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Expression, purification, and kinetic characterization of recombinant rat cysteine dioxygenase, a non-heme metalloenzyme necessary for regulation of cellular cysteine levels

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Abstract

Cysteine dioxygenase (CDO, EC 1.13.11.20) is a non-heme mononuclear iron enzyme that oxidizes cysteine to cysteinesulfinate. CDO catalyzes the first step in the pathway of taurine synthesis from cysteine as well as the first step in the catabolism of cysteine to pyruvate and sulfate. Previous attempts to purify CDO have been associated with partial or total inactivation of CDO. In an effort to obtain highly purified and active CDO, recombinant rat CDO was heterologously expressed and purified, and its activity profile was characterized. The protein was expressed as a fusion protein bearing a polyhistidine tag to facilitate purification, a thioredoxin tag to improve solubility, and a factor Xa cleavage site to permit removal of the entire N-terminus, leaving only the 200 amino acids inherent to the native protein. A multi-step purification scheme was used to achieve >95% purity of CDO. The ~40.3 kDa full-length fusion protein was purified to homogeneity using a three-column scheme, the fusion tag was then removed by digestion with factor Xa, and a final column step was used to purify homogeneous ~23 kDa CDO. The purified CDO had high specific activity and kinetic parameters that were similar to those for non-purified rat liver homogenate, including a V_{max} of ~1880 nmol min^{-1} mg^{-1} CDO ($k_{cat} = 43$ min^{-1}) and a K_m of 0.45 mM for L-cysteine. The expression and purification of CDO in a stable, highly active form has yielded significant insight into the kinetic properties of this unique thiol dioxygenase.

Keywords: Cysteine; Cysteine dioxygenase; Sulfur amino acids; Protein expression; Protein purification

Cysteine dioxygenase (CDO, EC 1.13.11.20)[1] is a mononuclear non-heme iron enzyme that catalyzes the addition of oxygen to the thiol group of cysteine to form cysteinesulfinate. CDO shows little sequence similarity with any other protein and appears to represent a unique "family" of iron-dependent dioxygenases. At the same time, CDO appears to be widespread in eukaryotes and eubacteria. CDO-like proteins or genes coding for putative CDO-like proteins are present in mammals, birds, amphibians, fish, invertebrates (flies and worms), fungi, slime molds, and eubacteria, but have not been reported in plants (National Center for Biotechnology Information Data Base). The ubiquitous presence of orthologs of CDO along with the lack of eukaryotic paralogs suggests that CDO serves a specialized but critical enzymatic function in both eubacteria and eukaryotes.

Within mammals, CDO mRNA is expressed in a tissue-specific manner. Usually, CDO activity is much more abundant in liver than in any other tissue, but appreciable levels of CDO activity are found in the kidney, lung, and brain [1–4]. CDO protein and activity levels correlate well with each other both within and among tissues and with CDO mRNA levels across tissues [4]. In liver, CDO protein

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[1] *Abbreviations used:* BCS, bathocuproine disulfonate; CDO, cysteine dioxygenase; IEX, ion exchange; IMAC, immobilized metal ion (nickel) affinity chromatography; OPA, *o*-phthaldialdehyde; ORF, open reading frame; MALDI-MS, matrix-assisted laser-desorption ionization-mass spectrometry; PEG, polyethylene glycol; rCDO, recombinant CDO; SEC, size-exclusion chromatography.

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or activity is not closely correlated with hepatic CDO mRNA because of the robust regulation of hepatic CDO at the level of protein turnover.

By catalyzing the first step in the oxidative metabolism of cysteine (Fig. 1), CDO plays a key role in cysteine catabolism, in provision of cysteine carbon (pyruvate) for gluconeogenesis or oxidative metabolism, in taurine biosynthesis, and in supply of inorganic sulfate for sulfation reactions. The end-products of the pathways of cysteine metabolism that are initiated by CDO, taurine and sulfate, are essential themselves for bile acid conjugation, osmotic regulation, synthesis of glycosaminoglycans, sulfation of xenobiotics, and other diverse functions.

The clinical literature suggests the association of impaired cysteine oxidation by CDO with the severity and progression of several disease states. Individuals with rheumatoid arthritis, liver diseases, Parkinson's disease, Alzheimer's disease, motor neuron disease and systemic lupus erythematosus frequently exhibit depressed levels of sulfate in plasma, elevated fasting plasma cysteine concentrations, and elevated plasma cysteine:sulfate ratios, and patients with rheumatic arthritis were found to have lower sulfate concentrations in synovial fluid [5–8]. Consistent with the low sulfate levels, these patients also displayed an impaired capacity for sulfation reactions in vivo. Although an insufficient supply of sulfate or taurine and impairment of sulfation reactions may be a contributory factor in the etiology of these diseases, it is also likely that the accumulation of cysteine in these patients has adverse effects. Large doses of cysteine or cystine have been shown to be neuroexcitotoxic in several species [9–11]. Cysteine is thought to be an excitatory amino acid, acting via effects on glutamate transport by systems X_C^- and X_{AG}^- and the *N*-methyl-D-aspartate subtype of the glutamate receptor [12,13], and cysteine can form toxins by reacting with other compounds [14].

Our laboratory has previously reported kinetic parameters and appropriate assay conditions for CDO activity in tissue preparations [15]. Hepatic CDO activity (V_{max}) was consistently highly correlated with the amount of CDO as measured by quantitative Western blot analysis using antibodies to purified rat liver CDO [4,16]; and both CDO activity and amount were highly correlated with measurements of cysteine metabolism to taurine and sulfate in isolated hepatocytes and in rats in vivo, demonstrating the physiological significance of CDO in regulating the overall cysteine catabolic flux [15,17,18].

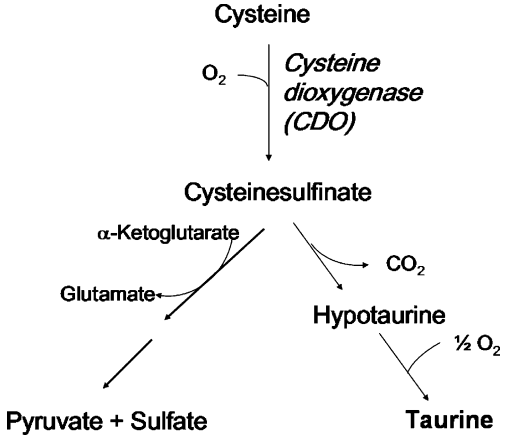

Fig. 1. Metabolism of cysteine via the cysteine dioxygenase/cysteinesulfinate pathway.

Efforts to examine the catalytic properties and cofactor requirements of cysteine dioxygenase have been hampered by difficulties in obtaining pure enzyme in active form.

Yamaguchi et al. [19] purified CDO from rat liver but found that this CDO became inactivated in the absence of an accessory protein, which they termed "protein A." More recently, Chai et al. [20] reported expression of recombinant rat CDO, but their recombinant enzyme appeared to have very low activity. We now report the expression and purification of recombinant rat liver cysteine dioxygenase that has a high specific activity and exhibits kinetic parameters similar to those previously reported for unpurified CDO in rat liver homogenates. This study demonstrates beyond doubt that CDO does not require an accessory protein for activity or for maintenance of its activity.

Materials and methods

Protein expression and purification

First strand rat liver CDO cDNA was prepared via RT-PCR with the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers were designed for use with the ligation independent cloning (LIC) vector pET-30 Xa/LIC (Novagen, San Diego, CA) using forward primer 5′-GGTATTGAGGGTCGCATGGAACGGACC GAGCTGCTG-3′ and reverse primer 3′-AGAGGAGAG TTAGAGCCCTATTAGTTGTTCTCCAGTGAACCTG AAG-5′. The PCR product was inserted into the vector using the Xa/LIC cloning kit. The construct was then used to transform Nova Blue *Escherichia coli* (Novagen). A portion of this construct was subsequently cloned into the pET32a vector using the *Bgl*II and *Nco*I restriction sites found on both plasmids, yielding a pET32a construct with a factor Xa cleavage site just before the CDO-ORF rather than the enterokinase site normally found on that plasmid. Vector pET32a contains a thioredoxin protein sequence 5′ of the CDO-ORF, causing a fusion protein to be formed. It also contains a 6× His tag between the thioredoxin sequence and the factor Xa site. The sequence of the construct in the pET32a vector was verified at the Cornell University BioResource Center. The encoded fusion protein, with numbered amino acid residues, is illustrated below.

The rCDO/pET32a vector was then amplified by transformation into *E. coli* strain XL1-Blue (Novagen). Plasmid was purified using a MidiPrep kit (Qiagen, Valencia, CA) and subsequently used to transform *E. coli* strain BL21(DE3) competent cells (Novagen) for expression of the rCDO fusion protein. For protein expression, cells