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Evidence for expression of a single distinct form of mammalian cysteine dioxygenase

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Summary. Cysteine dioxygenase (CDO) plays a critical role in the regulation of cellular cysteine concentration. Because multiple forms of CDO (~23 kDa, ~25 kDa, and ~68 kDa) have been claimed based upon separation and detection using SDS-PAGE/western blotting (with antibodies demonstrated to immunoprecipitate CDO), we further investigated the possibility of more than one CDO isoform. Using either rabbit antibody raised against purified rat liver CDO or against purified recombinant his_6-tagged CDO (r-his_6-CDO) and using 15% (wt/vol) polyacrylamide for the SDS-PAGE, we consistently detected the ~25 kDa band, but never detected a ~68 kDa band, in rat liver, kidney, lung and brain. Nondenatured gel electrophoresis of r-his_6-CDO yielded a molecular mass estimate of 25.7 kDa and no evidence of dimerization. Mass spectrometry of r-his_6-CDO yielded two peaks with molecular masses of 24.1 kDa and 24.3 kDa. Anion-exchange FPLC of r-his_6-CDO also gave two peaks, with the first containing CDO that was 7.5-times as active as the more anionic form that eluted second. When the two peaks recovered from FPLC were run on SDS/PAGE, the first (more active) CDO fraction yielded two bands (perhaps as an artifact of SDS/PAGE), whereas the second (less active) CDO fraction yielded only the ~23 kDa band. We conclude that the physiologically active form of CDO is the ~25 kDa (i.e., 23.5 kDa based on mass spectrometry) monomer and that this active form is probably derived by post-translational modification of the 23 kDa gene product.

Keywords: Cysteine dioxygenase – Cysteine – Isozymes – Molecular mass

Introduction

Cysteine dioxygenase (CDO) is emerging as a very important enzyme in the regulation of cellular cysteine concentration. CDO catalyzes the oxidation of cysteine to cysteinesulfinate, a reaction that commits cysteine to catabolism to either taurine and CO_2 or to pyruvate and inorganic sulfur. A robust increase in CDO concentration up to about 25-fold occurs rapidly in response to a three-fold increase in dietary protein or sulfur amino acid level. At the same time, the increases in steady-state cysteine and glutathione concentrations are only one- to two-fold basal levels (Bella et al., 1999a, 1999b; Stipanuk et al., 2002), demonstrating that CDO is regulated to effectively maintain low cellular cysteine levels.

The marked changes in hepatic CDO activity in response to dietary intake, along with the high K_m liver isozyme of methionine adenosyltransferase, provide a system for rapid removal of excess sulfur amino acids and their metabolites while, at the same time, maintaining a sufficiently high cellular cysteine level to ensure adequate rates of synthesis of glutathione, coenzyme A and proteins. Normal regulation of cysteine metabolism appears to play an important role in health because high levels of cysteine and low levels of sulfate, and reportedly low CDO activity, are associated with the occurrence of several chronic neurological and non-neurological diseases associated with aging and with less rapid progression of some disease states (Davies et al., 1995; Bradley et al., 1991; Emery et al., 1992; Heafield et al., 1990). Elevated levels of cysteine have been shown to be both cytotoxic and neurotoxic (Flynn and McBean, 2000; Li and Dryhurst, 1997; Montine et al., 1997; Reis et al., 2000). Evidence also exists that low levels of CDO can result in low rates of inorganic sulfate release from sulfur amino acids and, hence, impaired sulfation reactions (Davies et al., 1995; Bradley et al., 1991; Emery et al., 1992), and low or absent CDO activity is clearly the basis of the requirement of some species for dietary taurine (Stipanuk et al., 1992).

The rat, human and mouse CDO genes have been cloned and sequenced (Hirschberger et al., 2001;

Tsuboyama et al., 1996; Ramsden et al., 1997; Hosokawa et al., 1990; McCann et al., 1994; Tsuboyama-Kasaoka et al., 1999). CDO has little homology with any other known protein (BLASTP, version 3.2, Altschul et al., 1997). The nucleotide sequence of the CDO gene is highly conserved in mammals with the ORF of the rat and mouse genes coding for protein products that are 100% identical and with the human CDO gene coding for a protein with an amino acid sequence that is the same length and 92% identical. CDOs with varying numbers of amino acid residues and with lower degrees of similarity have been identified in the bony fish *Gillichthys mirabilis* (Gracey et al., 2001) and the flat worm *Schistosoma japonicum* (Fan and Brindley, 1998). Furthermore, CDO-like proteins or genes coding for putative CDO-like proteins are present in bacteria, purple bacteria, yeast, round worms, and insects are found in the National Center for Biotechnology Information data base (www.ncbi.nlm. nih.gov) with a BLAST search (Altschul et al., 1997).

Although only a few research groups have been extensively involved in the study of CDO, much confusion has arisen in the literature based on the apparent identification of more than one isoform of CDO. Yamaguchi and Hosokawa (1978) introduced the idea that CDO existed in both NAD^+-dependent and NAD^+-independent forms. Yamaguchi and Hosokawa (1978) claimed that the NAD^+-independent enzyme was found only in liver while the NAD^+-dependent form was found in liver and most extrahepatic tissues. More recently, Parsons and coworkers (Parsons et al., 1998a, 1998b, 2001a; Qusti et al., 2000) claimed that CDO exists physiologically as a 68-kDa protein, which they speculate represents CDO covalently bound to itself or another protein, perhaps the ''protein A'' referred to by Yamaguchi and coworkers (Yamaguchi et al., 1978; Sakakibara et al., 1973, 1976). Parsons, Ramsden, and coworkers (Parsons et al., 1998b; McCann et al., 1994; Ramsden et al., 1997) used CDO type I to refer to the CDO gene product and CDO type II to refer to the putative 68-kDa form of CDO. In addition, we have reported that CDO is resolved into at least two bands upon SDS-PAGE, with apparent molecular masses of ~25 kDa and ~23 kDa. The higher ~25 kDa band is always observed, and the ~23 kDa band is frequently observed, particularly in samples that contain a high concentration of CDO (Bella et al., 1999a, 1999b). We have speculated that the ~25 kDa CDO may represent a post-translationally modified protein whereas the ~23 kDa protein is not modified. Despite these differences, each group has cloned and characterized only the one homologous CDO gene or cDNA that codes for a protein containing 200 amino acid residues and having a predicted molecular mass of ~23 kDa (McCann et al., 1994; Ramsden et al., 1997; Hosokawa et al., 1990; Tsuboyama et al., 1996; Tsuboyama-Kasaoka et al., 1999; Hirschberger et al., 2001).

Because of the contradictory reports in the literature, we conducted additional experiments to ensure that we have not overlooked a physiologically active form of CDO that has quaternary structure – either a homopolymer or a heterodimer formed from the ~23 kDa product of the CDO gene. To do this, we further examined the specificity of the CDO antibody prepared by Hosokawa et al. (1990). We also expressed and purified recombinant rat liver CDO fused to an N-terminal polyhistidine tag and then used this r-his_6-CDO to assess the activity of the pure CDO gene product, to analyze its molecular mass, activity, and peptide fragmentation patterns, to test the possibility of its association as a dimer or trimer and to raise rabbit antibodies, which we also used to further purify anti-rCDO from the rabbit immune serum.

Materials and methods

Animals and dietary treatments

Male Sprague-Dawley rats (~200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed as previously described (Stipanuk et al., 2002). Rats were fed either a low protein (LP), moderate protein (MP), high protein (HP), low protein + high cysteine (LP + C) or low protein + high methionine (LP + HM) semipurified diet for 2 weeks. These diets have been described previously (Stipanuk et al., 2002) and were prepared by Dyets, Inc. (Bethlehem, PA). In brief, the LP diet contained 100 g casein/kg, the MP diet contained 200 g casein/kg, the HP diet contained 400 g casein/kg, the LP + C diet contained 100 g casein + 8 g cystine/kg, and the LP + M diet contained 100 g casein + 10 g methionine/kg. Liver, kidney, lung and brain were rapidly removed, rinsed with ice-cold saline, weighed and frozen in liquid nitrogen. Frozen tissues were stored at $-140^{\circ}C$ until analyses were performed. The experimental protocol was approved by the Cornell University Institutional Animal Care and Use Committee.

Expression and purification of recombinant CDO

The rat CDO open reading frame (ORF) was cloned into the Nco I and Bgl II sites of the pQE-60 protein expression vector (Quiagen) so that it was fused to an N-terminal hexahistidine (his_6)-coding sequence. The his_6-tagged construct was amplified in XL-Blue MRF′ *E. coli* and subsequently used to transform M15 *E. coli* for protein expression. The tagged protein was purified using a His-Bind Resin column and step-wise elution with imidazole buffer (Novagen, Inc.).

Antibodies to CDO

The purified IgG fraction from rabbit anti-CDO serum (Hosokawa et al., 1990) was a gift from Dr. Yu Hosokawa (National Institute of Health and

Nutrition, Tokyo, Japan). The reactivity and specificity of this antibody have been described previously (Bella et al., 1999b).

Rabbit antibody was raised against r-his$_6$-CDO (Center for Research Animal Resources, Cornell University), and anti-r-his$_6$-CDO was immunopurified using an NHS-HP HiTrap cartridge (Amersham Biosciences) to which purified r-his$_6$-CDO had been attached. The histidine-tagged CDO purified using the His-Bind Resin had been further purified on a HiLoad 26/60 Superdex 200 column prior to binding to the NHS-HP HiTrap cartridge.

SDS-PAGE and western blotting of CDO

A portion of each frozen tissue sample was homogenized in ice-cold 0.05 M 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.0. The homogenate was centrifuged at 20,000 × g for 30 min at 4°C, and the supernatant was further centrifuged at 100,000 × g for 60 minutes at 4°C. Protein concentration in the tissue supernatant fractions was determined by the method of Smith et al. (1985). Proteins were separated by one-dimensional SDS-PAGE (15% wt/vol polyacrylamide) and detected by staining with Coomassie brilliant blue or by western blotting with anti-CDO. Western blot analysis of CDO was done as described previously (Kwon and Stipanuk, 2001; Stipanuk et al., 2002).

Nondenatured protein molecular mass estimation

The molecular mass of native purified r-his$_6$-CDO was determined using a nondenatured protein molecular weight marker kit (Sigma MW-ND-500) and electrophoresis on gels of various polyacrylamide concentrations according to instructions provided by the manufacturer (Sigma).

Mass spectrometry

The molecular weight of r-his$_6$-CDO, purified with the His-Bind Resin, was determined by MALDI mass spectrometry (Bioresource Center, Cornell University). Also, the two CDO bands obtained upon SDS-PAGE of r-his$_6$-CDO were cut from the gel, and each band was further analyzed by tryptic digestion and MALDI mass spectrometry of the peptide fragments (Bioresource Center).

Anion-exchange chromatography

The r-his$_6$-CDO was separated by fast protein liquid chromatography (FPLC) on a MonoQ HR5/5 column (Amersham Biosciences) using a NaCl gradient. Buffer A was 20 mM Tris-HCl, pH 8.0, and Buffer B was 1 M NaCl, 20 mM Tris-HCl, pH 8.0. A linear gradient from 0% to 15% Buffer B was run over 2 column volumes of buffer and was followed by a linear gradient from 15% to 20% Buffer B over 20 column volumes of buffer to elute the CDO.

Cysteine dioxygenase activity

CDO activity was determined as described by Bagley et al. (1995). Protein was determined by the bicinchoninic acid method of Smith et al. (1985).

Results

Examination of immunodetectable proteins with anti-CDO raised against purified rat liver CDO

Results of SDS-PAGE and western blotting (Figs. 1 and 2) indicate that the major CDO species in liver, kidney, lung and brain is the ~23/25 kDa protein predicted from the

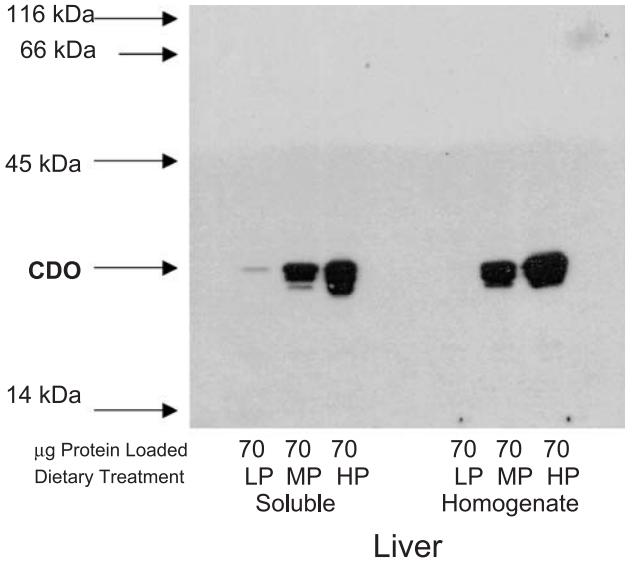

Fig. 1. Western blot analysis of CDO in the soluble (100,000 × g supernatant) fraction and in whole homogenate of liver of rats fed a low protein (*LP*), moderate protein (*MP*), or high protein (*HP*) diet. Location of molecular weight markers is shown on the left

nucleotide and amino acid sequences of CDO cDNA and CDO protein, respectively (Hosokawa et al., 1990). This molecular mass is consistent with our previous reports for both rat and mouse CDO (Bella et al., 1999a, 1999b; Kwon and Stipanuk, 2001; Hirschberger et al., 2001). As we have previously reported for liver CDO (Bella et al., 1999a, 1999b; Kwon and Stipanuk, 2001), anti-CDO reacted only with the ~23/25 kDa protein (i.e., CDO), with at least two bands of slightly different apparent molecular mass being resolved. Similar results were obtained for whole homogenate and the soluble fraction of liver (Fig. 1), and omission of DTT in the SDS mixture used to prepare samples for the SDS-PAGE gave similar results to those shown. Even with over-exposure of the film, no higher molecular mass bands were observed in either the soluble protein fraction or the whole liver homogenate. No evidence for the putative 68 kDa form of CDO reported by Parsons and coworkers (Parsons et al., 1998a, 1998b, 2001a, 2001b; Qusti et al., 2000) was observed with the antibody to rat liver CDO provided by Hosokawa (Hosokawa et al., 1990).

Both the ~25 and ~23 kDa forms of CDO were clearly detected in all kidney samples (Fig. 2). As in liver, no other immunoreactive proteins were observed. In lung samples, CDO was clearly detected. The anti-CDO also detected a 30 kDa protein in kidney, which was easily visualized even when only 10 μg of total protein was loaded. When higher amounts of total soluble protein were loaded, immunoreactive proteins with apparent

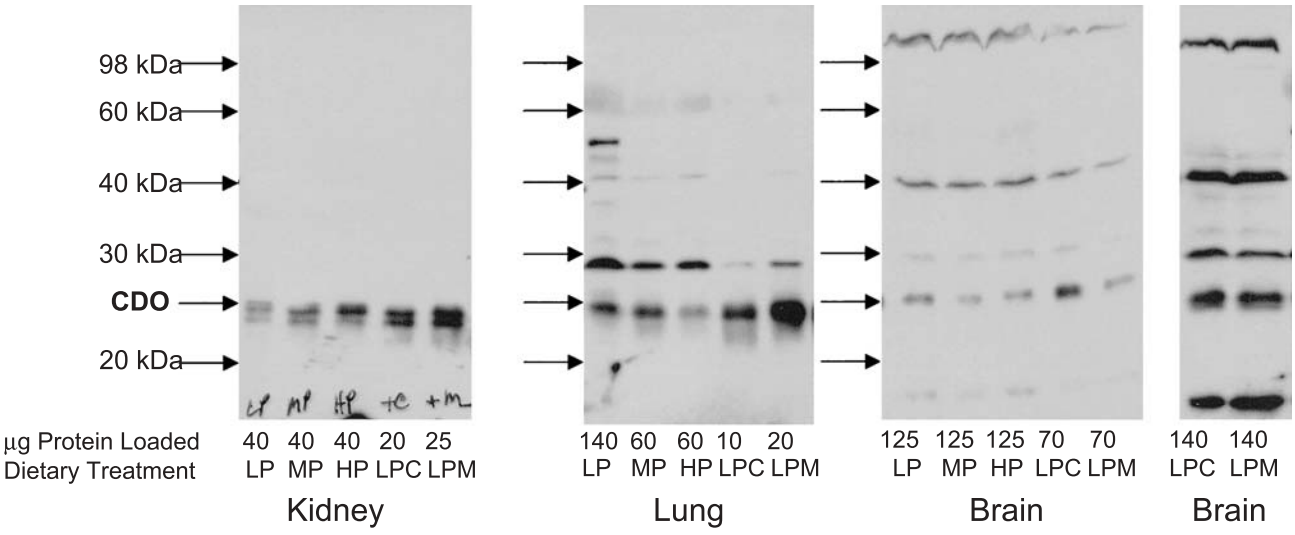

Fig. 2. Western blot analyses of CDO in the soluble (100,000 × g supernatant) fraction of kidney, lung and brain of rats fed a low protein (*LP*), moderate protein (*MP*), high protein (*HP*), low protein + high cysteine (*LPC*) or low protein + high methionine (*LPM*) diet. Location of molecular weight markers is shown on the left

molecular masses of ∼40 kDa, ∼50 kDa and ∼70 kDa were also observed. CDO concentration in brain is very low so a large amount of total protein had to be loaded in order to detect CDO. Several additional brain proteins reacted with anti-CDO: these proteins had apparent molecular masses of ≪20 kDa, ∼30 kDa, ∼40 kDa and > 100 kDa.

Studies with recombinant CDO

Using the rat cDNA cloned into an expression vector containing a his_6 tag, we have expressed and purified recombinant his_6-CDO. Recombinant his_6-CDO is enzymatically active, and the activity is stable at − 70°C for at least 3 weeks. Nondenatured gel electrophoresis of r-his_6-CDO yielded a molecular mass estimate of 25.7 kDa and no evidence of dimer or polymer formation (Fig. 3).

Recombinant CDO preparations typically gave two bands near 24 kDa (∼23 and ∼25 kDa) when analyzed by SDS-PAGE (Fig. 4), and thus gave results similar to those obtained for rat liver and hepatocytes. Similar results were obtained when the his_6 tag was fused to the C-terminus of CDO as when it was fused to the N-terminus. To further analyze the mass of the two r-his_6-CDO bands, we cut out the higher and lower bands from a gel and further analyzed each by tryptic digestion and mass spectrometry; the sizes and pattern of the peptide fragments were identical for the two CDO bands and consistent with identification of both as CDO.

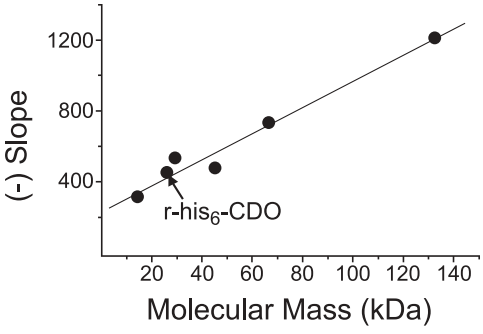

Fig. 3. Results of nondenatured gel electrophoresis of r-his_6-CDO and native protein molecular mass markers. Proteins were stained with Coomassie brilliant blue

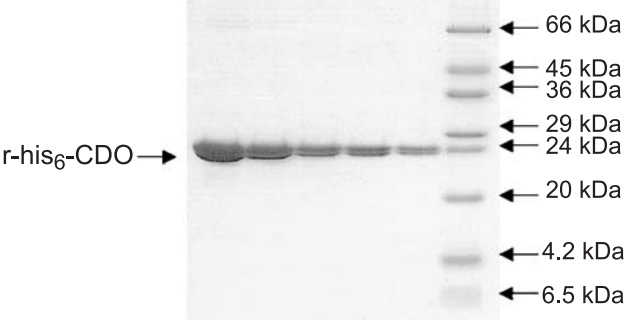

Fig. 4. SDS-PAGE of rCDO. Decreasing amounts of r-his_6-CDO were loaded in lanes 1–5: 25, 15, 9, 3 and 1.5 μg protein per lane. Molecular mass markers were run in lane 6. Proteins were stained with Coomassie brilliant blue

Both mass spectrometric analysis and anion-exchange FPLC of r-his$_6$-CDO gave two peaks. Mass spectrometry of the r-his$_6$-CDO gave peaks with masses of 24.1 and 24.3 kDa (not shown); these masses compare favorably with the calculated mass of 23,827 kDa for rCDO containing the added N-terminal his$_6$ tag. Anion-exchange FPLC separated r-his$_6$-CDO into two peaks (Fig. 5A). Fractions of these peaks were assayed for CDO activity; CDO in the first peak had 7.5-times as much activity as the CDO in the second, more slowly eluting peak. SDS/PAGE of the CDO that eluted more slowly from the anion-exchange column yielded only the lower (~23 kDa) band, but the more rapidly eluting, more active CDO resolved into two bands upon SDS/PAGE, suggesting that some conversion of the ~25 kDa to the ~23 kDa or less active state may occur as an artifact of SDS/PAGE (Fig. 5B).

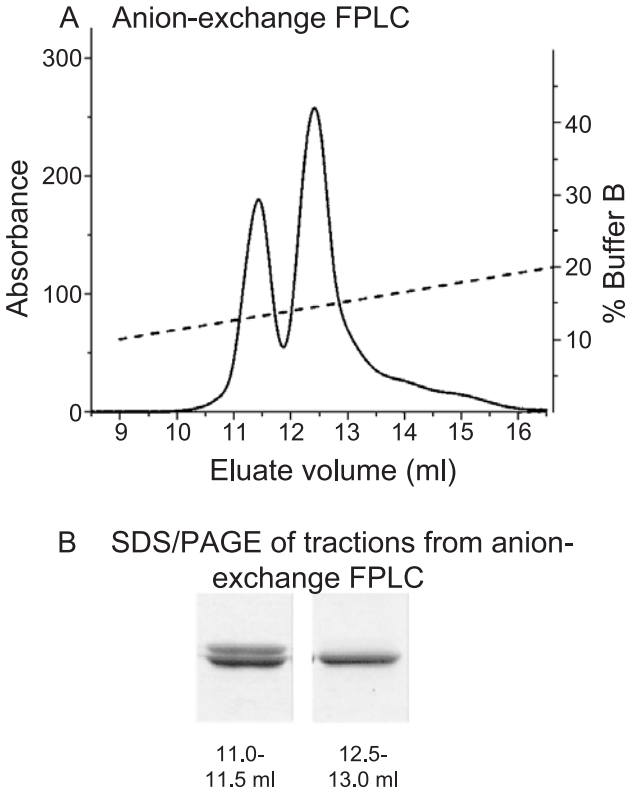

Fig. 5. A Anion-exchange FPLC of r-his$_6$-CDO on a MonoQ HR5/5 column (Amersham Biosciences) using a NaCl gradient; Buffer B contained 1 M NaCl. **B** SDS/PAGE of fractions from peak 1 (11.0–11.5 ml) and peak 2 (12.5–13.0 ml) obtained by anion-exchange FPLC of r-his$_6$-CDO

Studies with CDO antibody raised against r-his$_6$-CDO

Anti-r-his$_6$-CDO was raised in two rabbits, and immune serum was purified by rCDO-affinity purification to obtain anti-r-his$_6$-CDO. SDS/PAGE/western blot analysis of soluble protein fractions from liver and kidney with this anti-r-his$_6$-CDO is shown in Fig. 6. The anti-r-his$_6$-CDO easily detected the abundant CDO in liver from rats fed a high protein diet, but gave very light bands for liver of rats fed low protein diets or for kidney of rats fed either diet, consistent with the very low abundance of CDO in kidney and in liver of rats fed low protein diets. Interestingly, however, we observed an additional immunoreactive band, with an apparent molecular mass of ~80 kDa, in the liver, but not in the kidney, and the intensity of this band was similar in liver from rats fed low protein or high protein diets. If we had examined only the liver of rats fed the low protein diet, this would have been the most obvious band on the western blot even though it clearly is not CDO. The same results were obtained with anti-r-his$_6$-CDO from each of the two rabbits.

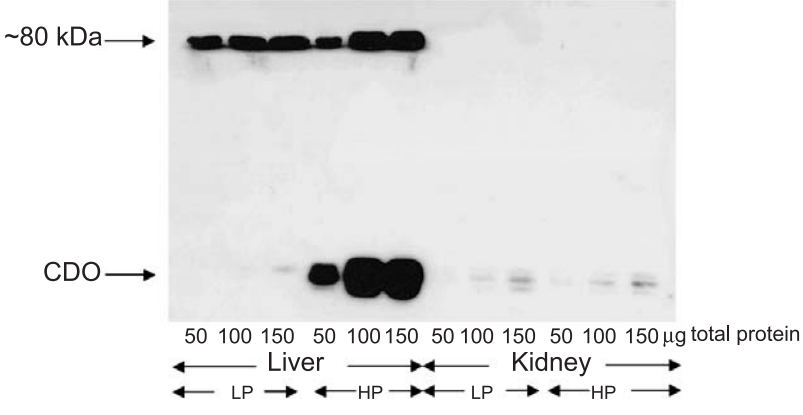

Fig. 6. SDS-PAGE and western blotting of rat liver and kidney soluble fractions from rats fed low protein (*LP*) or high protein (*HP*) diets. Western blotting was done with affinity-purified anti-r-his$_6$-CDO. For each sample, 50, 100 or 150 μg of total protein was loaded (right to left)

Discussion

CDO is a unique, highly conserved protein. No evidence of multiple genes or multiple mRNA transcripts (Hirschberger et al., 2001) has been reported. Likewise, there is evidence for only one gene product, although that product appears to undergo some type of posttranslational modification that increases its molecular mass by ~0.3 to 0.5 kDa. Reports of both NAD^+-dependent and NAD^+-independent forms of CDO and of both ~23 and ~68 kDa forms of CDO are most likely artifacts resulting from insufficiently specific methodological procedures.

Yamaguchi et al. (1978) first purified CDO, based on activity, and observed a single protein band with an estimated molecular mass of ~23 kDa when the purified CDO was subjected to either gel filtration (Sephadex G-75) or SDS/PAGE. The detection of a single CDO band by Yamaguchi et al. (1978) instead of the two bands we routinely observe upon SDS/PAGE is likely explained by their use of 10% (wt/vol) polyacrylamide gels instead of the 15% (wt/vol) polyacrylamide gels we use to obtain finer resolution of the two bands. Hosokawa et al. (1990) obtained the first cDNA sequence for CDO, using antibody to rat liver CDO (i.e., the same anti-CDO we used in Figs. 1 and 2) to screen for positive clones, and they confirmed the nucleotide sequence by comparing the predicted amino acid sequence to that obtained from several peptide fragments of their purified rat liver CDO. Thus, it is clear that the protein purified by Yamaguchi et al. (1978) was the product of a CDO gene coding for a 23 kDa polypeptide.

Yamaguchi and coworkers (Sakakibara et al., 1973; Yamaguchi et al., 1978; Yamaguchi and Hosokawa, 1987) reported that CDO in the cytosolic fraction of rat liver homogenates rapidly lost activity in the early purification steps. They subsequently found that addition of the cytosolic proteins that eluted in the first protein peak during DEAE-cellulose column chromatography restored CDO activity, and they referred to the putative active component of this cytosolic fraction as ''protein A.'' Although these findings suggested a role for a second protein in maintenance of CDO activity, CDO and ''protein A'' were readily separated and clearly not covalently linked. It should be noted that, in contrast to Yamaguchi and coworkers, we have not observed rapid inactivation of CDO and believe this may be explained by our preparation of tissue homogenates in suitable buffers rather than 0.25 M sucrose (Bagley et al., 1995).

In our earlier work with rat liver CDO, we demonstrated that NAD^+ enhanced activity but was not necessary for activity (Bagley et al., 1995). The activating role of NAD^+ is clearly non-coenzymatic, and NAD^+ can be replaced with NADH, NADP(H) or nicotinamide (Lombardini et al., 1969; Yamaguchi et al., 1971). However, the mechanism by which these pyridine nucleotides act in stimulating CDO activity is not understood, and no evidence has been published that demonstrates an effect of NAD^+ on one form, but not on another form, of CDO.

The early evidence for a NAD^+-independent form of CDO may have been an artifact due to measurement of product. In many assays, including those used in early studies in our laboratory, both [^{35}S]sulfate and [^{35}S]cysteinesulfinate were collected and counted in the eluate from the anion-exchange columns used to separate product and substrate (Loriette et al., 1979; Misra, 1979a, 1979b, 1983; Ida et al., 1985; Kuo and Stipanuk, 1984). CDO-independent (cysteinesulfinate-independent) pathways in which cyst(e)ine is substrate for cystathionase (β-cleavage) or aminotransferases can result in sulfate and pyruvate production from cysteine, and these pathways are favored under many *in vitro* assay conditions, especially in extrahepatic tissues in which CDO concentration is low (Stipanuk and Beck, 1982; Drake et al., 1987; Bagley et al., 1995; Stipanuk et al., 2002).

Parsons and coworkers (Parsons et al., 1998a, 1998b, 2001a; Qusti et al., 2000) have published results of SDS-PAGE and western blotting of liver, brain and kidney homogenates and immunohistochemical studies of CDO distribution in liver, brain and kidney that were done using antibody (''anti-H CDO'') against a linear epitope of rat CDO (residues 167–177). In these studies, they demonstrate that the anti-H CDO detects a protein with an apparent molecular mass of 68 kDa. A 68 kDa form of CDO is not consistent with any of our observations nor with those of Yamaguchi and coworkers. Parsons et al. (1998) claim that the 68 kDa protein detected by their antibody is the physiological form of CDO and hypothesize that this immunologically-detected protein represents a trimer of CDO or a dimer consisting of CDO plus a ~46 kDa protein, perhaps ''protein A'', arguing that the failure of the presumed quaternary structure to be disrupted by mercaptoethanol/SDS might be explained by covalent association of proteins. It seems quite unlikely that ''protein A'' or another protein could be linked covalently to CDO under conditions of SDS-PAGE when, in fact, they were clearly separated under the non-denaturing conditions Yamaguchi et al. (1978) used for enzyme purification. Furthermore, our work with CDO from rat liver and nonhepatic tissues demonstrates that CDO is not covalently linked to another subunit, and our studies of

recombinantly-expressed rat liver CDO reported here demonstrate that an accessory or subunit protein is not required for CDO activity and that CDO does not form a homodimer or trimer.

Both Parsons et al. (1998) and our group (Bella et al., 1999b) have demonstrated that our respective antibodies immunoprecipitate CDO activity, but reactivity of antibody with CDO does not mean it does not react with other proteins as well. If both the ~23/25 kDa and the ~68 proteins detected by our groups contain the CDO gene product, then we each should be observing reactive bands at both molecular masses or we should be observing a reactive band at the same molecular mass, neither of which is the case. We demonstrated (Fig. 5) that our highly purified anti-r-his_6-CDO is not entirely specific for CDO. It is quite possible that the epitope antibodies produced by Parsons and coworkers also detect other proteins besides CDO and that the ~68 kDa protein they detect is not CDO.

Our identification of the 23/25 kDa species as the physiological form of CDO is strongly supported by the very close correlation of our measures of CDO activity and CDO protein concentration across tissues and in liver of rats fed diets with different protein concentrations (Bella et al., 1999a, 1999b; Stipanuk et al., 2002). The correlation of CDO activity with CDO concentration (~23/25 kDa CDO), calculated for previously published data (Stipanuk et al., 2002), is >0.99. Furthermore, similar treatment effects and correlation of CDO activity and protein measurements were observed in studies with hepatocytes cultured in medium with and without sulfur amino acid supplementation (Kwon and Stipanuk, 2001). Although Parsons et al. (1998a, 1998b) have also reported that anti-H-CDO-detectable protein expression (i.e., the 68 kDa protein) in liver and brain appeared to correlate with CDO activity when levels before and after induction by addition of methionine to the drinking water were compared, quantitative analysis of the western blots was not reported and the magnitude of response appeared to be vastly less than we have observed in our studies of dietary induction of CDO in rat liver (Bella et al., 1999a, 1999b).

The question of whether the product of the CDO gene is posttranslationally modified is a question that remains unanswered. Our mass spectrometry and anion-exchange FPLC results with r-his_6-CDO suggest that the active form of CDO has been posttranslationally modified in a manner that increases its molecular mass by ~0.3 to 0.5 kDa and that makes it less negatively charged (less strongly retained on an anion-exchange column). The polypeptide chain appears to remain intact as both mass species were observed on SDS/PAGE of either N- or C-terminally his_6-tagged CDO that had been purified by Ni-chelation chromatography. An intact 200-amino acid polypeptide chain in both species is also consistent with our earlier finding of only one 5′-RACE/PCR product coding for CDO (Hirschberger et al., 2001) and with our present finding of identical peptide fragment sizes and patterns in tryptic digests of the two r-his_6-CDO bands.

We are continuing to explore the nature of the apparent modification of CDO that is related to its activity state, and the anion-exchange FPLC promises to be a useful tool for this process. The observation that active CDO obtained from anion-exchange FPLC yielded two bands upon SDS/PAGE suggests that some conversion of the upper to the lower band may occur as an artifact of SDS/PAGE and, thus, limit the usefulness of this technique. Given the likelihood of some artifactual appearance of the lower band, the presence of the lower CDO band upon SDS/PAGE of tissue or recombinant CDO should be interpreted cautiously. Nevertheless, it is clear that the physiologically active form of CDO is a monomer with a mass very close to 23.5 kDa.

Acknowledgments

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