Escherichia coli L-Serine Deaminase Requires a [4Fe-4S] Cluster in Catalysis*

Received for publication, April 21, 2004
Published, JBC Papers in Press, May 19, 2004, DOI 10.1074/jbc.M404381200

Robert M. Cicchillo‡, Melissa A. Baker‡, Eric J. Schnitzer‡, Elaine B. Newman§, Carsten Krebs§‡, and Squire J. Booker‡**

From the Departments of ‡Biochemistry and Molecular Biology and §Chemistry, The Pennsylvania State University, University Park, PA 16802 and the ¶Department of Biology, Concordia University, Montreal, Quebec H3G 1M8, Canada

L-Serine deaminases catalyze the deamination of l-serine, producing pyruvate and ammonia. Two families of these proteins have been described and are delineated by the cofactor that each employs in catalysis. These are the pyridoxal 5′-phosphate-dependent deaminases and the deaminases that are activated in vitro by iron and dithiothreitol. In contrast to the enzymes that employ pyridoxal 5′-phosphate, detailed physical and mechanistic characterization of the iron-dependent deaminases is limited, primarily because of their extreme instability. We report here the characterization of L-serine deaminase from Escherichia coli, which is the product of the sdaA gene. When purified anaerobically, the isolated protein contains 1.86 ± 0.46 eq of iron and 0.670 ± 0.019 eq of sulfide per polypeptide and displays a UV-visible spectrum that is consistent with a [4Fe-4S]0 cluster. Reconstitution of the protein with iron and sulfide generates considerably more of the cluster, and treatment of the reconstituted protein with dithionite gives rise to an axial EPR spectrum, displaying $g_\perp = 2.03$ and $g_\parallel = 1.93$. Mössbauer spectra of the $^{57}$Fe-reconstituted protein reveal that the majority of the iron is in the form of [4Fe-4S]2+ clusters, as evidenced by the typical Mössbauer parameters-isomer shift, $\delta = 0.47$ mm/s, quadrupole splitting of $\Delta Q = 1.14$ mm/s, and a diamagnetic ($S = 0$) ground state. Treatment of the dithionite-reduced protein with L-serine results in a slight broadening of the feature at $g = 2.03$ in the EPR spectrum of the protein, and a dramatic loss in signal intensity, suggesting that the amino acid interacts directly with the cluster.

---

1. L-Serine deaminase can be deaminated by a variety of enzymes of varying degrees of specificity, producing pyruvate and ammonia (see Scheme 1). As described in the Swiss-Prot data base, these enzymes fall into two families, the serine/threonine deaminases (EC 4.3.1.19) and the bacterial L-serine deaminases (EC 4.3.1.17). The first of these, the serine/threonine deaminases, require pyridoxal 5′-phosphate as a cofactor in catalysis. Enzymes within this family that have been characterized to any significant extent include the mammalian liver L-serine deaminase, which serves a gluconeogenic function (1, 2), and the biosynthetic threonine deaminase (EC 4.3.1.18), which is encoded by the ilvA gene of Escherichia coli. This enzyme deaminates either serine or threonine (3) and is essential for iso-leucine biosynthesis. A second threonine deaminase, the biodegradative enzyme, which is encoded by the tdcB gene of E. coli, uses the same cofactor, as does L-serine deaminase of E. coli (4.3.1.18) (4–6). Moreover, serine is deaminated by enzymes of varying physiological function, including cystathionine γ-synthase (7) and the β subunit of tryptophan synthase (8). The mechanism of all these enzymes is well understood. The role of the pyridoxal 5′-phosphate cofactor is to facilitate removal of the α-proton of the bound amino acid, allowing for a β-elimination of the hydroxyl group as water. Tautomerization of the resulting α-aminoacrylate results in 2-aminopropionic acid, which is hydrolyzed to ammonia and pyruvate (9).

By contrast, the bacterial L-serine deaminases (LSDs) are activated by iron and are not as well characterized (10, 11). This family of enzymes is exclusively prokaryotic and widespread in bacteria, although not omnipresent. In fact, many bacteria elaborate one to three LSDs. In E. coli these proteins are encoded by the sdaA, sdaB, and tdcG genes (12–14); the corresponding protein products are designated as LSD1, LSD2, and TdcG, respectively. All three proteins are very similar in primary structure; LSD2 and TdcG exhibit 77 and 78% identity with LSD1. In contrast to LSD1 and LSD2, TdcG is synthesized only during anaerobic growth (12).

The manner in which expression and/or function of these enzymes is regulated is known in some detail, but the mechanism of catalysis is not. LSD1 has been moderately characterized (13, 15, 16). It contains 454 amino acids, including 9 cysteines (13). When purified aerobically, the enzyme is inactive until incubated with iron and DTT under aerobic conditions. These characteristics make it very similar to the LSDs of Clostridium acidiurici (17), Peptostreptococcus microslyticus (18), and Clostridium propionicum (19), which are all activated in the same manner. Electron paramagnetic resonance (EPR) spectroscopic studies on the P. asaccharolyticus enzyme indicate the presence of [3Fe-4S]0 clusters (20). It is believed, however, that the catalytically active form of the enzyme contains a [4Fe-4S]0 cluster, in light of the striking similarities between the proposed reaction mechanism of the

---

* This work was supported by Grant GM-63847 from the National Institutes of Health (to S. J. B.) and funds from the Center for the Study of Biometals in Health and Disease supported by the Tobacco Settlement Formula (417-12HY TSF) (to C. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence may be addressed: Penn State University, 306 S. Frear Laboratory, University Park, PA 16802. Tel.: 814-865-6099; Fax: 814-863-7024; E-mail: ckrebs@psu.edu.

*** To whom correspondence may be addressed: Penn State University, 330 S. Frear Laboratory, University Park, PA 16802. Tel.: 814-865-8793; Fax: 814-863-7024; E-mail: sjb14@psu.edu.

1 The abbreviations used are: LSD, L-serine deaminase; BSA, bovine serum albumin; DTT, dithiothreitol; EPPS, N-(2-hydroxyethyl)piperazine-N′-3-propanesulfonate; EPR, electron paramagnetic resonance; FeS, iron-sulfur; LDH, lactate dehydrogenase; SAM, S-adenosyl-L-methionine; PFL, pyruvate formate-lyase; Tricine, N-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.
Iron-dependent LSDs and aconitase, which has been studied in detail using a variety of spectroscopic techniques (10, 21). Herein, we use a variety of spectroscopic methods to show that treatment of LSD1 with iron and sulfide results in formation of a [4Fe-4S] cluster on the protein and that this state of the protein is essential for catalysis. Reconstituted LSD1 catalyzes the deamination of L-serine with a first-order rate constant (kcat) of 436 s⁻¹ and a Km value of 2.67 ± 0.25 mM. It will also deaminate l-threonine and l-allo-threonine, albeit with greatly reduced efficiency; however, d-serine and l-cysteine are not substrates but serve as competitive inhibitors. Additionally, analyses of EPR spectra recorded on the dithionite-reduced protein in the presence of various substrates and inhibitors are consistent with a direct role in catalysis for the iron-sulfur (FeS) cluster as opposed to a simple structural element.

EXPERIMENTAL PROCEDURES

Materials—L-Serine and dithiothreitol (DTT) were obtained from ICN Biomedicals Inc. L-Threonine, L-cysteine, lactate dehydrogenase (LDH), sodium fluoride (nonahydrate), molecular weight standards for gel filtration, and ferric chloride were obtained from Sigma. Sodium hydrosulfite (dithionite), potassium ferricyanide, and L-allo-threonine were obtained from Aldrich. Bovine serum albumin (BSA) standard and the Bradford reagent for protein quantification were purchased from Bio-Rad (22). As determined in this work, this method overestimates the true concentration of LSD1 by a factor of 1.35. Routine chemical analyses of iron and sulfide were carried out by the methods of Beinert (23) and von Hippel (26) was used to establish an extinction coefficient for apo-LSD1, which in turn was used to correct the Bradford dye-staining protein assay.

Routine gel-filtration of LSD1 was carried out in a Coy Laboratory Products (Grass Lake, MI) anaerobic chamber using Sephadex G-25 resin (Amersham Biosciences). When it was necessary to concentrate the protein, it was carried out using an Amicon stirred-cell ultrafiltration device (Millipore, Billerica, MA) in combination with a YM-10 membrane, which has a molecular mass cutoff of 10 kDa. Ultrafiltration was carried out inside of the anaerobic chamber by threading the tubing from the nitrogen source through one of the chamber’s ports.

Overexpression and Purification of LSD1—A 200-ml overnight culture of E. coli BL21(DE3) containing plasmid psdaH6 in terrific broth (100 μg ml⁻¹ ampicillin) was used to inoculate 16 liters of the same medium, which was divided equally among four 6-liter bioreactors. Plasmid psdaH6 contains the sdaA gene cloned as a fusion construct with a carboxyterminal 6× histidine tag that is separated from the last amino acid of the protein by a linker of 2 amino acid residues. The cultures were grown at 37°C with gentle shaking (200 rpm) and induced at an A600 of 0.5 by addition of solid isopropyl-β-D-thiogalactopyranoside to a final concentration of 400 μM. At induction, solid FeCl₃ was also added to each flask to a final concentration of 50 μM. Expression of the sdaA gene was allowed to proceed for 4 h at 37°C, after which, the cultures were placed in an ice-water bath for 30 min. The bacteria were harvested by centrifugation for 20 min at 10,000 × g and 4°C, yielding ~30 g of cells from 16 liters of cell culture after freezing in liquid nitrogen.

LSD1 was purified by immobilized metal affinity chromatography using a nickel-nitrotriacetic acid matrix (Qiagen, Valencia, CA). All steps were carried out inside of the anaerobic chamber under an atmosphere of N₂ and H₂ (95%/5%), wherein the O₂ concentration was maintained below 1 ppm via the use of palladium catalysts. Steps involving centrifugation were carried out outside of the anaerobic chamber; however, samples were loaded into appropriate centrifuge bottles and tightly sealed before removing them. In a typical purification, 30 g of cells was thawed in 60 ml of Buffer A (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 20 mM imidazole) and allowed to incubate with lysozyme (1 mg ml⁻¹) for 30 min at room temperature. Subsequent steps were carried out on ice. FeCl₃ was added by sonic disruption in 3–5 min bursts, and the lysate was centrifuged at 50,000 × g for 1 h at 4°C. The supernatant was loaded onto a nickel-nitrotriacetic acid column (2.5 × 7 cm) that was equilibrated in Buffer A, and the column was washed with 50 ml of Buffer A containing 10% glycerol and 40 mM imidazole. Finally, the protein was eluted with Buffer A containing 10% glycerol and 250 mM imidazole. Fractions that displayed a brown color were pooled and concentrated using an Amicon stirred-cell and exchanged into Buffer B (50 mM EPPS, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT) by anaerobic gel filtration. The protein was routinely frozen in small aliquots and stored in liquid N₂ until ready for use.

Reconstitution of Serine Deaminase—Reconstitution of LSD1 with iron and sulfide was carried out inside of the anaerobic chamber with buffers and solutions that were prepared with deoxygenated water. A typical reconstitution reaction contained 100 μM LSD1, and 8-fold molar excesses of FeCl₃ and Na₂S₅ in a final volume of 2 ml. The protein was initially treated with a 50-fold molar excess of DTT for 10 min on ice. The FeCl₃ was added, and then a solution of Na₂S₅ was added dropwise over 10 min. The mixture was allowed to stir gently on ice for 4 h in the anaerobic chamber. It was then placed in centrifuge tubes, capped, and centrifuged at 20,000 × g for 20 min at 4°C. It was brought back into the anaerobic chamber, and the supernatant was removed and exchanged into Buffer B by anaerobic gel filtration.

Activity Determination for Serine Deaminase—LSD1 was first diluted to a final concentration of 5 μM in Buffer B containing 50% glycerol. A typical reaction contained in a final volume of 1 ml: 0.2 mM NADH, 10 units of LDH, 0–20 mM l-serine, 100 mM EPPS, pH 8.0, and 55 mM LSD1. The reaction mixture was prepared inside the anaerobic chamber; all of its components were anaerobic except for LDH. However, the volume of LHD added to the reaction mixture was 1% of the reconstituted LSD1 volume. The reaction was initiated by addition of substrate and monitored at 3–5 min at 37°C in a septum-sealed anaerobic cuvette (Starna, Atascadero, CA), and the reaction was initiated by injection of LSD1 via a gas-tight syringe. Enzyme turnover was monitored by a time-dependent decrease in absorption at 340 nm (εmax = 6.22 × 10³ M⁻¹ cm⁻¹), which is due to NADH oxidation. One unit of activity is defined as 1 μmol of NADH produced per minute. Values for the kinetic parameters Kₐ and Vmax were obtained from fits of initial rate data as a function of serine concentration according to Equation 1. In some in-
stances, data were fitted to Equation 2, which accounts for substrate cooperativity. When assays were carried out in the presence of inhibitors, the data were fitted to Equation 3 by multiple non-linear regression using the GraFit software program (27). This equation describes competitive inhibition.

\[
v = \frac{V_{\text{max}} [S] (K_s + [S])}{V_{\text{max}}^* (K + [S]^*)}
\]

(Eq. 1)

\[
v = \frac{V_{\text{max}}[S]^* (K + [S]^*)}{V_{\text{max}}^* (K_s + [S])}
\]

(Eq. 2)

\[
v = \frac{V_{\text{max}} [S]^* (K + [S]^*)}{V_{\text{max}}^* (K_s + (1 + (JK))) \cdot [S])}
\]

(Eq. 3)

Molecular Sieve Chromatography of LSD1—Molecular sieve chromatography of LSD1 was performed under anaerobic conditions with an AKTA (Amersham Biosciences) fast-performance liquid chromatography system, which was maintained inside a Coy anaerobic chamber. The fast-performance liquid chromatography system was equipped with a HiPrep 16/60 Sephacryl S-200 HR column (Amersham Biosciences) and was controlled with the associated software program UNICORN, which was also used for data collection and analysis. The column was equilibrated in an anaerobic solution of Buffer B, and samples (250 μm) and standards (2–10 mg ml⁻¹) were chromatographed at a flow rate of 1 ml min⁻¹ over a time span of 120 min. The proteins, cytochrome c (12.4 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa), were used to generate a standard curve of known molecular masses, and the void volume (Ve) of the column was determined using blue dextran (2000 kDa). The elution volumes (Ve) of the standards were obtained, and the ratios of Ve/Ve⁻¹ were plotted against the log of their respective molecular masses. The standard curve was then used to extrapolate the apparent molecular weight of reconstituted LSD1 from its elution volume.

Preparation of Samples for EPR and Mössbauer Spectroscopy—Samples of LSD1 suitable for EPR or Mössbauer spectroscopy were prepared inside the anaerobic chamber. EPR samples contained 200–300 μm enzyme in Buffer B, were treated with 2 mm (final concentration) sodium dithionite, and frozen immediately in cold isopentane (−150 °C) sitting in a Dewar of liquid nitrogen. In some samples, t-serine, l-threonine, or l-cysteine (20 mM each) was added to the reduced protein before freezing. In all cases, the amount of time that elapsed between the addition of dithionite (and the substrate when appropriate) and the freezing of the sample was less than 30 s.

Mössbauer spectra were recorded on proteins that contained ⁵⁷Fe in place of natural-abundance iron. In all samples, the ⁵⁷Fe was incorporated into the protein via reconstitution, as described above, and samples contained 250–350 μm protein, and 20 mM l-threonine when appropriate. Samples were frozen within 30 s after adding the substrate to the enzyme.

**RESULTS**

Purification and Characterization of Serine Deaminase—The iron-dependent serine deaminases have been reported to be unstable, losing activity quickly during purification, and to some extent, even in the cell (28–30). To maximize purification of active enzyme, the protein was expressed as a fusion construct with a carboxyl-terminal 6× histidine appendage and purified under anaerobic conditions inside an anaerobic chamber. The eluted protein was light brown in color, and its UV-visible spectrum displayed features, although not prominent, that are consistent with the presence of an Fe/S cluster (Fig. 1, solid line). Among these features, the shoulder at 320 nm and the hump at 416 nm are the most defining, as is the broad tailing that extends beyond 700 nm.

An extinction coefficient was determined using apo-LSD1 and the procedure of Gill and von Hippel and was used to correct the Bradford dye-binding protein assay. It was found that the Bradford assay, using BSA as a standard, overestimates the concentration of LSD1 by a factor of 1.35. Analysis of subsequent aliquots of the as-isolated protein indicated the presence of 1.86 ± 0.46 eq of iron and 0.670 ± 0.019 eq of sulfide per poly peptide using the Bradford correction factor.

Reconstitution of Serine Deaminase—Treatment of as-isolated LSD1 with 5 mM DTT and 8 eq each of FeCl₃ and Na₂S resulted in a pronounced change in its color after anaerobic gel filtration, affording a protein that was intensely brown. Analytical methods revealed that the reconstituted protein contained (8.07 ± 0.03) iron and (7.44 ± 0.46) S²⁻ per monomer. The quoted uncertainties are the standard deviation of three independent analyses, and they reflect the individual uncertainties of the protein concentration and the iron and sulfide concentration. The UV-visible spectrum of the reconstituted protein is also shown in Fig. 1 (dashed line). It displays prominent features at 316 and 407 nm. Importantly, the ratio of the feature at 278 nm to that at 407 nm (2.56) decreased significantly from its value in the as-isolated protein (8.33). These features, in combination with the overall spectral envelope of the protein, are most consistent with the formation of [4Fe-4S] clusters upon reconstitution, although it must be emphasized that the types and relative amounts of different Fe/S cluster species cannot be determined with great accuracy from UV-visible spectra. To quantitatively determine the composition of the Fe/S cluster(s) in reconstituted LSD1, Mössbauer and EPR spectroscopic experiments were carried out as described below.

Spectroscopic Characterization of Reconstituted Serine Deaminase—Shown in Fig. 1A is the Mössbauer spectrum of ⁵⁷Fe-reconstituted LSD1 recorded at 4.2 K in an externally applied magnetic field of 40 millitesla oriented parallel to the γ-beam. The prominent features of this spectrum are two sharp lines of similar intensity at −0.13 and +1 mm/s, and a broad peak at 2.7 mm/s. In addition, there is a featureless component extending from −6 to +6 mm/s, and a pronounced shoulder at 0.55 mm/s, which is denoted by an arrow. The two lines at −0.13 and +1 mm/s belong to a quadrupole doublet of isomer shift, δ = 0.47 mm/s, and quadrupole splitting, ΔE_Q = 1.14 mm/s. These parameters are consistent with the presence of [4Fe-4S]²⁺ clusters associated with the protein. The shoulder at +0.55 mm/s is at a position that is typical of the high energy line of a quadrupole doublet originating from [2Fe-2S]²⁺ clusters, thus raising the possibility that this cluster form is also present in the sample. The broad component ranging from −6 to +6 mm/s, and the peak at 2.7 mm/s, are often caused by adventitiously bound iron species in samples of reconstituted Fe/S proteins. Alternatively, these features could arise from paramagnetic Fe/S cluster species, such as [2Fe-2S]⁰, [3Fe-4S]³⁻, and [4Fe-4S]⁴⁻ clusters. It is not possible to distinguish and quantify small amounts of these Fe/S cluster forms using Mössbauer spectroscopy alone if they are present. However, the [2Fe-2S]⁰, [3Fe-4S]³⁻, and [4Fe-4S]⁴⁻ clusters all have an S = 1/2 electronic ground state and exhibit characteristic EPR spectra. Consequently, we recorded the EPR spectrum of reconsti-

![FIG. 1. UV-visible spectra of as-isolated LSD1 (solid line) and reconstituted LSD1 (dashed line).](http://...
Fig. 2. Mössbauer spectra of ⁵⁷Fe-reconstituted LSD1 recorded at 4.2 K in an externally applied field (oriented parallel to the γ-beam) of 40 millitesla (A) and 8 tesla (B). The solid line overlaid with the experimental data (hashed marks) are simulations representing the contribution from the [4Fe-4S]²⁺ and [2Fe-2S]²⁺ clusters. The individual spectral components of these two clusters are plotted as dashed ([4Fe-4S]²⁺) and dotted ([2Fe-2S]²⁺) lines above the experimental data for clarity. The parameters used for the simulation are given in the text.

Fig. 3. X-band EPR spectra of reconstituted LSD1 reduced with 2 mM dithionite. Conditions of measurement were: microwave power, 5 milliwatts; receiver gain, 10 G; temperature, 13 K; microwave frequency, 9.85 GHz. Inset: reconstituted LSD1 before reduction with dithionite, obtained under identical conditions.

diamagnetic (S = 0) ground states, resulting in no internal magnetic fields at 4.2 K. Therefore, it is expected that the effective magnetic field at the ⁵⁷Fe nuclei would equal the externally applied field. The solid line overlaid with the experimental data in Fig. 2B is a simulation using the parameters described above and assuming diamagnetism (S = 0). The individual components are shown as dashed ([4Fe-4S]²⁺) and dotted lines ([2Fe-2S]²⁺) above the data. The quality of the simulation is excellent, and it corroborates the presence of [4Fe-4S]²⁺ and [2Fe-2S]²⁺ clusters.

EPR spectra of the reconstituted enzyme are consistent with the configurations and stoichiometries of Fe/S species determined by Mössbauer spectroscopy. In the absence of dithionite, the enzyme displays a weak and fairly isotropic signal centered at 

Inset: reconstituted LSD1 before reduction with dithionite, obtained under identical conditions.

Quantification of the spin concentration of the signal indicates that it accounts for 78 μM protein, or 0.3 eq with respect to the protein. A study of the temperature dependence of the signal at 5 milliwatts of microwave power (data not shown) indicates that it is maximal at 13 K and then broadens considerably such that it is no longer visible at temperatures above 45 K. This behavior suggests that the paramagnetic signal derives from a [4Fe-4S]⁺ cluster and that the [2Fe-2S]²⁺ clusters that are observed by Mössbauer spectroscopy are not reduced under the described experimental conditions. This conclusion stems from the inability to observe a signal above 45 K and the generally accepted dictum that most [2Fe-2S] clusters are slow relaxing and observable by EPR at temperatures above 77 K (31).

Quantification of Turnover of LSD1—Because the two products of the LSD1 reaction are pyruvate and ammonia, enzyme
activity was measured by monitoring the LDH-dependent reduction of pyruvate to lactate at the expense of NADH. LDH will also act on α-ketobutyrate, producing 2-hydroxybutyrate (32), and allowing turnover of L-threonine or L-allo-threonine to be monitored by the same method. In the presence of 55 nM enzyme and varying concentrations of L-serine, the reactions were quite fast and, depending on the substrate concentration, usually complete (all of the substrate was consumed) within 3 min of initiation. In some cases, small, but discernible lags were present; however, proper controls indicated that they did not derive from an insufficient amount of the coupling enzyme in the assay. We attribute the lags to either assembly of Fe/S clusters onto polypeptides that are insufficiently reconstituted, or a slow oligomerization of polypeptides to the active quaternary structure, which is facilitated in the presence of substrate.

In Fig. 4, a plot of the initial rate as a function of L-serine concentration is displayed, the inset depicting the Lineweaver-Burk representation of the data. A fit of the data to Equation 1, as compared with 128 kDa, consistent with a dimeric quaternary structure

In contrast to the reconstituted enzyme, as-isolated LSD1 behaved erratically, making it difficult to obtain good kinetic data on this state of the enzyme. In general, however, its activity was more than 5-fold lower than that of the reconstituted enzyme. It was absolutely critical that dilutions of the reconstituted enzyme be made in Buffer B containing 50% glycerol. We discovered that at lower concentrations of glycerol, the enzyme rapidly lost activity \( t_{1/2} \sim 30\) min, even while sitting on ice in the anaerobic chamber. When 50% glycerol was not part of the diluent, the kinetic profiles displayed cooperative behavior at low concentrations of substrate, followed by what appeared to be substrate inhibition. This behavior can be rationalized by a combination of the instability of the enzyme and performing the assays in sequence from low substrate to high substrate. At concentrations of substrate near \( V_{\text{max}} \), wherein the observed rate does not change significantly as a function of substrate concentration, what appears to be substrate inhibition is simply activity loss that is due to enzyme instability.

**Fig. 4. Turnover of LSD1 as a function of substrate concentration.** Assays were carried out as described under “Experimental Procedures.” The concentration of LSD1 in each assay was 55 nM, and the concentration of L-serine was varied between 0 and 20 mM. The solid line represents a fit to Equation 1, yielding \( V_{\text{max}} = 1.41 \mu\text{mol min}^{-1} \) and \( K_m = 2.67 \pm 0.25 \mu\text{M} \). The inset is a Lineweaver-Burk plot of the data.

**Fig. 5. Inhibition of LSD1 by D-serine (A) and L-cysteine (B).** Assays were carried out as described under “Experimental Procedures.” The concentration of LSD1 in each assay was 55 nM. A, L-serine was varied between 1 and 15 mM at 0 (open triangles), 3 (open circles), 6 (closed circles), 12 (open squares), and 24 (closed squares) mM D-serine. B, L-serine was varied between 2 and 20 mM at 0 (open triangles), 2 (open circles), 5 (closed circles), 10 (open squares), and 20 (closed squares) mM cysteine. Lineweaver-Burk plots were constructed by multiple non-linear regression analysis of the data to Equation 3 using the GraFit software program.
Interaction of the [4Fe-4S] Cluster with Substrate and Substrate Analogues—To determine whether the [4Fe-4S] cluster participates in catalysis or simply acts as a structural element of the protein, we assessed whether the addition of substrate would perturb the Mössbauer or EPR spectra of the samples. Unexpectedly, upon addition of 20 mM L-serine to dithionite-reduced LSD1, the intensity of the EPR spectrum drastically decreased in the < 30 s that it took to freeze the sample (Fig. 7A); the residual spectrum shows a very slight broadening of the feature at \( g = 2.03 \) (Fig. 7A, inset). By contrast, the spectra in the presence of L-cysteine, L-threonine, and D-serine are not significantly reduced in intensity but also show the slight broadening in their features at \( g = 2.03 \), which is indicated by the arrows in Fig. 7 (B and C). The spectrum in the presence of L-cysteine is not displayed, because it is identical to those in the presence of L-threonine and D-serine. The marked change in spectral intensity upon addition of the true substrate, but not upon addition of inhibitors or poor substrates, suggests that the amino acid binds to or near the Fe/S cluster and that the reduction in spectral intensity might be related to turnover.

Mössbauer spectroscopy was also used to probe for interactions between the substrate and the Fe/S cluster. Because small substrate-induced perturbations in the spectrum of the Fe/S cluster are more easily detected in species giving rise to a quadrupole doublet rather than a magnetically split spectrum, the experiment was carried out using reconstituted LSD1 that was not treated with dithionite and that contained 50 ± 5% of the iron in the form of [4Fe-4S]^{2+} clusters. L-Threonine was employed as the added substrate, because it is turned over significantly slower than L-serine (see above) but gives rise to the same perturbation of the EPR spectrum of the [4Fe-4S]^{2+} cluster. The Mössbauer spectrum of reconstituted LSD1 (350 \( \mu \)M) in the presence of 20 mM L-threonine was identical to the spectrum of reconstituted LSD1 in the absence of substrate (i.e., the Mössbauer spectrum is not perturbed by L-threonine).

**DISCUSSION**

In this study we described the biochemical and biophysical characterization of \( E.\ coli \) L-serine deaminase containing an N-terminal 6\( \times \) His tag. When overexpressed in \( E.\ coli \), the isolated protein contained small amounts of iron and sulfide \((1.86 \pm 0.46 \text{ iron and } 0.670 \pm 0.019 \text{ S}^{2-} \text{ per monomer}) \) and displayed a weak UV-visible spectrum in the 300- to 700-nm region that is consistent with the presence of [4Fe-4S] clusters. Anaerobic treatment of the protein with FeCl_3 and Na_2S resulted in the uptake of substantial amounts of iron and sulfide, as well as a dramatic increase in the activity of the protein. We used a combination of UV-visible, EPR, and Mössbauer spectroscopies together with analytical methods to assess the configuration of all species of iron on the protein subsequent to reconstitution, as well as their relative stoichiometries. We found that the predominant configuration was [4Fe-4S]^{2+} \((1.25 \pm 0.25 \text{ eq per polypeptide}) \), accounting for greater than 50% of the total iron associated with the protein. Other Fe/S-cluster forms detected included 0.25 eq of [2Fe-2S]^{2+} and 0.02 eq of [3Fe-4S]^{2+} clusters per polypeptide. The large uncertainty in each value reflects the individual errors of three different methods, which are the determination of protein and iron concentrations, and the quantification of various configurations of iron by Mössbauer spectroscopy.

We argue, as has been previously proposed (10, 11), that the catalytically active form contains one [4Fe-4S]^{2+} cluster and that the other cluster species are artifacts of the reconstitution
procedure. Indeed, the protein contains 9 cysteine residues, which can contribute to the coordination of adventitious iron, especially if any of the cysteines are on the surface of the protein. The thermodynamic stability of Fe/S clusters is well documented, and [4Fe-4S] and most likely [2Fe-2S] clusters, which are coordinated by exogenous thiolate ligands (e.g. DTT) are present in solution under reconstitution conditions (33). Spectroscopically, such species are not expected to be discernible from genuine Fe/S cofactors. Potentially, these clusters may bind to surface-exposed cysteine residues of the protein, forming clusters of the general formula [4Fe-4S]DTT_Cys_{4-9}, which may not be separated from the protein by gel filtration chromatography.

We demonstrated that the reconstituted enzyme is catalytically active, converting L-serine to pyruvate and ammonia with a \( k_{\text{cat}} \) of 436 s\(^{-1}\). L-Threonine and L-\(\alpha\)-allo-threonine were also turned over, albeit significantly slower, whereas L-cysteine and D-serine were found to be competitive inhibitors. In contrast, the as-isolated enzyme did not yield reliable kinetic data, which we attribute to its lability and cofactor deficiency. These studies support our contention that the catalytically active state of LSD1 contains a [4Fe-4S] cluster.

Our model for the deamination of L-serine by LSD1 is based on the elegant work of Beinert, Kennedy, Emptage, München, and collaborators on the enzyme aconitate (21), which catalyzes the interconversion of citrate and isocitrate. The first step involves the dehydration of citrate to form cis-aconitate, which is subsequently rehydrated, affording isocitrate. In its resting state, the enzyme contains a [4Fe-4S]\(^{2+}\) cluster that is ligated to the protein via three cysteine residues that are coordinated to three of the iron of the cluster, termed Fe\(_a\). The fourth iron, Fe\(_a\), is not coordinated by a cysteine residue, but contains a bound hydroxyl (water) group. In addition, the hydroxyl group of the substrate, as well as one of the substrate carboxylate groups, ligate iron Fe\(_a\) in a bidentate fashion, resulting in hexacoordination at this site. The Fe/S cluster is thought to facilitate loss of the hydroxyl group by acting as a Lewis acid (21).

A similar role has been proposed for the Fe/S cluster of LSD1 (10, 11), and a mechanism for deamination of L-serine is shown in Scheme 2. Coordination of the hydroxyl group of L-serine (1) to the unique iron of the Fe/S cluster (2) allows elimination of the hydroxyl group, presumably with general acid assistance, and formation of 2-amino-2-propenoic acid. Tautomerization of this species gives rise to 2-iminopropionic acid (3), which is subsequently hydrolyzed to ammonia and pyruvate.

In aconitase, a wealth of spectroscopic evidence supports the role of the unique iron site in binding substrate and facilitating loss of the hydroxyl group. Upon addition of substrate, the electronic structure of the [4Fe-4S]\(^{2+}\) cluster changes significantly, as evidenced by changes of the Mössbauer parameters of site Fe\(_a\), which depend on the nature of the substrate bound: \( \delta = 0.44 \) mm/s and \( \Delta E_Q = 0.83 \) mm/s (no substrate), \( \delta = 0.84 \) mm/s and \( \Delta E_Q = 1.26 \) mm/s (isocitrate), and \( \delta = 0.89 \) mm/s and \( \Delta E_Q = 1.83 \) mm/s (citrate) (21, 34–36). In addition, the EPR spectrum of the [4Fe-4S]\(^{2+}\) cluster changes significantly upon substrate addition, with the principal g values shifting from 2.06, 1.93, and 1.86, to 2.04, 1.85, and 1.78, respectively (21, 35). The exact mode of binding of substrates to the Fe/S cluster was established by \(^{57}\)Fe, \(^{33}\)S, \(^{15}\)N, and \(^{17}\)O electron-nuclear double-resonance spectroscopy (37–40) and was subsequently confirmed by x-ray crystallography (41). Other enzymes containing a [4Fe-4S] cluster, in which only three iron sites are coordinated by cysteinate ligands from the protein, include the enzymes of the radical S-adenosyl-l-methionine (SAM) superfamily. The cosubstrate or cofactor, SAM, coordinates to the fourth iron site in a bidentate fashion via the amino and carboxylate substituents of the molecule (42, 43), causing significant changes of the EPR and Mössbauer spectroscopic properties of the cluster. For example, in pyruvate formate-lyase (PFL) activase, the Mössbauer parameters of Fe\(_a\) of the [4Fe-4S]\(^{2+}\) cluster change from \( \delta = 0.42 \) mm/s and \( \Delta E_Q = 1.12 \) mm/s to \( \delta = 0.72 \) mm/s and \( \Delta E_Q = 1.15 \) mm/s upon addition of SAM. Binding of SAM also perturbs the EPR spectrum of the [4Fe-4S]\(^{2+}\) cluster of PFL activase; the observed \( g \) values shift from 2.01 and 1.94 to 2.01, 1.89, and 1.88 (44, 45). In biotin synthase, another member of the radical SAM superfamily, addition of

---

\( 2 \) In accord with this hypothesis, site-directed mutagenesis studies indicate that only 3 of the 9 cysteine residues are essential for catalysis, because their substitution with non-sulfur-containing amino acids results in abolishment of catalytic activity. These cysteines presumably are those that ligate the [4Fe-4S] cluster. Similar substitutions of the remaining cysteine residues either produce relatively slight decreases or increases in activity (X. Zhao, Q. Sun, Y. M. Tang, S. J. Booker, and E. Newman, submitted for publication).

---

\( \text{SCHEME 2. Role of the Fe/S cluster in the serine deaminase reaction.} \)

---

An Iron-Sulfur Cluster in Serine Deaminase
SAM perturbs the Mössbauer parameters of two iron sites of the \([4\text{Fe}-4\text{S}]^{2+}\) cluster. In the absence of SAM, both sites have \(\delta = 0.47\) mm/s and \(\Delta E_Q = 1.10\) mm/s, which convert to \(\delta = 0.40\) mm/s and \(\Delta E_Q = 0.86\) mm/s, and \(\delta = 0.64\) mm/s and \(\Delta E_Q = 1.26\) mm/s, respectively, in the presence of SAM. In analogy to PFL active asaconitase, the EPR spectrum of the \([4\text{Fe}-4\text{S}]\) cluster of biotin synthase is sensitive to the presence of SAM; the principal \(g\) values shift from 2.04 and 1.94 to 2.00, 1.93, and 1.85 (46).

In contrast to the significant changes in the EPR and Mössbauer spectra ofaconitase and the radical SAM enzymes upon binding of substrate, we observe comparatively small changes in the EPR spectra of the \([4\text{Fe}-4\text{S}]\) cluster in the presence of L-serine, L-threonine, L-\(\alpha\)-threonine, or D-serine. The changes, however, are nearly identical for all of these substrates, suggesting that they interact with the \([4\text{Fe}-4\text{S}]\) cluster in a similar way. The Mössbauer spectrum of LSD1 in the presence of L-threonine revealed no discernable changes compared with the spectrum of reconstituted LSD1. We point out, however, that for aconitase and the radical SAM enzymes, the unique iron site has coordination numbers of 6 and 5, respectively, when the substrate is bound. In the absence of substrate, their Mössbauer parameters for \(\Theta^0\) are similar to those typically observed for cysteinate-ligated iron sites of \([4\text{Fe}-4\text{S}]^{2+}\) clusters. The changes in the Mössbauer parameters upon substrate binding are thus likely to be a consequence of the increased coordination number. Our combined EPR and Mössbauer spectroscopic studies suggest that L-serine might displace the fourth ligand coordinated to \(\Theta^0\) in LSD1, which is presumably a water (hydroxide) molecule, and coordinates the iron in a monodentate fashion (Scheme 2). In support of this hypothesis, it has been proposed that coordination of L-serine to the Fe/S cluster of LSD1 is most likely monodentate, involving only the hydroxyl group of the substrate, because simultaneous coordination of the hydroxyl group and either the amino or the carboxylate group would not permit proper orbital alignment for effective elimination of water (hydroxide) from the substrate (10).

Three related serine deaminases have been described in some detail. These are the enzymes from Pestiptreptococcus asaccharolyticus (10), Clostridium propionicum (19), and Clostridium sticklandii (47). Each of these proteins is activated by iron and DTT; however, the nature of the iron-containing co-factor has been demonstrated only in the enzyme from P. asaccharolyticus. Upon purification of the enzyme under anaerobic conditions, it displayed a rhombic EPR signal \((g_1 = 2.001, g_2 = 2.014, g_3 = 2.028)\) in phosphate buffer at pH 5 and a fairly axial signal around \(g = 2.01\) in Tricine-KOH buffer (pH 8.0). Both signals broadened considerably as the temperature was raised, such that they were no longer visible above 30 K. These \(g\) values and temperature-dependent properties were taken to indicate the presence of \([3\text{Fe}-4\text{S}]\) clusters (20).

Interestingly, the addition of high concentrations of L-serine in the EPR spectra of the \([4\text{Fe}-4\text{S}]\) cluster ofaconitase and the radical SAM enzymes upon substrate (10). The principal \(g\) values shift from 2.04 and 1.94 to 2.00, 1.93, and 1.85 (46).
