this buffer does not need to be adjusted.] Refrigerate.
5. **1× Tris-buffered with Tween-20 (TBST):** The final composition of the 1× solution should be 150 mM NaCl and 0.1% (v/v) Tween-20 in 50 mM Tris-HCl, pH 7.5. To prepare 1 liter of a 10× stock of TBST, dissolve 60.6 g Tris base and 87.7 g NaCl in 900 ml of water and adjust pH to 7.5 with 6 N HCl; add 10 ml Tween-20, and bring volume to 1 liter. Refrigerate. To prepare a 1× solution, dilute 1 volume of 10× TBST with 9 volumes of water.
6. **Blocking buffer (5% w/v non-fat dry milk, 1× TBST).** Dissolve 5 g non-fat dry milk in 100 ml of 1× TBST. Prepare fresh on the day of use.

Perform SDS-PAGE

Perform denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli gel method.

1. Load the protein molecular weight marker according to the manufacturer's instructions or the desired amount of each prepared sample (i.e., in 1× loading buffer, heated to 95 °C and cooled) onto each well of the SDS-polyacrylamide gel.
2. For standard sized large gels, run at 20-25 mA per gel until the bromophenol blue dye enters the separating gel, then increase to 30-35 mA per gel. Continue the electrophoresis until the 20 kDa molecular weight marker is ~2 cm from the bottom of the gel (~4.5 h). Additional details on how to prepare and run one dimensional gel electrophoresis of proteins and the recipes for buffers can be found in Unit 10.2A.

Perform immunoblotting

Following separation of proteins by electrophoresis, the proteins are transferred to a PVDF membrane and immunoblotting is done using primary antibody to CDO.

1. Following electrophoresis, electroblot overnight to transfer the proteins onto a 0.45 mm Immobilon-P PVDF membrane (Millipore) at 30 V (0.1 A) using the transfer buffer described above (i.e., 25 mM Tris base, 193 mM glycine, 15 % methanol). After transfer, the membrane can be stained using Ponceau S to visualize proteins (Unit 10.8.7), if desired.
2. Block membranes with blocking buffer (5% w/v non-fat dry milk in 1× TBST for 1 hour at room temperature with gentle rocking.
3. Replace blocking buffer with the primary antibody solution diluted into blocking buffer (see supplier's suggestion for dilution factor) and incubate for 2 hours at room temperature with gentle rocking.
4. After incubation with primary antibody, wash membranes 3 times for 10 min each with 1× TBST at room temperature with rocking.
5. Dilute horseradish peroxidase-conjugated goat anti-rabbit secondary antibody in blocking buffer (5% w/v non-fat dry milk in 1× TBST) according to dilutions recommended by the manufacturer. Add this solution to the washed membrane, and incubate the membrane for 1 hour at room temperature.
6. Wash immunoblotted membrane 3 times for 10 min each with 1× TBST at room temperature and with rocking.
7. Develop signal using chemiluminescent substrate (such as Supersignal West Pico or Supersignal West Dura, Pierce) according to the manufacturer's directions, and visualize proteins that cross-reacted with the primary antibody by exposure to X-ray film. Additional details on electroblotting and immunodetection procedures can be found in Unit 10.8. CDO should migrate with an apparent molecular mass of ~22-23 kDa.
8. Membranes can be stripped using commercially available reagents such as Restore Western Blot stripping buffer (Pierce), and the western blotting procedure can be repeated for detection of additional proteins including the loading/transfer control (e.g., actin).

Quantify developed blot

For quantification of amounts of CDO, the density of the CDO band on the film can be quantified.

1. Scan developed films using a desktop scanner. With the obtained electronic images, perform two-dimensional quantitative densitometric analysis of the detected CDO and control protein (e.g., actin) bands using appropriate software (such as NIH Image 1.63 software). A rough idea of relative amounts of CDO can be obtained by dividing the density units for CDO by those for the actin (or other) control. This assumes that the samples being compared were all loaded on the same gel and contain amounts of CDO that fall within a linear range (usually less than a 3-fold difference).
2. For more accurate quantification, a standard curve can be run on each gel using purified recombinant CDO as a true standard, or a relative standard curve can be generated on each gel by loading graded amounts of a selected liver preparation (usually from a rat fed a moderate or high protein diet). If the same standards are run on each gel, it is also then possible to compare CDO amounts between different gels/blots.

3. For either approach, the measure of CDO should be divided by the amount of total protein loaded per lane.

Reagents and Solutions

1. **50 mM MES buffer, pH 6.1:** Dissolve 1.066 g MES monohydrate in deionized water (<100 ml). Adjust pH to 6.1. Adjust final volume to 100 ml. Refrigerate.
2. **200 mM MES buffer, pH 6.1:** Dissolve 4.264 g MES monohydrate in deionized water (<100 ml). Adjust pH to 6.1. Adjust final volume to 100 ml. Refrigerate.
3. ***Fe (II), NAD, and hydroxylamine premix:** 2.5 mM ferrous sulfate heptahydrate plus 10 mM NAD plus 25 mM hydroxylamine hydrochloride, pH 5.9. Dissolve 0.1327 g NAD⁺ and 0.0347 g hydroxylamine hydrochloride in 18 ml deionized water (in a 25 ml beaker so that a pH probe can be immersed in the solution). While stirring with a small stir bar, slowly add increments of 0.5 M KOH until the pH reaches 5.9. [Do not exceed pH 5.9 as this will promote oxidation and precipitation of the iron that will be added last.] Keep track of the volume and add water to make the final volume up to 20.0 ml. Then, just before using, add 0.0139 g ferrous sulfate heptahydrate and stir to dissolve. [*Prepare just prior to use because the iron will otherwise oxidize and precipitate over time.]
4. ***Cysteine and BCS solution:** 20 mM L-cysteine (free base) plus 0.25 mM bathocuproine disulfonate (BCS). Dissolve 0.0485 g L-cysteine (free base) and 0.0028g bathocuproinedisulfonic acid (disodium salt) in 20 ml deionized water. [*Prepare just prior to use because cysteine oxidizes to cystine and precipitates over time. It is permissible to prepare BCS as a stock solution and dilute it into the cysteine solution; e.g., use 50 µl of a 100 mM BCS solution per 20 ml.]
5. **Sulfosalicylic acid (SSA), 5% (w/v):** Dissolve 5 g sulfosalicylic acid in deionized water and bring volume up to 100 ml.

Dowex 50 WX8 resin, 200-400 mesh (H⁺) form: A 1:1 (v/v) suspension of the Dowex resin with deionized water is prepared and kept at 4°C.

0.10 mM L-asparagine: Prepare a 100 mM stock of asparagine (dissolve 132 mg L-asparagine in water and bring volume up to 10.0 ml). Dilute this stock asparagines solution 1:1000 with water to obtain a 0.10 mM solution.

0.2 M borate buffer, pH 10.4: Dissolve 12.35 g boric acid and 10 g potassium hydroxide pellets in 900 ml water. Titrate to a pH of 10.4 with 10 M KOH and bring to a volume of 1 L with water.

OPA derivatization mixture (5 mM o-phthalaldehyde + 27.5 mM 2-mercaptoethanol in ~0.2 M borate buffer). Dissolve 3.5 mg o-phthalaldehyde (OPA) in 150 µl 100% ethanol; add 5 ml of 0.2 M borate buffer (pH = 10.4), and 10 µl of 2-mercaptoethanol. Mix by stirring for ~20 minutes, protected from light. Store the solution in the dark (or in an opaque bottle) at 4°C for no longer than 3-4 days prior to use. This derivatization solution should be prepared fresh and kept in the dark; it should be stable for ~36 hr at room temp or for 3 or 4 days at 4°C when kept in the dark or in an opaque bottle.

1.0 M potassium phosphate, pH 7.0: Prepare a 1.0 M solution of KH₂PO₄ (136.09 g/L in water) and a 1 M solution of K₂HPO₄ (174.18 g/L in water). Mix the 2 solutions to obtain a final solution with a pH of 7.0 (approximately a 1:1 mixture). Filter the buffer through a 0.45 µm filter.

Buffer A (50 mM potassium phosphate with 3% THF, pH 7): Mix 50 ml of 1.0 M potassium phosphate buffer, pH 7.0, 30 ml of tetrahydrofuran, and 920 ml of milli-Q water.

Buffer B (50 mM potassium phosphate with 3% THF and 60% acetonitrile, pH 7): Mix 50 ml of 1.0 M potassium phosphate buffer, pH 7.0, 30 ml of tetrahydrofuran, 600 ml of acetonitrile, and 320 ml of milli-Q water.

100 mM L-cysteinesulfinic acid (CSA) standard: Dissolve 0.153 g CSA in water and bring up to 10.0 ml. Prepare a range of standards (10, 25, 100, 500 and 1000 μ M) by serial dilution of the stock in 0.2 M borate buffer, pH 10.4.

100 mM L-cysteic acid (CA) standard: Dissolve 0.169 g CA in water and bring up to 10.0 ml. Prepare a range of standards (10, 25, 100, 500 and 1000 μ M) by serial dilution of the stock in 0.2 M borate buffer, pH 10.4.

1. **50 mM MES buffer, pH 6.1:** Dissolve 1.066 g MES monohydrate in deionized water (<100 ml). Adjust pH to 6.1. Adjust final volume to 100 ml. Refrigerate.
2. **200 mM MES buffer, pH 6.1:** Dissolve 4.264 g MES monohydrate in deionized water (<100 ml). Adjust pH to 6.1. Adjust final volume to 100 ml. Refrigerate.
3. ***Fe (II):** 2.5 mM ferrous sulfate heptahydrate. Dissolve 0.0139 g ferrous sulfate heptahydrate in 20 ml of water. [*Prepare just prior to use because the iron will otherwise oxidize and precipitate over time. If premature precipitation is a problem, check pH of deionized water which should be ~5.5.]
4. ***Cysteine and BCS solution:** 12 mM L-cysteine (free base) plus 0.25 mM bathocuproine disulfonate (BCS). Dissolve 0.0291 g L-cysteine (free base) and **0.0028 g** bathocuproinedisulfonic acid (disodium salt) in 20 ml deionized water. [*Prepare just prior to use because cysteine oxidizes to cystine and precipitates over time. It is permissible to prepare BCS as a stock solution and dilute it into the cysteine solution; e.g., use 50 μ l of a 100 mM BCS solution per 20 ml.]

Commentary

Background Information

CDO catalyzes the initial step in the oxidation of the sulfur atom of cysteine and commits cysteine to conversion to either pyruvate plus sulfite/sulfate (with transfer of the amino group) or to hypotaurine/taurine (with loss of CO₂), depending on whether the product, CSA (cysteinesulfinic acid), undergoes transamination or decarboxylation. CDO activity and CDO concentration are high in liver of rats fed normal or high protein diets but low in liver of rats fed low protein diets (Bagley and Stipanuk, 1994; Bella et al., 1999; Stipanuk et al., 2002; Lee et al., 2004). CDO activity and concentration are also low in most extrahepatic tissues, although relatively high levels are observed in adipose tissue and moderate levels are observed in kidney (Stipanuk et al., 2002; Ueki and Stipanuk, 2006). Within some other nonhepatic tissues, specific cell types do express high levels of CDO despite the overall abundance in the tissue being low (Ueki and Stipanuk, 2006). Most cell lines that we have studied, including hepatocyte-derived cell lines, express minimal amounts of CDO protein, and CDO mRNA and protein expression in primary hepatocytes decreases with time in culture (Ohta et al. 2000; Kwon and Stipanuk, 2001). In contrast, a significant

induction of CDO mRNA and protein occurs during differentiation of 3T3-L1 preadipocytes into adipocytes (Ueki and Stipanuk, unpublished results).

CDO activity in tissues is highly responsive to the cysteine availability and intracellular cysteine concentration, being very low when cysteine levels are low but increasing dramatically within hours of exposure to high levels of cysteine. At least two cysteine-responsive mechanisms are involved in the regulation of CDO activity in tissues. The first mechanism involves the regulation of CDO ubiquitination and proteasomal degradation in response to cysteine. When cysteine is low, CDO undergoes rapid ubiquitination and degradation and does not accumulate in the cell. However, when cysteine is high, ubiquitination is minimized and the rate of CDO degradation is decreased, allowing CDO to accumulate. The second mechanism involves the slow “maturation” of CDO that involves formation of a thioether linkage between Cys⁹³ and Tyr¹⁵⁷ (Dominy et al., 2008). This thioether linkage has been observed in the crystal structures of rat, mouse, and human CDOs (Simmons et al., 2006a; McCoy et al., 2006; Ye et al., 2007). Although the “immature” CDO lacking the cysteinyl-tyrosine crosslink is catalytically active, this moiety acts as a protein-derived cofactor and increases the catalytic efficiency (k_{cat}/K_m) of CDO by about 10-fold. In addition, formation of the thioether linkage results in an enzyme that has a longer catalytic half-life, which means it can undergo more catalytic cycles before it becomes inactivated. The formation of the cysteinyltyrosine linkage is dependent upon catalytic turnover in the presence of Fe(II), O₂, and cysteine and occurs inefficiently, requiring many catalytic cycles. Thus, enzyme maturation occurs more readily in tissues or cells with high cysteine concentrations, and “immature” enzyme can undergo “maturation,” with its associated increase in catalytic activity, during the course of an enzyme activity assay. [We have found CDO maturation in vitro to be essentially complete within 10 min of incubation in the presence of 10 mM cysteine (Dominy et al., 2008). As such, thioether formation could give rise to non-linearity (i.e., activation over time) during assays of CDO activity.]

Formation of a thioether linkage between Cys⁹³ and Tyr¹⁵⁷ in rat/mouse CDO results in migration of CDO as two bands upon SDS-PAGE, with the upper band representing the immature CDO and the lower band representing the mature CDO with the thioether crosslink (Dominy et al., 2008). Thus, two bands are often seen in Western blots of CDO if the SDS-PAGE gels are run under conditions allowing separation of the two species (Dominy et al., 2008; Stipanuk et al., 2004). Wild-type (non-mutated) CDO expressed in *Escherichia coli*, which have a relatively high intracellular cysteine concentration, exists almost entirely as the thioether form. CDO from liver of rats fed a low protein diet exists mainly as the upper immature band, whereas that from liver of rats fed a high protein diet is approximately evenly distributed between the immature and mature (thioether-linked) forms. CDO from rat kidney, which has a much higher cysteine concentration than most other rat tissues, exists mainly as the mature (thioether-linked) form.

Other investigators have reported using some dramatically different assay conditions than those described here. We have previously published (Bagley et al., 1995) an extensive comparison of our procedures with those used by Yamaguchi et al. (1978). Major variations in reported assays are whether or not exogenous ferrous ions are added, the concentration of cyst(e)ine that is used and whether oxidation of cysteine to cystine is minimized, the pH at which the assays are reportedly run, how accurately the product is measured, and most likely whether CDO was inactivated during sample preparation. For convenience, assays for CDO are routinely run under air-saturated conditions, but the activity observed under air-saturated conditions is less than is observed if oxygen tension is increased.

Little activity is observed without the addition of exogenous Fe(II). Active CDO contains one mole of Fe(II) per mole enzyme. However, CDO appears to bind iron very loosely, and

purified CDO has less than 10% iron occupancy. Even with tissue homogenates, addition of ferrous ions dramatically increases CDO activity. Fe(III) will not substitute for Fe(II).

Cysteine solutions should always be prepared immediately prior to use to minimize oxidation of cysteine to cystine. Cystine does not serve as a substrate for CDO, and cystine readily precipitates out of solution due to its limited solubility. The routine addition of the copper chelator bathocuproine disulfonate (BCS) to substrate solutions and assay mixtures is recommended because this will minimize the oxidation of cysteine to cystine (Coloso et al., 1990). BCS has been shown to maintain at least 70% of the total cyst(e)ine in thiol form throughout a 20-min incubation in air-saturated medium. Maintenance of cysteine in its thiol form is especially important for assays to determine kinetic parameters of CDO. It should also be noted that very high concentrations of cysteine inhibit CDO activity, and this is especially noted when purified CDO is assayed. Additionally, it should be noted that some apparently aberrant results obtained with early assays for CDO may have been due to the use of extremely high cysteine levels (e.g., 50 to 80 mM) which promoted cyst(e)ine metabolism by other pathways, producing anionic metabolites which were not separated by the crude chromatographic methods used to measure radiolabeled product (Daniels and Stipanuk, 1982, Kuo and Stipanuk, 1984; De La Rosa and Stipanuk, 1985).

The pH optimum for CDO from all sources that we have tested (mammalian, bacterial, and fungal; tissue extracts or purified enzyme) is in the range of 6.0 to 6.2. Lack of activity at higher pHs could possibly be due to increased oxidation of Fe(II) to Fe(III) in the in vitro assay mixture, which is readily visible by the naked eye. Other pH optima have been reported in the literature, some of which are pHs at which we observe no activity. One should check the pH of the final assay mixture to ensure that the reported pH is the true pH of the assay. Metabolism of cyst(e)ine by CDO-independent reactions, especially by transamination is favored at more alkaline pH (Stipanuk and Beck, 1982).

Hydroxylamine is routinely included in the incubation mixtures for assay of CDO in tissue or cell preparations to prevent cysteinesulfinate from being further metabolized. Hydroxylamine inhibits pyridoxal 5'-phosphate-dependent enzymes, including cysteine sulfinate decarboxylase which catalyzes the decarboxylation of cysteinesulfinate, glutamate decarboxylase which catalyzes the decarboxylation of both glutamate and cysteinesulfinate in brain, and aspartate aminotransferase or other aminotransferases that catalyze the transamination of cysteinesulfinate. Omission of hydroxylamine from assays of liver for CDO activity resulted in little accumulation of product. Apparent activity in the presence of hydroxylamine was ~7-times higher than in incubations without hydroxylamine. Because solutions of purified CDO do not contain enzymes that catabolize cysteinesulfinate, hydroxylamine can be omitted when the activity of purified CDO is being assayed.

Almost all of the assay protocols for CDO activity that were published prior to 2006 included NAD in the reaction mixture. Although substantial CDO activity is observed in rat liver or hepatocyte preparations in the absence of exogenous NAD, addition of NAD at a final concentration of 2 mM in the incubation medium increased activity up to 180% of baseline activity. Moreover, NAD can have a dramatic effect on the linearity of the assay over time, appearing to increase the catalytic stability of CDO in assays with tissue homogenates. Despite the apparent effect of NAD, no one has shown an absolute requirement for NAD and more recent work with purified CDO has shown that addition of NAD has no effect on the reaction. Furthermore, the crystal structure of CDO shows no room for the enzyme to accommodate NAD in the active site. Judgment should be used on whether or not NAD is included. We have included it in the standard assay for tissue homogenates, which often need to be carried out over longer incubation times because of low activity. On the other hand, we omit it from assays of purified recombinant CDO or of

bacterial expression system lysates because CDO activity should be high enough in these preparations to be able to use short incubation times and, also, because we have not shown any effect of NAD when added to assays of purified CDO.

The product, cysteinesulfinate, is measured following its separation from other compounds by HPLC. The application of the assay is limited by the sensitivity of available methods for measurement of cysteinesulfinate formation, and a radiolabeled assay may be necessary to assay CDO activity in samples that have low CDO activity. When radiolabel is used, the eluate corresponding to the CSA elution time is counted by liquid scintillation counting and this value is used to determine CSA produced over time. Otherwise, the CSA is measured as its o-phthalaldehyde fluorescent derivative using either pre- or post-column derivatization and comparison to CSA standards.

In general, there is good correlation between CDO activity and CDO protein abundance in tissues. Rat and mouse CDO are identical proteins of 200 amino acid residues, with a predicted molecular mass of 23 kDa; the human protein is identical except for 16 amino acid substitutions. Upon SDS-PAGE, mammalian CDO runs as two main bands that are readily detectable with antibody raised against recombinant rat/mouse CDO. [An additional ~22 kDa band, which has not been characterized, is sometimes observed.] The isoforms of CDO that run with apparent molecular masses of ~23 kDa and ~22.5 kDa both have catalytic activity, but the catalytic efficiency of the ~22.5 kDa band is much higher. It should be noted that all three bands can be observed in the western blots that we previously published although we did not consistently correctly distinguish the three bands in that paper (Stipanuk et al., 2004). Furthermore, with “over-exposure” of immunoblots of tissue preparations, a ladder of higher molecular mass bands that have been shown to be ubiquitinated forms of CDO can be detected with some, but not all, preparations of antibody to recombinant rat/mouse CDO (Stipanuk et al., 2004; Stipanuk et al., 2006; Dominy et al., 2006a).

The protein modification underlying the resolution of CDO into two bands was recently elucidated (Dominy et al., 2008). Crystal structures of mouse, rat and human CDO all show a thioether linkage between Cys⁹³ and Tyr¹⁵⁷. Most of the CDO present in liver of a rat fed a high protein diet contains the thioether crosslink, and this form runs with a lower apparent molecular mass (~22.5 kDa) upon SDS-PAGE (Dominy et al., 2008). On the other hand, much of the protein in liver of a rat fed a low protein diet will migrate as the upper, unmodified, band (~23 kDa). Tissues that have a high intracellular cysteine level contain mainly the thioether crosslinked form of CDO (e.g., kidney), whereas tissues with lower intracellular cysteine levels contain mainly the unmodified form of CDO (e.g., the brain) (Stipanuk et al., 2004).

Critical Parameters

The activity of purified CDO is strongly inhibited by cysteine concentrations above 5 mM (Dominy et al., 2008), whereas this phenomenon is not typically observed in assays of CDO activity in tissue preparations or cell lysates where the effective cysteine concentration may be modulated by the presence of other proteins in the reaction mixture. Nevertheless, the substrate inhibition of CDO does occur at non-physiological cysteine concentrations and especially in assays of purified CDO. Thus, we recommend, as a general practice, the use of the lowest cysteine concentration that will avoid substrate depletion (and lower apparent activity) over the time course of the assay. This approach might require dilution of samples with very high activity prior to assay.

Not surprisingly, given the activation and inactivation of CDO that occurs in response to the presence of substrate, strict linearity with time can be difficult to achieve in assays of CDO, and the relationship between enzyme activity and enzyme concentration is complex. To

avoid changes in the ratio of mature to immature CDO and/or the inactivation of CDO, one should use the shortest incubation times and the lowest concentrations of cysteine that will allow for measurement of product formation. We typically obtain reliable results using 5 mM L-cysteine for assays of CDO in tissue preparations and 3 mM L-cysteine for assays of purified CDO. In general we use total incubation times between 5 and 10 min. As an additional check on nonlinearity of the assay, we recommend running the assay using various enzyme concentrations and difference reaction times (i.e., half as much enzyme for twice the time). If the replicates containing half as much enzyme but incubated for twice as much time consistently have more specific activity than those containing twice as much enzyme but incubated for half as much time, this would suggest that conversion to the thioether form (i.e., activation of the enzyme) might be taking place. Alternatively, if replicates incubated for twice as much time consistently have less specific activity than those incubated for half as much time, this would suggest that inactivation of CDO might be occurring.

The major CDO species in tissues or cells migrates at ~23 kDa, as predicted, on SDS-PAGE. Some epitope antibodies (e.g., residues 167-177) used by other investigators reportedly did not react with 23 kDa CDO, leading to the false conclusion that the physiological form of CDO involved quaternary structure that was not disrupted by SDS-PAGE and had a molecular mass of ~ 68 kDa (Parsons et al., 1998a, 1998b, 2001a, 2001b; Qusti, 2000). Based on crystal structures now published for CDO (McCoy et al., 2006; Simmons et al., 2006a; Ye et al., 2007, it may be possible to choose peptide antigens that will yield better antibodies.

Troubleshooting

Because some tissues, cell types or cell lines contain very little CDO, little or no CDO activity or protein may be detected. This may occur even when CDO mRNA is present, because CDO amount is regulated largely at the level of its degradation. Thus, we recommend that one use liver tissue from a rat fed a standard non-purified rodent diet, a diet containing a moderate to high level of protein (e.g., 20-40% casein), or a diet supplemented with sulfur amino acids when first using this assay and before applying it to samples with unknown amounts of CDO activity.

Anticipated Results

CDO activity and protein are most abundant in liver, with hepatic CDO activity ranging from $0.13 \text{ nmol} \pm 0.01 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for rats fed a low protein (10% casein) diet to $4.4 \pm 1.1 \text{ nmol} \pm 0.01 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for rats fed a high protein (40% casein) diet (Stipanuk et al., 2002). CDO activity in non-hepatic tissues is less influenced by diet and was 0.05 ± 0.01 , 0.05 ± 0.01 , and $0.04 \pm 0.01 \text{ nmol} \pm 0.01 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for kidney, lung, and brain, respectively, of rats fed a moderate protein (20% casein) diet (Stipanuk et al., 2002). Western blotting for CDO in rat and mouse tissues indicated that CDO protein is most abundant in liver, followed by kidney and lung, with little CDO being detected in skeletal muscle, heart, testis, spleen, small intestine, or brain (Hirschberger et al., 2001). Western blotting of rat and mouse adipose tissues in our laboratory has indicated abundant levels of CDO protein in both white and brown adipose tissues.

Time Considerations

Assays for cysteine dioxygenase activity can easily be done in two separate steps; the assay can be run and the acid supernatant from the stopped reaction mixture can be stored frozen until it is convenient to quantify product by HPLC. Allow about 3 h to set up and run a set of CDO assays (~ 30 tubes). The HPLC analysis requires ~1/2 h per sample, but this can be largely automated. For western blotting of CDO, allow about 5 h to pour a gel, load samples,

run the electrophoresis, and transfer proteins to the membrane. Membranes can be immunoblotted immediately or stored in 1× TBST at 4 °C for up to several weeks prior to immunoblotting. Immunoblotting requires about 6 h in total.

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Figure 1. Chromatogram from HPLC determination of cysteinesulfinate production

Asparagine (Asn) is an internal standard. No cysteic acid is apparent in this sample. The sample being chromatographed is from an assay, according to protocol, of CDO activity in liver from a rat fed a high protein diet.

Figure 2. Example of use of relative standard curve for quantification of Western blots

In this example, a liver preparation from a rat fed a diet with 20% casein (moderate protein, MP) was used as the “relative standard.” Various aliquots were loaded on the gel, and immunoblotting was done using anti-CDO to generate a standard curve (see left side of blot). The right side of the blot shows several samples: 15 ug of total protein were loaded from liver preparations from rats fed diets that contained either 10% casein (low protein, LP), 20% casein (moderate protein, MP), 40% casein (high protein, HP), LP plus 0.3% L-methionine (moderate methionine, MM), or LP plus 1.0% L-methionine (high methionine, HM). On this gel, only the MP and MM samples fall within the range of the standard curve. For accurate quantification, more total protein could be loaded for the LP sample and less total protein could be loaded for the HM and HP samples. [For examples of relative quantification using this approach, see Bella et al., 1999; Stipanuk et al., 2002. For examples of quantification using purified protein as the standard, see Lee et al., 2006.]

Figure 3. Effect of cysteine concentration on CDO activity

Panel A shows a graph for the effect of cysteine concentration on CDO activity of rat liver homogenate. The assay was done using 0.03 or 0.06 g rat liver per ml assay mixture and incubation times of 24 or 12 min, respectively. *Panel B* shows a graph for the effect of cysteine concentration on activity of purified rat CDO. The assay was done using a 1 μM concentration of CDO and a reaction time of 10 min for the assay. Based on data of Bagley et al., 1995, and Dominy et al., 2008.

Figure 4. Assay of CDO activity in rat liver supernatant

Assays were done with either 60 or 120 μ l of supernatant from a homogenate (0.2 g liver per ml) of liver of a rat that had been fed a high protein diet with 400 g casein/kg for 5 days.

Figure 5. Western blots showing hepatic CDO protein expression in rats

Each lane contains a sample from a separate animal; rats were fed a semi-purified diet containing either 10% casein (low protein, LP) or 40% casein (high protein, HP) and no supplemental sulfur amino acid for one week prior to tissue collection. Each lane on the gel was loaded with liver supernatant containing 30 μ g of total protein, and the polyacrylamide concentration of the gel was 12% (w/v). Exposure times (20 or 60 s) are indicated to the left of the blots. The major CDO species that are typically seen in rat hepatic tissue are indicated to the right of each blot: 1, non-cofactor containing CDO; 2, cofactor-containing CDO; 3, unknown modification of CDO protein. [Whereas species 1 and 2 are clearly visible in recombinantly expressed rat CDO, species 3 is not. Preliminary data suggest that it is a modification that occurs only *in vivo*.]

Table 6.15.1

Example Table for Setting up Assay Tubes and Starting/Stopping Reactions						
Sample incubations	200 mM MES buffer	Fe(II), NAD, and NH ₂ OH	Homogenate or Supernatant	50 mM MES buffer	Start (add 100 µl cysteine + BCS substrate solution) at	Stop (add 200 µl 5% SSA) at
1 (120 µl for 5 min)	100 µl	80 µl	120 µl		0 min	5 min
2 (120 µl for 5 min)	100 µl	80 µl	120 µl		0.5 min	5.5 min
3 (120 µl for 5 min)	100 µl	80 µl	120 µl		1.0 min	6 min
4 (120 µl for 5 min)	100 µl	80 µl	120 µl		1.5min	6.5 min
5 (120 µl for 5 min)	100 µl	80 µl	120 µl		2.0 min	7 min
6 (120 µl for 5 min)	100 µl	80 µl	120 µl		2.5 min	7.5 min
1 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	8 min	18 min
2 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	8.5 min	18.5 min
3 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	9 min	19.0 min
4 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	9.5 min	19.5 min
5 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	10 min	20.0 min
6 (60 µl for 10 min)	100 µl	80 µl	60 µl	60 µl	10.5 min	20.5 min

Example table for setting up assay flasks and starting/stopping reactions.						
Sample incubations	200 mM MES buffer	Fe(II), NAD, and NH ₂ OH	CDO solution	50 mM MES buffer	Start (add 100 μ l cysteine + BCS substrate solution)	Stop (add 200 μ l 5% SSA)
1 (0.3 ml) for 10 min	100 μ l	50 μ l	50 μ l	100 μ l	0	10 min
2 (0.3 ml) for 10 min	100 μ l	50 μ l	500 μ l	100 μ l	0.5 min	10.5 min
3 (0.3 ml) for 10 min	100 μ l	50 μ l	50 μ l	100 μ l	1.5 min	11.0 min
4 (0.3 ml) for 10 min	100 μ l	50 μ l	50 μ l	100 μ l	2.0 min	11.5 min
5 (0.3 ml) for 10 min	100 μ l	50 μ l	50 μ l	100 μ l	2.5 min	12.0 min
6 (0.3 ml) for 10 min	100 μ l	50 μ l	50 μ l	100 μ l	3.0 min	12.5 min
1 (0.6 ml) for 5 min	100 μ l	50 μ l	100 μ l	50 μ l	0	5.0 min
2 (0.6 ml) for 5 min	100 μ l	50 μ l	100 μ l	50 μ l	0.5 min	5.5 min
3 (0.6 ml) for 5 min	100 μ l	50 μ l	100 μ l	50 μ l	1.5 min	6.0 min
4 (0.6 ml) for 5 min	100 μ l	50 μ l	100 μ l	50 μ l	2.0 min	6.5 min
5 (0.6 ml) for 5 min	100 μ l	50 μ l	100 μ l	50 μ l	2.5 min	7.0 min
6 (0.6 ml) for 50 min	100 μ l	50 μ l	100 μ l	50 μ l	3.0 min	7.5 min