The first major step in cysteine catabolism, the oxidation of cysteine to cysteine sulfenic acid, is catalyzed by cysteine dioxygenase (CDO). In the present work, we utilize recombinant rat liver CDO and cysteine derivatives to elucidate structural parameters involved in substrate recognition and x-ray absorption spectroscopy to probe the interaction of the active site iron center with cysteine. Kinetic studies using cysteine structural analogs show that most are inhibitors and that a terminal functional group bearing a negative charge (e.g. a carboxylate) is required for binding. The substrate-binding site has no stringent restrictions with respect to the size of the amino acid. Lack of the amino or carboxyl groups at the α-carbon does not prevent the molecules from interacting with the active site. In fact, cysteamine is shown to be a potent activator of the enzyme without being a substrate. CDO was also rendered inactive upon complexation with the metal-binding inhibitors azide and cyanide. Unlike many non-heme iron dioxygenases that employ α-keto acids as cofactors, CDO was shown to be the only dioxygenase known to be inhibited by α-ketoglutarate.

The first major step in cysteine catabolism involves its conversion to cysteine sulfenic acid by cysteine dioxygenase (CDO), which is a non-heme iron-containing dioxygenase present in mammalian tissues. CDO plays an important role in the formation of essential metabolites such as taurine and sulfate. Because cysteine is toxic at high levels (1), CDO assists in maintaining low intracellular cysteine levels without compromising its availability for incorporation into proteins and synthesis of major metabolites.

Since the early studies on CDO by Sörbo and Ewetz (2), there have been great advances in understanding the regulation and biochemical properties of this enzyme. However, there is little information regarding the active site of CDO and its specificity for the only known substrate, cysteine. Lack of the amino or carboxyl groups at the α-carbon does not prevent the molecules from interacting with the active site. In fact, cysteamine is shown to be a potent activator of the enzyme without being a substrate. CDO was also rendered inactive upon complexation with the metal-binding inhibitors azide and cyanide. Unlike many non-heme iron dioxygenases that employ α-keto acids as cofactors, CDO was shown to be the only dioxygenase known to be inhibited by α-ketoglutarate.

interaction with CDO could aid in unraveling thiol homeostasis and regulation.

MATERIALS AND METHODS

Chemicals—Cysteine, cysteine sulfenic acid, and heptafluorobutyric acid were obtained from Aldrich Chemical Co. Sodium phosphate, sodium chloride, and ferrous sulfate were purchased from Fisher Scientific. Yeast extract, Tryptone, phenylmethylsulfonyl fluoride, ampicillin, chloramphenicol, and isopropyl β-D-thiogalactopyranoside were obtained from Sigma.

Overexpression, Purification, and Activity Measurement of the Recombinant CDO—BL21(D3)pLysS cells containing the pET-14b/CDO-ORF plasmid were grown in LB media at 22 °C, and the recombinant CDO was purified from the cells by immobilized nickel affinity chromatography as previously reported (9). Enzyme activity was determined by cysteine sulfenic acid quantitation using the ion-pair reversed-phase high-performance liquid chromatography method as described by Chai et al. (9). All assays were performed at pH 7.5 in 50 mM phosphate buffer. Gas chromatographic analysis with atomic emission detection was performed on derivatized samples using the procedure reported by Uden et al. (10).

CDO Treatment with Iodoacetamide—An aliquot (120 μl) of a solution containing 0.22 mg/ml apo- or holo-CDO was treated with 50 μl of 10.2 mM iodoacetamide solution for 4 h at room temperature. The sulfhydryl-specific alkylating reagent iodoacetamide was removed by ultrafiltration followed by incubation of the enzyme with 30 mM cysteine for 4 h at 37 °C. The apo-CDO was reconstituted with ferrous ammonium sulfate prior incubation with the substrate, as previously described (9).

X-ray Absorption Spectroscopy—XAS data for all samples were acquired at beamline X9B at the National Synchrotron Light Source at Brookhaven National Laboratory. CDO samples treated with thrombin to remove the His tag were obtained as previously described (9). A fraction of the purified enzyme was incubated with 5.3 mM cysteine under anaerobic condition in a Coy chamber, which is approximately the concentration needed to reach V_{max}. The samples were contained in polycarbonate holders that were inserted into a slotted aluminum holder and held near 50 K using a helium cryostat. The XAS data were collected under dedicated conditions at ~2.8 GeV and 160–260 mA as previously described (11), except that a sagittally focusing Si(111) double-cry Monochromator was used for these studies. The x-ray energy of the focused monochromatic beam was internally calibrated to the first inflection point of iron foil (7112.0 eV).

X-ray fluorescence data were collected using a 13-element germanium detector (Canberra) over the range from ~6.9 to 8.1 KeV, with the vertical primary aperture of 1.0 mm. Harmonic rejection was achieved by use of a nickel focusing mirror flat. An average of 15 scans for resting CDO and 19 scans for the CDO ES complex was used for EXAFS analysis. The summed, energy-calibrated data files were background-corrected using two third-order polynomial fits and normalized. Single-scattering EXAFS arising from atoms in the first coordination shell of the iron was analyzed using WinXAS (12) according to standard procedures (13).
Theoretical phases and amplitudes for EXAFS analyses were obtained from FEFF 8.2 calculations of crystallographic characterized model compounds, \((\text{Et}_4\text{N})_2[\text{Fe}(p-\text{SC}_6\text{H}_4\text{CH}_3)_4]\) (14) and catena-(hexakis(2-imidazoyl-N,N,N')bisimidazole) tri-iron (15). Integer values for the number of scattering atoms in a shell were used in the fits without refinement. This led to the following running parameters for each shell in the first coordination sphere: the distance \((r)\), the disorder parameter \((\sigma^2)\), and phase shift parameter \((\Delta E_0)\). Models incorporating histidine imidazole ligands were used to fit data due to scattering from atoms in the second and third coordination spheres. These models used imidazole parameters, including multiple scattering pathways contributing >20% intensity that were calculated using FEFF 8.2, as previously described (13).

RESULTS

L-Cysteine is the only known substrate for cysteine dioxygenase (3). Nonetheless, kinetic studies of the inhibition of the enzyme with molecules structurally analogous to the substrate can provide insights into the requirements for substrate binding (Scheme 1). The effect of cysteine analogs on CDO activity was assessed using an in vitro assay that monitors cysteine sulfenic acid formation from cysteine in the presence of varying concentrations of cysteine analogs, where 100% activity is described as CDO activity in the absence of the analogs tested.

Our initial characterization of recombinant CDO showed that the enzyme is inhibited by homocysteine but not by methionine (9). Assays with homocysteine indicated that alkyl chain extension by the addition of an extra methylene in the backbone does not prevent binding to the active site. However, the terminal methyl group attached to the sulfhydryl prevents inhibition by methionine. Studies with S-methylcysteine (data not shown) also show no inhibition, as in the case with methionine. On the other hand, S-carboxymethylcysteine (addition of a terminal carboxylate group) exhibited 50% inhibition at a concentration of 2.3 mM (Fig. 1A).

The studies reported above indicated that a charged side chain was required for inhibitor binding and that a sulfhydryl might not be required for inhibition of the enzyme. This was confirmed in studies using aspartic acid, which decreases enzyme activity by half at a concentration of 1.5 mM (Fig. 1B). However, replacing the sulfhydryl by the uncharged hydroxyl group of serine did not affect enzyme activity (data not shown).

The inhibition of CDO by aspartate ion suggested that the enzyme might be inhibited by \(\alpha\)-ketoglutarate, which is a common cofactor for non-heme iron dioxygenases (16). In fact, \(\alpha\)-ketoglutarate was found to inhibit CDO with 50% inhibition at 6.8 mM (Fig. 1C).

We also examined the importance of the functional groups at the \(\alpha\)-carbon of the amino acid. Mercaptopropionic acid is a cysteine analog lacking the amine group, and at a concentration of 1.2 mM inhibited CDO activity by 50% (Fig. 2A). Cysteamine (2-aminoethanethiol) is synthesized in mammalian tissues from pantetheine (17). In contrast to mercaptopropionic acid, it was surprising to discover that cysteamine enhances the activity of recombinant significantly (Fig. 2B). At a reaction time of 45 min, CDO activity increases almost 20 times in the
presence of 5.8 mM cysteamine. Cysteamine is not an alternative substrate for the enzyme, because cysteine sulfinic acid can be detected by the assay when similar concentrations of cysteine and cysteamine are present. Furthermore, no oxidation product of cysteamine was detected by the reversed-phase high-performance liquid chromatography method or by gas chromatography analysis with an atomic emission detector. In fact, we did not observe product formation on any of the cysteine analogs tested, including D-cysteine (data not shown).

The observation that cysteamine is an activator of CDO but not a substrate suggested a possible redox role. The possibility that an active site sulfhydryl group might be oxidized to a disulfide, but could be reduced by cysteamine, was an attractive possibility given the literature on the activity of the enzyme with preincubation with cysteine (18). However, CDO treated with the sulfhydryl-binding agent iodoacetamide had no effect on the enzyme activity. The same result was obtained by incubating apo-CDO with iodoacetamide after removal of the reagent and reconstitution with iron. We conclude that there is no essential sulfhydryl group in the active site.

CDO is a metalloenzyme that requires ferrous ion to be active (18). We also investigated the inhibition of recombinant CDO using metal-binding anions. Azide (Fig. 3A) and cyanide (Fig. 3B), also inhibit CDO with a 50% activity reduction at 1.4 and 2.7 mM, respectively, suggesting that these anions bind to the active site iron.

X-ray absorption spectroscopy was employed to examine the interaction of the iron center with cysteine. The data obtained are summarized in Table 1 and Fig. 4. X-ray absorption near edge structure analysis shows that the iron K-edge occurs at 7123.1 and 7121.2 eV in the resting and substrate-bound enzyme. The pre-edge feature assigned to a 1s → 3d transition has an area of 9.0 ± 1003 102 eV in the resting enzyme and 8.1 ± 1002 eV in the oxidized enzyme, indicating that both contain six coordinate iron atoms (19). The results of EXAFS analysis of the first coordination sphere show that the ligand environment of the resting enzyme is composed of six N/O-donor ligands. Upon binding substrate, the structure of the iron site is perturbed but is still consistent with six O/N donors, indicating that cysteine may bind to the iron center, but is not bound via the sulfur atom. Models incorporating histidine imidazole ring atoms showed that the data are consistent with the presence of two or three histidine ligands.

**DISCUSSION**

The in vitro assays of CDO activity employing structural analogs of cysteine show that a negatively charged side chain is required for binding at the active site. This negative charge may be supplied by a thiolate, as in the substrate l-cysteine or the inhibitor, homocysteine, or by a carboxylate, as in S-carboxymethylcysteine, aspartic acid, and α-ketoglutarate. Analogous having an uncharged side chain, such as serine or the methylthioethers of cysteine or homocysteine, have no effect on the enzyme activity.

S-Carboxymethylcysteine is a mucoactive drug used in the treat-
ment of chronic obstructive pulmonary disease (20). Even though initially speculated that CDO might be responsible for the S-oxidation of S-carboxymethylcysteine (21), it is noteworthy to consider

TABLE 1
Selected fits of iron K-edge EXAFS data for resting CDO and ES complex
Unfiltered data over the range \( k = 2–12.5 \ \text{Å}^{-1} \); \( N \) is the number of bonds at a specific distance; \( r(\text{Å}) \) is the bond distance; \( s^2 \) is the root mean square disorder in the bond distance, and \( \Delta E_0 \) is the phase shift parameter. The accuracy of distances determined by EXAFS for atoms in the first coordination sphere of the metal are limited to \( \pm 0.02 \ \text{Å} \) by the theoretical phase parameters. The refinements generally show precisions that are \( <0.02 \ \text{Å} \) for well ordered atoms in the first coordination sphere. For imidazole carbon and nitrogen atoms in the second and third coordination sphere, the error is estimated to be \( \sim 0.05 \ \text{Å} \) from model studies.

| Sample/fit no. | \( N \) | \( r(\text{Å}) \) | \( s^2 \) | \( \Delta E_0 \) | Residual |
|---------------|-------|-------------|--------|--------------|----------|
| **Resting**   |       |             |        |              |          |
| R01           | 6     | 2.04        | 11     | 3.4          | 39.3     |
| R02           | 3     | 2.11        | 6.4    | 7.0          | 36.6     |
| R03           | 5     | 1.94        | 6.4    | 4.8          |          |
| R04           | 1     | 1.93        | 6.9    | 53           |          |
| R05           | 3     | 2.06        | 0.4    | 4.1          |          |
| R06           | 3     | 2.08        | 6.9    | 6.7          | 25.4     |
| **ES Complex**|       |             |        |              |          |
| ES01          | 6     | 2.12        | 5.7    | 5.7          | 43.0     |
| ES02          | 3     | 2.21        | 6.5    | 6.5          | 39.8     |
| ES03          | 3     | 2.06        | 5.5    | 5.5          |          |
| ES04          | 5     | 2.14        | 11     | 6.9          | 40.5     |
| ES05          | 1     | 2.01        | 10     | 59           |          |
| ES06          | 2     | 2.42        | 3.7    | 7.7          | 38.8     |
|               | 2     | 2.04        | 1.6    | 4.7          |          |
|               | 1     | 2.16        | 0.4    | 7.0          |          |
|               | 1     | 2.31        | 6.9    | 6.9          | 14       |
|               | 3     | 2.04        | 3.3    | 3.5          | 28.2     |
|               | 3     | 2.17        | 3.0    | 3.1          |          |
|               | Fe–C | 2.98        | 8.8    | 3.4          |          |
|               | Fe–C/N| 4.24       | 12     | 4.6          |          |

The effects of this drug on cysteine metabolism due to its interaction with CDO.

Additional support for the essentiality of an anionic side chain came from kinetic studies of CDO in the presence of \( \alpha \)-ketoglutarate. There are several non-heme iron dioxygenases that make use of \( \alpha \)-ketoglutarate as an essential cofactor (16, 22). These oxygenases are classified as intermolecular dioxygenases, because one of the oxygen atoms of dioxygen is inserted into the substrate, whereas the \( \alpha \)-keto acid acts as the second oxygen acceptor. CDO is clearly not a member of this group of enzymes, because CDO incorporates both oxygen atoms in the substrate (23). To the best of our knowledge, CDO is the first known dioxygenase that has been shown to be inhibited by \( \alpha \)-ketoglutarate. This inhibition fits with the other inhibitors in that \( \alpha \)-ketoglutarate is an amino acid with a negatively charged side chain.

The iron center plays an essential role in catalysis. Previously we have studied enzyme deactivation by the chelating agents EDTA and \( \alpha \)-phenanthroline (9). Our current work illustrates enzyme deactivation by binding of azide or cyanide, anions that typically bind to metals. One possible mechanism that accounts for the requirement that the substrate have an anionic side chain is that the anionic side chain is involved in binding to the active site iron center. The interaction of cysteine with the active site iron was addressed using XAS. This experiment suffered from the facts that the enzyme does not bind iron tightly, cannot be purified from iron-containing solutions, and cannot retain more than 10% of stoichiometric iron (9). Nonetheless, information regarding the structure of the primary coordination sphere of the iron could be obtained from analysis of the XAS arising from the iron that was bound to the enzyme following removal of unbound and nonspecifically bound iron with Chelex.

Fits of the EXAFS arising from the iron center in the resting enzyme are consistent with six O- or N-donors bound to the iron at an average distance of 2.04 Å (Table 1, fit R01). Separating the single shell of scattering atoms into two shells at 2.11 Å and 1.94 Å gives a modest improvement in the fit (Table 1, fit R02). More improvement in the fit can be obtained by incorporating scattering atoms at three different distances (2.18 Å, 2.03 Å, and 1.85 Å) (Table 1, fit R04), although the shift in \( E_0 \) for the shortest distance is rather large. Attempts to incorpo-
rate an S-donor ligand in the fits led to unreasonably large shifts in $E_0$, unrealistically short Fe–S distances, or poorer fits compared with those with an equivalent number of shell but lacking S-donors (Table 1, fits R03 and R05). Thus, there is no evidence to support S-coordination in the resting enzyme.

The fits obtained from the ES complex are also consistent with a six-coordinate iron center with only O- or N-donors in the primary coordination sphere. A single shell fit (Table 1, fit ES01) gives an average Fe-O/N distance of 2.12 Å. Splitting the single shell of scattering atoms into two shells at 2.21 Å and 2.06 Å gives a modest improvement in the fit (Table 1, fit ES02). Incorporation of three shells of O- or N-donors does not significantly improve the fit (Table 1, fit ES04). The distances obtained for the ES complex are longer than those obtained for the resting enzyme, showing that the iron site is perturbed by substrate binding. The data are consistent with binding of cysteine to the iron center via the carboxylate group, but because there is no change in coordination number or donor-atom set, the data are also consistent with a binding site close to the metal that changes the M-L distances. However, incorporation of an S-donor ligand into any fit leads to poorer residuals compared with fits with the same number of shells but lacking S-donors. Thus, there is no evidence to support that the cysteine is bound to the iron via the sulfur atom. Another difference between the EXAFS data from the resting enzyme and the ES complex is the intensity of features near 2.3 Å (uncorrected for phase shift) in the Fourier-transformed spectra. Whereas this might indicate the presence of a long Fe–S distance (~2.7 Å), no fits with a long Fe–S bond were obtained.

The crystal structure of cysteine dioxygenase from Mus musculus was recently obtained (PDB code 2ATF), with a bound nickel ion instead of a non-heme ferrous ion (24). The structure indicates a nickel center formed by three histidine ligands and three solvent-derived ligands. Our XAS data from the resting state CDO are consistent with three N-donor ligands and three O-donor ligands at typical Fe-aqua distances. Although the data do not show a significant peak in the Fourier-transformed spectra near 4 Å that is typical of coordinated histidine imidazole side chains, the data are not inconsistent with histidine coordination. Models incorporating imidazole rings show that the features near 2.3 Å in the Fourier-transformed ES spectrum can be accounted for by three histidine ligands (Table 1, fit ES06, and Fig. 4). Similarly, incorporation of three histidine ligands in the model for the resting enzyme also leads to a successful fit of the data. The smaller intensity of the features arising from the imidazole ligands in the resting enzyme result in a larger value of $\sigma^2$, suggesting that the intensity differences between the two sets of data are due to greater disorder in the resting enzyme iron site (Table 1, fit R06, and Fig. 4). We conclude that the iron site in CDO is consistent with the crystal structure reported for the nickel-substituted enzyme.

None of the substrate derivatives tested gave any indication of product formation. However, the fact that L-cysteine is the only known substrate that undergoes oxidation suggests that a specific enzyme-substrate geometry at the active site is required for the oxidative chemistry to occur. According to our observations, D-cysteine was neither a substrate nor an inhibitor, suggesting the importance of the chirality at the $\alpha$-carbon for active site binding. One possibility is that the geometry and distances between the sulfur atom of cysteine, the ferrous center, and oxygen are crucial. The data are consistent with a model where the cysteine binds at or near the iron center in a way that positions the sulfur atom near to where the oxygen molecule binds to the ferrous center.
Perturbations in the EXAFS spectrum are consistent with substrate binding to iron via a N/O-donor atom. Such binding could be used to position the sulfur atom of cysteine. Inhibition by aspartate would result from the replacement of the S-atom, whereas inhibition by homocysteine would result from misplacement of the sulfur atom due to the longer side chain (Scheme 2). The three protein derived N-donors and three solvent ligands depicted on the resting state in Scheme 2 were based on the observations from the reported crystal structure (PDB code 2ATF) and our current XAS studies.

In contrast to the other cysteine structural analogs, cysteamine was shown to greatly enhance the enzyme activity but was not a substrate. How cysteamine activates CDO is not clear, although a redox role is attractive. One possible redox role would be to reduce or maintain an active site cysteine sulphydryl group. This possibility is not consistent with the fact that CDO activity is unaffected by thiol-modifying agents. Recombinant CDO was treated with iodoacetamide, a thiol-modifying reagent used in a prior investigation of residual thiols that are critical for enzyme function (25). After treating holo-CDO with iodoacetamide followed by its removal by ultrafiltration, enzyme activity was unaffected. The same results are observed after treatment of apo-CDO with iodoacetamide followed by reagent removal and subsequent reconstitution with ferrous ion. It is unlikely that cysteine residues play a role in catalysis, even though Khan et al. (21) reported that CDO is inhibited by sulfhydryl-modifying reagents added to the assay solution. In our hands, we observed that reduction of the cysteine pool by thiol-modifying agents could lead to the erroneous interpretation of an apparent enzyme activity reduction.

The role of cysteamine in enhancing the activity of CDO is intriguing in several respects. Cysteamine seems to play a role in cysteine homeostasis at the cellular and molecular level. Lysosomes are responsible for the intracellular protein digestion, and the products of the degradation process leave the organelle by specific transporters for reuse or excretion (26). Nephropathic cystinosis is an autosomal recessive lysosomal storage disorder caused by a defective transport of cystine out of the lysosomes due to mutations in the transporter cystinosin (27, 28). Lysosomal accumulation of the poorly soluble cystine results in crystal formation that in turn leads to disorders that include renal failure, growth retardation, and crystal formation in the cornea (29). Cysteamine has been used to treat patients suffering from cystinosis, because it lowers intralysosomal cystine concentrations and diminishes the symptoms associated with the disease (30). Cystine is believed to be removed from the lysosome as cysteamine-cysteine mixed disulfide complexes, but “the subsequent fate” of cyst(e)ine in the cystos is unknown (31). Cysteine being transferred from the lysosome to the cytoplasm should be removed by excretion out of the cells or converted to other metabolites by catabolism.

Besides increasing CDO activity at the molecular level, cysteamine seems to play a cellular role in the regulation of hepatic CDO concentration. Up-regulation of hepatic CDO concentration was observed in animals fed with a high protein diet or sulfur amino acids, but no change in CDO mRNA level was detected (32). Studies with rat hepatocytes indicated a similar relationship between cysteine level in culture medium and CDO concentration (33). It was suggested that CDO is not regulated at the transcriptional level, but by adjusting its degradation rate. In a cysteine-deficient environment, CDO is ubiquitinated and targeted for proteolysis by the 26 S proteasome (33). Although cysteine analogs or thiol-containing molecules fail to block CDO ubiquitination, cysteamine was shown to be as effective as cysteine in preventing CDO degradation. The specific signal mechanism for CDO degradation by the ubiquitin-proteasome pathway is not known, but it was speculated that conformational changes upon cysteine or cysteamine binding could interfere with polyubiquitination. Although cysteamine might play a role in preventing CDO degradation, it may also concomitantly enhance its activity for cysteine removal.

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