Mechanism of Cysteine Desulfurase Slr0387 from *Synechocystis* sp. PCC 6803: Kinetic Analysis of Cleavage of the Persulfide Intermediate by Chemical Reductants†

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ABSTRACT: Cysteine desulfurases (CDs) are pyridoxal-5′-phosphate (PLP)-dependent enzymes that cleave sulfur from cysteine via an enzyme cysteinyl persulfide intermediate. In vitro studies of these enzymes have generally employed dithiothreitol as a cosubstrate to reductively cleave the persulfide intermediate, and it has been suggested that persulfide cleavage is the rate-limiting step for catalysis. In this study, the kinetics and mechanisms of cleavage of the persulfide intermediate in Slr0387 (CD-0387), a sequence group I (NifS/IscS-like) cysteine desulfurase from *Synechocystis* sp. PCC 6803, by physiological and nonphysiological reductants have been examined, and the extent to which this step is rate-limiting for catalysis has been determined. The observations that dithiols such as dithiothreitol (DTT) cleave the persulfide with ∼100-fold greater efficiency than structurally similar monothiols such as 2-mercaptoethanol (2-ME), that cleavage by DTT exhibits saturation kinetics, and that the dependence of the observed first-order rate constant for persulfide cleavage by DTT on the concentration of the dithiol corresponds precisely with that for formation of a complex between DTT and the PLP cofactor of the resting enzyme suggest that persulfide cleavage by dithiols occurs by prior formation of a complex, in which addition of one thiol to the cofactor positions the second thiol for attack. This conclusion and the observation that a second molecule of L-cysteine can bind to the cofactor in the persulfide form of CD-0387 explain why several CDs are subject to potent inhibition by L-cysteine during turnover with DTT: binding of L-cysteine prevents formation of the PLP−DTT adduct and renders the dithiol no better than a monothiol, which must react with the persulfide in bimolecular fashion. Consistent with this rationale, catalysis by CD-0387 with 2-ME as cosubstrate, while less efficient, is not subject to potent inhibition by L-cysteine. The similarity of the maximum rate constant for persulfide cleavage by DTT to k_{cat} suggests that persulfide cleavage is, in fact, primarily rate-determining, and this conclusion is confirmed by the observation that k_{cat} is ∼10-fold greater when tris-(2-carboxyethyl)phosphine (TCEP), the most efficient persulfide cleaver identified, is used as the reducing cosubstrate. The faster turnover with TCEP provides a chemical model for activation of CD-0387 and other CDs by the presence of accessory factors that serve as efficient acceptors of the persulfide sulfur.

Cysteine desulfurases (CDs)[1] catalyze the desulfuration of L-cysteine to yield L-alanine and free sulfur (*1*). They have been shown to provide sulfur for the biosynthesis of cofactors and macromolecules such as molybdopterin, thiamin, thionucleotides in tRNA, and iron−sulfur clusters (*1*−*23*). The CD reaction involves formation of an enzyme cysteinyl persulfide intermediate and L-alanine from the CD resting enzyme and substrate L-cysteine (*24*, *25*). The enzyme bound S^0 could, in principle, be reductively released as sulfide prior to incorporation into the aforementioned organic and inorganic cofactors or, alternatively, could undergo direct covalent transfer to additional small-molecule or protein accessory factors. In vitro studies of the desulfurase reaction initially employed small-molecule thiols to effect reductive cleavage of the persulfide (*1*, *17*, *24*, *26*−*28*), but a wealth of recent evidence indicates that the latter fate is operant in vivo. Thus, proteins such as NifU and IscU have been implicated as sulfur acceptors for the sequence group I NifS and IscS CDs, respectively (*29*, *30*). More recently, formation of a complex between the sequence group II SufS CD and the SufE protein (*31*, *32*) and subsequent transfer of the persulfide sulfur to a cysteine residue on SufE have been demonstrated (*31*, *33*). In several studies, it has been proposed that the persulfide cleavage step is rate-determining in the steady state (*17*, *27*, *33*). Indeed, early work suggested that the presence of a target protein for iron−sulfur cluster assembly by *Azotobacter vinelandii* NifS (apo dinitrogenase reductase or NifH) activates the CD (*16*), and recent work has shown conclusively that the sulfur-accepting (persulfide-cleaving) SufE protein activates SufS (*31*, *32*). However,

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[1] Abbreviations: CD, cysteine desulfurase; PLP, pyridoxal-5′-phosphate; DTT, dithiothreitol; TCEP, tris-(2-carboxyethyl)phosphine; 2-ME, 2-mercaptoethanol.

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interactions with other proteins could also accelerate persulfide formation, and the preceding paper indicates that persulfide formation is, in fact, exceedingly slow in cysteine desulfuration by SufS from *Synechocystis* sp. PCC 6803 (*34*). The purposes of the work described herein were (1) to define the reactivity of the persulfide species and the mechanism(s) for its cleavage and (2) to assess the extent to which this step is rate-limiting for catalysis with different reducing cosubstrates.

The fully sequenced genome of the photosynthetic, freshwater cyanobacterium, *Synechocystis* sp. PCC 6803, has three genes, *slr0387*, *sll0704*, and *slr0077*, that encode CDs (*35*, *36*). The first two are more similar to the sequence group I, IscS and NifS CDs, whereas the last is a sequence group II, SufS CD (*21*, *37*). In general, the group I enzymes are considerably more active in the absence of accessory factors than are those in group II (*1*, *17*, *19*, *24*, *26*, *27*, *31*−*34*, *37*, *38*), suggesting that, if persulfide cleavage is rate-determining in both cases, then it is more facile in the group I CDs. In this work, we report kinetic characterization of the cleavage of the persulfide intermediate in the group I desulfurase Slr0387 from *Synechocystis* sp. PCC 6803 (hereafter, CD-0387) by a series of chemical reductants.

MATERIALS AND METHODS

Materials. DNA modifying enzymes and reagents for the polymerase chain reaction (PCR) were purchased from New England Biolabs (Beverly, MA). Oligonucleotides primers were purchased from Invitrogen (Carlsbad, CA). [^{35}S]-L-Cysteine (1075.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The RC58 regenerated cellulose membranes with pore size of 0.2 μm were purchased from Schleicher & Schuell (Keene, NH). Glutathione was purchased from Boehringer Mannheim (Indianapolis, IN). Tris-(hydroxymethyl)aminomethane (Tris), ammonium sulfate, sodium chloride, L-cysteine, Sephadex G-25 resin, pyridoxal-5′-phosphate (PLP), pyridoxal hydrochloride, and streptomycin sulfate were purchased from Sigma-Aldrich. Dithiothreitol (DTT) was obtained from United States Biochemicals (Cleveland, OH). Tris-(2-carboxyethyl)phosphine (TCEP) was purchased from Pierce (Rockford, IL).

Construction of CD-0387 Overexpression Strain. A 1181-bp DNA fragment containing the *slr0387* gene was amplified by PCR using *Synechocystis* sp. PCC 6803 genomic DNA as template and primers slr0387N-Terminus (5′-GAGGAAATCCATATGGAACGGCCTCTTTACTTCG- 3′) and slr0387C-Terminus (5′-GGGAATTCAACTCTGGTTAGAAGCATC-3′). The PCR fragment was digested with *Nde*I and *Eco*RI and ligated into the *Nde*I and *Eco*RI sites of pET22b (Novagen) to generate the expression plasmid pET22bslr0387. The integrity of the coding region was verified by DNA sequence determination at the Penn State Nucleic Acid Facility.

Overexpression and Purification of CD-0387. Expression of CD-0387 was achieved in BL21(DE3) cells. Cells were grown at 37 °C in rich LB medium (3.5% tryptone, 2% yeast extract, and 0.5% sodium chloride) containing 10 μM pyridoxal hydrochloride and 150 mg/L ampicillin to an OD_{600} of 0.7, induced by addition of 20 μM IPTG, and grown for an additional 20−22 h at 15 °C. The cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80 °C.

The frozen cells were resuspended in 50 mM Tris-HCl, pH 7.6, containing 0.25 mM phenylmethylsulfonylfluoride (PMSF). Cell lysis was accomplished by passage through a French pressure cell. Cell debris was removed by centrifugation at 12 000*g* for 10 min. The supernatant was brought to 0.1 M NaCl and 0.25% poly(ethylene glycol), and the resulting suspension was centrifuged for 10 min at 12 000*g*. To the supernatant was added 0.2 volume of a 6% (w/v) solution of streptomycin sulfate, and the resulting suspension was centrifuged for 10 min at 12 000*g*. To the supernatant was added 127 g/L ammonium sulfate (50% of saturation), and the suspension was centrifuged at 12 000*g* for 10 min. The yellow pellet was dissolved in a minimum volume of 50 mM Tris-HCl, pH 7.6, and the solution was dialyzed overnight against 4 L of the same buffer containing 50 μM PLP. The dialyzed protein was loaded onto a Mono-Q HR 10/10 column (Pharmacia) and eluted with a 220 mL linear gradient of 0−1 M NaCl at a flow rate of 1 mL/min. Fractions containing CD-0387 were pooled, concentrated, and dialyzed overnight against 4 L of 50 mM Tris-HCl, pH 7.8, containing 50 μM PLP. A typical purification yielded 25 mg of CD-0387 per gram of wet cell mass. The purity of the protein was estimated to be >95% by denaturing polyacrylamide electrophoresis with Coomassie staining.

Cysteine Desulfurase Assays. Desulfurase activity of CD-0387 was measured by two different assays. The standard assay mix for the first method had, in a total volume of 60 μL, [^{35}S]-L-cysteine (50 000−100 000 cpm per assay), 100 mM Na-HEPES, pH 7.8, a reductant (DTT, 2-ME, or TCEP), and 1 μM CD-0387. The reaction was terminated by addition of 1 mL of 0.74 mM H_2SO_4, and the hydrogen sulfide volatilized was trapped as zinc sulfide on a RC-58 membrane that had been saturated with 3.75 μM zinc chloride at pH 9.5 before being placed in an open-faced cap on the scintillation vial in which the reaction was carried out. Volatile hydrogen sulfide trapped as zinc sulfide was quantified separately from unreacted cysteine using a liquid scintillation counter. A second assay, which has been described (*1*), was used to measure L-alanine produced by the CD-0387 reaction. In this case, the reaction was terminated by heating at 100 °C for 1 min.

Stopped-Flow Kinetic Measurements. The reaction at 21 °C of DTT, 2-ME, or TCEP with CD-0387 or its persulfide form was monitored in an Applied Photophysics diode-array stopped-flow spectrofluorimeter. The persulfide species of CD-0387 was prepared by addition of 80 or 120 μM L-cysteine to 200 μM CD-0387. Nonlinear regression analysis of equilibrium and kinetic data was carried out with Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

Spectral Change Associated with Formation of the CD-0387 Persulfide Species. Addition of substoichiometric cysteine to CD-0387 at pH 7.8 causes a shift in the spectrum of the cofactor from λ_{max} = 385 nm (resting enzyme) to λ_{max} = 417 nm (Figure 1). A similar change has been reported in studies on other CDs (*1*, *17*, *19*). There is chemical precedent to suggest that the spectral shift might be due to the formation of an external cysteine or alanine aldimine (*39*). However,

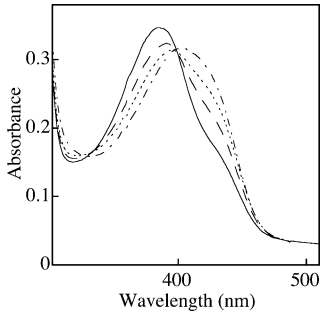

FIGURE 1: Spectral shift associated with formation of the persulfide form of CD-0387 upon addition of stoichiometric L-cysteine. L-Cysteine was added at 21 °C to 100 μM CD-0387 in 50 mM Tris, pH 7.8, to final concentrations of 0 (—), 20 (− − −), 40 (···), and 100 μM (− · −) L-cysteine.

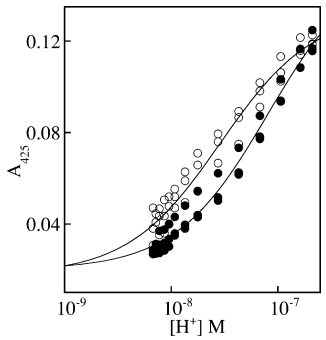

FIGURE 2: pH-dependence of the absorbance at 425 nm of a solution of 50 μM CD-0387 (●) and its persulfide form (○). The persulfide was prepared by addition of 1 equiv of L-cysteine. The solid traces, which are fits of the equation for a hyperbola, correspond to pK_a values of 7 and 7.5 for resting CD-0387 and its persulfide form, respectively.

chemical and spectral analyses indicate that the red shift results not from formation of a stable external aldimine but from a 0.5 unit increase in the pK_a of a weakly acidic group on PLP (possibly the iminium moiety of the internal aldimine) associated with formation of the persulfide species. This increase in pK_a increases the fraction of the 425-nm-absorbing protonated form of the cofactor and decreases the fraction of the 380-nm-absorbing unprotonated form. The dependencies of the absorbance at 425 nm on pH (Figure 2) indicate that this group has pK_a values of 7.0 and 7.5 in resting (open circles) and cysteine-treated (1 equiv) CD-0387 (filled circles), respectively.

Chemical analyses to confirm that the cysteine-treated enzyme is, in fact, a persulfide species were carried out as follows. The enzyme was treated with 0.6 equiv of [^{35}S]-L-cysteine. The majority of the radiolabel (70−80%) was found to coelute with the protein during size exclusion chromatography on G-25. The absence of 20−30% of radiolabel from the protein fraction was shown by thin-layer chromatography of the [^{35}S]-L-cysteine to be due to an impurity in the substrate. After G-25 chromatography of the cysteine-treated CD-0387, the absorption spectrum of the enzyme was acquired and found still to be red-shifted. Analysis for alanine before the chromatography step yielded 0.5 ± 0.1 equiv, whereas only 0.05 ± 0.01 equiv was detected after the chromatography step. Thus, removal of the majority of substrate (by conversion to product) and product (by chromatography) does not revert the absorption spectrum. By contrast, treatment of the cysteine-treated, chromatographed protein with a few equivalents of DTT led to reversion of the spectrum to that of the resting enzyme and volatilization of the radiolabel. Denaturation of the enzyme with 6 M guanidine prior to treatment with DTT did not prevent volatilization of the radiolabel, indicating that the red-shifted form of CD-0387 has the L-cysteine sulfur associated with the protein via a DTT-labile bond. These results confirm that the red shift in the spectrum coincides with formation of the persulfide species.

Table 1: Second-Order Rate Constants (k_2) for Reduction of the CD-0387 Persulfide by Mono- and Dithiols at 21 °C and pH 7.8

reducing agent	k_2 (M^{-1} s^{-1})
1,2-ethanedithiol	170 ± 10
(*R*,*S*)-2,3-dimercapto-1-propanol	120 ± 7
dithiothreitol (DTT)	340 ± 20
2-mercaptoethanol (2-ME)	2.0 ± 0.1
(*R*,*S*)-3-mercapto-1,2-propanediol	1.2 ± 0.1
glutathione	2.4 ± 0.1
DL-homocysteine	2.1 ± 0.1

Reactivity of Persulfide toward Thiol and Nonthiol Reductants. The chemical reactivity of the persulfide form of CD-0387 (obtained by addition of 0.4 equiv of L-cysteine to 100 μM CD-0387) toward different monothiols and dithiols was examined (Table 1). Except for DTT, 2-ME, and glutathione, for which the reaction was examined both spectrophotometrically and radiometrically, persulfide cleavage was generally monitored by the decrease in absorbance at 417 nm (Figure 3A). As explained earlier, the observed spectral change is not a direct result of persulfide cleavage but instead reflects a change in the protonation state of the cofactor, which is coupled to the cleavage. Comparison of the second-order rate constants for persulfide cleavage (Table 1) indicates that dithiols are at least 100-fold more efficient than structurally similar monothiols.

By contrast to the monothiols, which exhibit strictly second-order kinetics in persulfide reduction, the observed rate constant for reduction by DTT reaches a maximum value of 2.5 s^{-1} (Figure 3C). The observation of "saturation" could indicate either that the change in protonation state that occurs after persulfide reduction and gives the spectral change becomes rate-limiting at high DTT concentration or that DTT binds to the persulfide form of the enzyme prior to rate-limiting reduction. The former possibility can be excluded on the basis of the observation that the spectral change occurs much more rapidly (k_{max} = 120 s^{-1}) when the nonthiol reductant TCEP is used (Figure 4). Additional evidence for the latter possibility was provided by examination of the interaction of DTT with the resting enzyme. CD-0387 forms a complex with DTT that gives rise to a shift in the PLP absorption to λ_{max} = 342 nm (Figure 3B). This complex presumably has at least one thiol group bound to the electrophilic C4′ position of the cofactor, removing the exocyclic conjugation of the internal aldimine and blue-shifting the absorption feature. The dependence of the formation of this adduct on DTT concentration precisely tracks the dependence of the observed rate constant for persulfide cleavage (Figure 3C). This correlation is strong evidence that cleavage of the persulfide by DTT (and, by analogy, other dithiols) occurs by prior formation of a complex with the enzyme cofactor. Formation of a complex could explain the 100-fold greater efficiency of dithiols over monothiols because it could position the second thiol for attack on the persulfide.

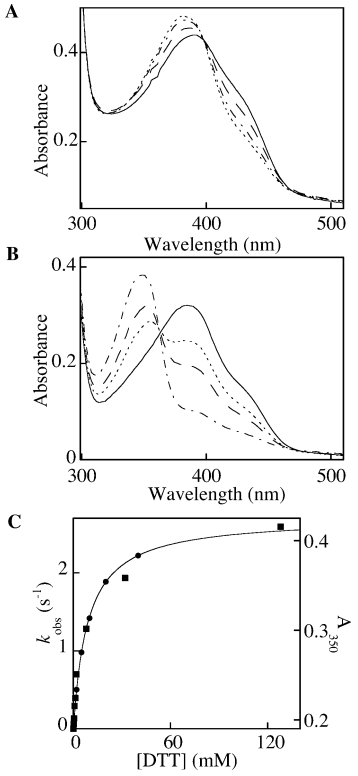

FIGURE 3: Evidence for complexation of DTT with PLP prior to the cleavage of the persulfide. Panel A shows time-dependent spectra illustrating cleavage of the persulfide by 200 μM DTT at 21 °C. The spectra were acquired immediately after mixing (—) and 200 (− − −), 400 (− · −), and 500 s (···) later. The persulfide was prepared by addition of 60 μM L-cysteine to 100 μM CD-0387 (in 100 mM Na-HEPES, pH 7.8). Panel B shows changes in the absorption spectrum of CD-0387 associated with formation of the PLP−DTT adduct. The spectra are of samples with 100 μM CD-0387 and 0 (—), 5 (···), 10 (− − −), and 40 mM (− · −) DTT. Panel C shows matching dependencies of the observed first-order rate constant for cleavage of the persulfide (■, left-hand axis) and complex formation between DTT and the cofactor of the resting enzyme (●, right-hand axis) on the concentration of DTT. The solid line is a fit of the equation for a hyperbola to the data.

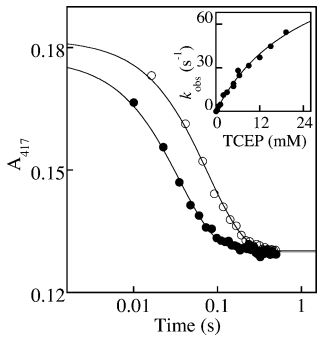

FIGURE 4: Absorbance-versus-time traces illustrating cleavage of the persulfide by TCEP at 21 °C. The traces shown are for reactions with 3 (○) and 9 mM (●) TCEP. The persulfide was prepared by addition of 120 μM L-cysteine to 200 μM CD-0387 (in 100 mM Na-HEPES, pH 7.8). The inset shows the dependence of k_{obs} for cleavage on TCEP concentration and a fit of the equation for a hyperbola to the data.

Dependence of Steady-State Rate on Concentrations of L-Cysteine and DTT. The dependence of the rate of CD-0387-catalyzed L-cysteine desulfuration on the L-cysteine and DTT concentrations is quite complex (Figure 5). DTT actually inhibits catalysis at concentrations above 5 mM

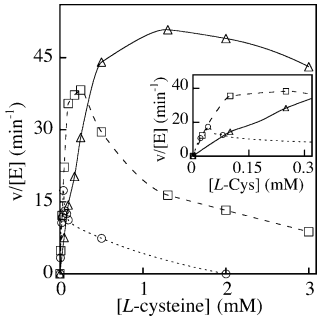

FIGURE 5: Dependence of the steady-state rate on concentrations of L-cysteine and DTT. The reaction was carried out in 100 mM Na-HEPES, pH 7.8, with either 1 (○, ···), 5 (□, − − −), or 50 mM (△, —) DTT as the cosubstrate in the presence of L-cysteine ranging from 50 μM to 3 mM. The inset shows the low L-cysteine concentration region expanded.

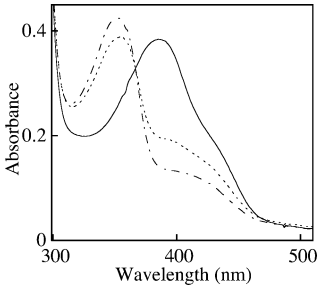

FIGURE 6: Spectra of CD-0387 in the presence of superstoichiometric L-cysteine demonstrating the formation of a 350-nm-absorbing complex between a second molecule of L-cysteine and the CD-0387 persulfide. The spectra are of samples with 100 μM CD-0387 in 100 mM Na-HEPES, pH 7.8, and either 0 (—), 2 (···), or 8 mM (− · −) L-cysteine.

when the L-cysteine concentration is less than 300 μM. At the lower DTT concentrations examined (1 and 5 mM), L-cysteine becomes severely inhibitory at concentrations exceeding 100 μM. Inhibition by DTT at low L-cysteine concentration is explained by the ability of the dithiol to bind to the cofactor and block binding by L-cysteine. Conversely, L-cysteine can also bind to the cofactor of the persulfide form of the enzyme. Formation of this CD-0387 persulfide−L-cysteine complex gives rise to a blue shift in the PLP spectrum (Figure 6) very similar to that given by binding of DTT. For this reason, we hypothesize that, in this complex, L-cysteine interacts with the cofactor via its thiol function, either alone or along with the amine group in the precedented cyclic hemithioaminal adduct (*40*). The binding of L-cysteine to the persulfide species could inhibit persulfide cleavage by one of two mechanisms. It could induce a conformational change in the protein that renders the persulfide moiety less accessible to attack by DTT. Alternatively (or in addition), it could block formation of the enzyme−DTT complex in which the second DTT thiol is positioned for attack on the persulfide.

Dependence of Steady-State Rate on Concentrations of L-Cysteine and 2-ME. The dependence of the steady-state rate on the concentrations of L-cysteine and the monothiol, 2-ME, indicates that inhibition by L-cysteine with DTT as reducing cosubstrate results primarily from the ability of L-cysteine to block addition of DTT to the cofactor. Only mild, if any, inhibition by L-cysteine is observed for catalysis with 2-ME as the reducing cosubstrate (Figure 7A). The maximum turnover rate constants measured at different

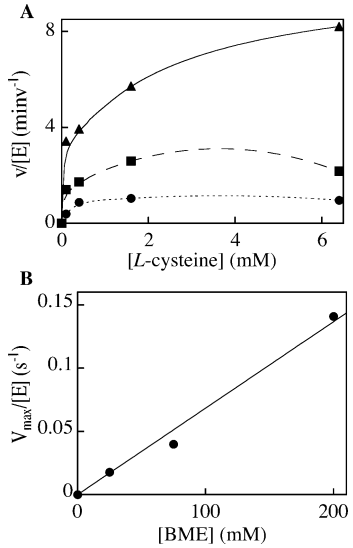

FIGURE 7: Dependence of the rate of CD-0387 catalysis on concentrations of L-cysteine and 2-ME. Panel A shows the dependence of the turnover number on concentrations of L-cysteine and 2-ME. The reactions were carried out at 21 °C in 100 mM Na-HEPES, pH 7.8, with 1 μM CD-0387 and 25 (●, ⋯), 75 (■, − − −), or 200 mM (▲, —) 2-ME. Panel B shows the plot of the maximum turnover number at each concentration of 2-ME versus 2-ME concentration. The solid trace is a linear fit to the data. Its slope corresponds to the apparent second-order rate constant for persulfide cleavage by 2-ME under the assumption that this step is completely rate-limiting.

concentrations of 2-ME are comparable to those for cleavage of the persulfide at the same concentrations. The second-order rate constant for cleavage of the persulfide bond with 2-ME (2.0 M^{-1} s^{-1}) is in reasonable agreement with the value of 0.72 M^{-1} s^{-1} obtained from analysis of the steady-state data (Figure 7B). The <3-fold discrepancy could be explained by heterogeneity (e.g., impurity, less than stoichiometric cofactor content, etc.), half-of-sites reactivity of the CD-0387 enzyme (which is expected to be a homodimer (*28*)), or both. Thus, potent inhibition by L-cysteine is unique to turnover with the dithiol cosubstrate. This observation provides support for the notion that the greater efficiency of the dithiol is related to its ability to bind to the cofactor. Blocking of this interaction by prior binding of L-cysteine renders the dithiol essentially equivalent to the monothiol.

Dependence of Steady-State Rate on Concentrations of L-Cysteine and the Nonthiol Reductant TCEP. As illustrated by Figure 4, TCEP cleaves the persulfide bond with a second-order rate constant of 4.9 × 10^3 M^{-1} s^{-1} and a maximum (saturating) first-order rate constant of 120 s^{-1}. Turnover with TCEP as the reducing cosubstrate (Figure 8) is faster and less sensitive to inhibition by L-cysteine than is turnover with DTT. The latter observation provides additional evidence for the proposed mechanism of mutual inhibition by L-cysteine and DTT. The maximum turnover number with TCEP exceeds 8 s^{-1}. Characterization of the first half reaction, persulfide formation, by stopped-flow absorption spectrophotometry indicates that the slowest step is product (L-alanine) release at a rate constant of 20 s^{-1} (unpublished data from our laboratory). Thus, the catalytic rate with TCEP as cosubstrate is within a factor of 2.5 of the expected maximal rate imposed by the kinetics of persulfide formation. (The observation that the variant protein, Slr0387-C326A, is inactive under identical conditions confirms that turnover

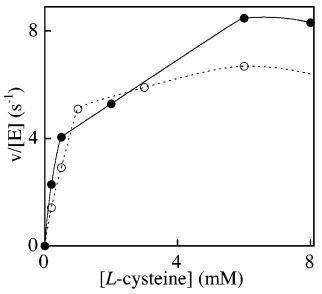

FIGURE 8: Dependence of the rate of CD-0387 turnover on the concentrations of L-cysteine and TCEP. The reactions were carried out in 100 mM Na-HEPES, pH 7.8, at 21 °C with 0.5−1 μM CD-0387 and 10 (○) or 20 mM (●) TCEP. CD-0387 activity was measured by quantifying the product L-alanine as described in the Materials and Methods section.

proceeds via the persulfide species.) If heterogeneity, half-of-sites reactivity, or both are again assumed, one may conclude that persulfide cleavage by TCEP is not primarily rate-limiting in the steady state under these conditions. Support for this conclusion is provided by the observation that an increase in TCEP concentration from 10 to 20 mM has a minor effect on the turnover rate (Figure 8) even though it increases the observed first-order rate constant for persulfide cleavage by nearly 2-fold (inset to Figure 4). Conversely, the observation that turnover with TCEP is much faster than with DTT as the reducing cosubstrate proves that persulfide cleavage is primarily rate-limiting for turnover with DTT.

DISCUSSION

The results presented herein provide precise chemical rationales for a number of features of CD-catalyzed cleavage of L-cysteine that had previously been reported without explanation. The red shift in the absorption factor of the cofactor upon treatment with substoichiometric L-cysteine was previously shown to occur in *A. vinelandii* NifS and IscS (*1*, *19*). This shift, which in principle might have been indicative of bound substrate or product, is in fact due to a change in the pK_a of some group on the cofactor associated with persulfide formation. Which group on the cofactor is affected and how the chemical state of the nucleophilic cysteine is communicated to the cofactor remain unknown. Severe substrate inhibition by L-cysteine during turnover with DTT as reducing cosubstrate was previously shown for a number of CDs, including CD-0387 from *Synechocystis* sp. PCC 6803 and its paralogous group I CD, Sll0704 (*26*, *28*). The demonstrations herein (1) that severe inhibition is unique to turnover in the presence of the dithiol cosubstrate, (2) that dithiols are 100-fold more efficient at persulfide cleavage than structurally similar monothiols, (3) that the dependence of k_{obs} for persulfide cleavage on DTT concentration coincides with that for formation of a DTT−PLP adduct, and (4) that a second molecule of L-cysteine can bind to the cofactor of the persulfide form all suggest that the greater efficiency of dithiols arises from their ability to interact with the cofactor, fixing the reductant in the active site and positioning the second thiol for attack on the persulfide, and that inhibition by L-cysteine arises from its blocking of the DTT−PLP interaction.

Finally, whereas it has often been suggested or implied that persulfide cleavage is rate-limiting for CD turnover (*17*, *27*, *33*), kinetic evidence to support this hypothesis has been

lacking. The demonstration that the sequence group I IscS CDs may transfer the persulfide sulfur to the IscU (*29*, *30*) and ThiI proteins (*6*−*8*) and the more recent observations that the sequence group II SufS CDs are activated by binding to the cognate SufE proteins (*31*, *32*) and that SufE can accept the sulfur from the SufS persulfide (*31*, *33*) are consistent with the notion that persulfide cleavage is rate-limiting and is accelerated by direct transfer to the proper accessory factor, but the presence of these factors could just as well affect the kinetics of the first half-reaction, persulfide formation. Thus, the demonstration herein that turnover of CD-0387 with TCEP as the reducing cosubstrate, which cleaves the persulfide by a different mechanism and with much greater efficiency, is faster by nearly an order of magnitude is perhaps the first solid kinetic evidence that persulfide cleavage is rate-limiting for CD turnover with DTT as the reducing cosubstrate. By contrast, the preceding paper shows that, in catalysis by the paralogous sequence group II CD Slr0077/SufS, persulfide formation is very slow in the absence of accessory factors (*34*). Thus, it seems that activation of CDs by accessory proteins can involve effects on either persulfide formation or its subsequent cleavage (or both).

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