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The iron–sulfur cluster in the L-serine dehydratase TdcG from *Escherichia coli* is required for enzyme activity

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Abstract The anaerobically inducible L-serine dehydratase, TdcG, from *Escherichia coli* was characterized. Based on UV–visible spectroscopy, iron and labile sulfide analyses, the homodimeric enzyme is proposed to have two oxygen-labile $[4Fe–4S]^{2+}$ clusters. Anaerobically isolated dimeric TdcG had a k_{cat} of 544 s^{-1} and an apparent K_M for L-serine of 4.8 mM. L-threonine did not act as a substrate for the enzyme. Exposure of the active enzyme to air resulted in disappearance of the broad absorption band at 400–420 nm, indicating a loss of the $[4Fe–4S]^{2+}$ cluster. A concomitant loss of dehydratase activity was demonstrated, indicating that integrity of the $[4Fe–4S]^{2+}$ cluster is essential for enzyme activity.

Keywords: L-Serine dehydratase; Iron–sulfur protein; $[4Fe–4S]^{2+}$ cluster; Anaerobic metabolism; *Escherichia coli*

1. Introduction

L-Serine can be irreversibly deaminated to pyruvate and ammonia by L-serine dehydratase [1]. The initial dehydration step results in the β-elimination of water. This is followed by tautomerization and hydrolysis reactions. Unlike L-threonine dehydratases, which catalyze the dehydration of L-threonine to 2-ketobutyrate and ammonia and which are pyridoxal phosphate (PLP)-dependent enzymes, bacterial L-serine dehydratases do not require PLP. Evidence has been presented that prokaryotic L-serine dehydratases have catalytically active $[4Fe–4S]^{2+}$ clusters [2–5]. In the PLP-dependent L-threonine dehydratases, a Schiff base is formed with the β-hydroxyamino acid and consequently activation of the proton on the α-carbon means that it can be simultaneously removed along with the β-hydroxyl group. The use of an Fe–S cluster in L-serine dehydratase, rather than PLP, as an electron-withdrawing center suggests that the β-hydroxyl group might be removed prior to the α-hydrogen [1], in analogy to aconitase [6], although this has yet to be demonstrated. Like aconitase, the three L-serine dehydratases characterized to date have an exchangeable iron atom in their $[4Fe–4S]^{2+}$ cluster and this is proposed to complex the hydroxyl group of L-serine [1].

The first iron–sulfur cluster-containing L-serine dehydratase to be characterized was isolated from the strict anaerobe *Peptostreptococcus asaccharolyticus* [2]. This heterodimeric enzyme has a low K_M for L-serine and exhibits a high catalytic activity. Electron paramagnetic resonance (EPR) analysis of the enzyme identified a $[3Fe–4S]^{+}$ cluster; however, it is proposed that the active enzyme has a $[4Fe–4S]^{2+}$ [3]. Recently, a homodimeric iron–sulfur cluster-containing L-serine dehydratase has been characterized from *Campylobacter jejuni* and was shown to be specific for L-serine (K_M = 14.6 mM) [4]. In another study, one of the three L-serine dehydratases encoded on the *Escherichia coli* genome, L-serine dehydratase 1, was shown by EPR to have a $[4Fe–4S]^{2+}$ cluster [5]. The anaerobically isolated enzyme had a low Fe and labile sulfide content and negligible enzyme activity. Reconstitution of the protein in the presence of iron and sulfide regenerated the $[4Fe–4S]^{2+}$ cluster and the enzyme exhibited a k_{cat} of 436 s^{-1} and a K_M of 2.67 mM for L-serine [5].

The three L-serine dehydratases present in *E. coli* share a high degree of amino acid sequence similarity [7]. Of particular interest is the anaerobically inducible L-serine dehydratase, TdcG, which is encoded by the multicistronic *tdcABCDEFG* operon [7]. As well as encoding TdcG, the operon also codes for a PLP-dependent L-threonine dehydratase, TdcB [8], together with a number of other enzymes that allows *E. coli* to utilize L-serine and L-threonine as an energy source [7,9]. In this study, we characterize anaerobically purified TdcG and demonstrate that it has a high specific activity with L-serine. Enzyme activity is oxygen-labile and is strictly dependent on the presence of an iron–sulfur cluster.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *E. coli* strains used in this study were MC4100 F^- *araD139*Δ(*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB530 rpsL150* λ^- [10] and BL21(DE3) F *ompT gal [dcm][lon] hsdS*$_B$ [11]. *E. coli* strains were grown in LB medium (per l, 10 g peptone, 5 g yeast extract and 8 g NaCl). Carbenicillin, when required for plasmid maintenance, was added at a final concentration of 100 μg ml^{-1}. For overproduction of His-tagged TdcG, BL21(DE3) containing pET100-G was grown in LB supplemented with 20 mM glucose. Induction of gene expression was initiated by adding 0.5 mM isopropyl-γ-D-thiogalactopyranoside (IPTG).

2.2. Cloning and overexpression of tdcG

The *tdcG* gene was PCR-amplified from the genome of MC4100 using the oligonucleotides G1 (5′-CACCATTAGTGCATTCGATATTTTCAAAATTGGG-3′) and G2 (5′-TCAGCCGCAGACTT-

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TAATGGC-3′) and Pfu DNA polymerase (Stratagene). The approximately 1.2 kbp amplified DNA fragment was cloned using a TOPO cloning kit into pET100 (Invitrogen). In the resulting plasmid, pET100-G, expression of the *tdcG* gene is under the control of the inducible T7 promoter and the *tdcG* gene product carries a polyhistidine (6× His) tag. DNA sequencing of pET100-G revealed that through the cloning procedure the recombinant protein lacked one of the C-terminal valines at amino acid position 452.

2.3. Purification of His-tagged TdcG

All buffers used in the anaerobic purification of TdcG were sparged with dinitrogen. Cell paste (4 g wet weight) derived from growth of BL21(DE3) pET100-G was resuspended in 10 ml of buffer A [20 mM Tris–HCl, 250 mM NaCl, 20 mM imidazole, and 6 mM β-mercaptoethanol, pH 8.0] containing 0.1 mM EDTA, 1 mM Pefabloc protease inhibitor (Roche), and a crude cell extract was prepared exactly as described in [7]. The crude cell extract (10 ml) was then flushed with dinitrogen and transferred to an anaerobic glove box (Miller Howe Ltd.) and allowed to equilibrate for 1 h before being applied to a 5 ml HiTrap-chelating column (Amersham Biosciences) charged with Ni^{2+} ions. The column was attached to an Äkta protein purification system (Amersham Biosciences) and TdcG was eluted by developing the column with a 0–100% gradient of buffer B [20 mM Tris–HCl, 250 mM NaCl, 1 M imidazole, and 6 mM β-mercaptoethanol, pH 8.0] over a period of 45 min, at a flow rate of 1 ml min^{-1}. Fractions of 1 ml were collected. The purity of the protein was monitored by SDS–PAGE and protein concentration was determined using the Folin phenol method as described in [12]. To avoid loss of enzyme activity and precipitation of protein, pooled fractions were exchanged into 20 mM Tris–HCl, pH 8.0, immediately after elution from the Hi-Trap chelating column by passage over a PD-10 column (Amersham Biosciences) and concentrated using a Centriprep concentrator (Amicon; 10 kDa cut-off). Generally, from 1 g wet weight of cell paste, 2 mg of purified TdcG was obtained.

2.4. L-Serine dehydratase assay

In an anaerobic glove box, a quartz cuvette was filled with anaerobic assay solution (50 mM Tris–HCl, pH 8.0, containing 0.2 mM NADH and 5 units of D-lactate dehydrogenase from *Lactobacillus leichmanii*) and an appropriate amount of TdcG. The assay was performed at RT in a final volume of 1 ml. The cuvette was sealed with a Suba-seal (W. Freeman Ltd., Barnsley, UK), removed from the anaerobic glove box and the baseline determined in a λ18 diode array spectrophotometer (Perkin–Elmer). The reaction was started by adding 1–50 mM anaerobic L-serine solution and the time-dependent oxidation of NADH was measured at 340 nm ($\varepsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1}). One unit of enzyme activity is equivalent to 1 μmole of L-serine dehydrated min^{-1} ml^{-1}. Michaelis–Menten kinetic constants (K_M and V_{max}) were obtained from fits of initial rate data as a function of L-serine concentration.

2.5. Other analytical techniques

UV–visible spectra were taken using a λ18 diode array spectrophotometer. Scans were typically taken from 200 to 750 nm. Measurements were taken at room temperature in a quartz cuvette with a pathlength of 10 mm.

The iron content of TdcG was determined using inductively coupled plasma emission (ICPE) [13] (Southern Analytical, Brighton, UK) using 1 mg of enzyme. Iron content of TdcG was also estimated from the molar extinction coefficient of the enzyme at 400 nm using a molar extinction coefficient for non-heme iron of 4000 M^{-1} cm^{-1} [14].

Sulfide analysis was determined using the method of Brumby et al. [15].

Dynamic light scattering (DLS) was used to assess the polydispersity of TdcG, as well as to estimate the molecular mass and give an indication of oligomeric state. DLS experiments were performed using a Dynapro MSTC (Protein Solutions Inc.) with a temperature-controlled microsampler (Protein Solutions Inc.). TdcG was filtered through a 0.1 μm centrifugal filter (Millipore) prior to taking measurements.

3. Results and discussion

3.1. TdcG is an iron–sulfur protein

The anaerobic enzyme TdcG shares 78% amino acid identity with the two L-serine dehydratases from *E. coli* [7], 46% identity with L-serine dehydratase from *C. jejuni* [4] and 26% identity with the enzyme from *P. asaccharolyticus* [5]. In particular, there are 4 cysteinyl residues that are totally conserved between the 5 polypeptides. Moreover, L-serine dehydratase 1 and the enzymes from *P. asaccharolyticus* and *C. jejuni* are iron–sulfur proteins [2,4,5]. Taken together, these features suggested that TdcG is probably an iron–sulfur protein. A His-tagged version of TdcG was purified anaerobically by Ni^{2+}-chelating affinity chromatography to near homogeneity, as judged by SDS–PAGE analysis (Fig. 1). The deduced subunit molecular mass of the His-tagged TdcG protein is 52.91 kDa (48.46 kDa for the polypeptide lacking the His-tag). Purified His-tagged TdcG migrated slightly faster in SDS–PAGE than anticipated with an estimated subunit molecular mass of 46 kDa (Fig. 1). DLS analysis of purified TdcG yielded a peak radius of 4.33 ± 0.28 corresponding to a native mass for the anaerobically purified enzyme of 101.7 ± 15 kDa, suggesting that His-tagged TdcG is a dimer (data not shown). The degree of polydispersity of TdcG was estimated to be 17%.

It was noted that the protein, when purified anaerobically, was brown in color, consistent with the presence of an iron–sulfur cluster. Indeed, UV–visible spectroscopic analysis of the protein revealed a broad shoulder from 395 to 420 nm (Fig. 2), which is characteristic of iron–sulfur proteins [14]. When TdcG was purified in the presence of oxygen and dialyzed to exchange the buffer to 50 mM Tris–HCl, pH 8, the broad shoulder at 395–420 nm was not observed (Fig. 2), indicating that the iron–sulfur cluster is oxygen-labile. Assuming a molecular mass of 105 940 Da for dimeric, His-tagged TdcG, this indicates an extinction coefficient at 400 nm of 27 499 M^{-1} cm^{-1}. This suggests the presence of an average of ~7 non-heme iron atoms per dimer [14]. ICPE analysis determined that anaerobically purified TdcG had 7.7 ± 0.6 mol iron per mol dimer, while aerobically purified TdcG had only 1.8 mol per mol dimer. Acid-labile sulfide analyses yielded values of

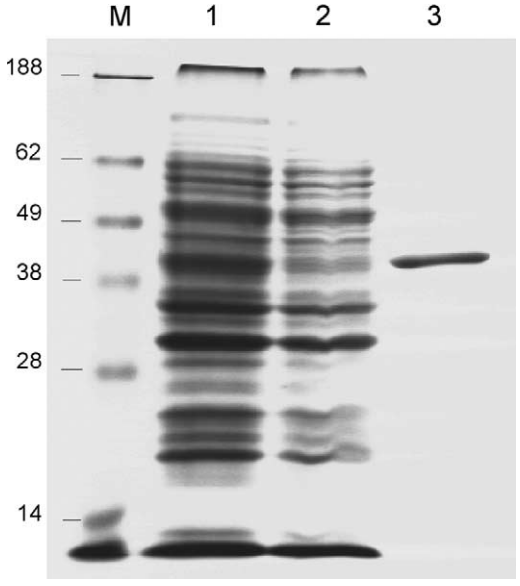

Fig. 1. Purification of His-tagged TdcG. A SDS–polyacrylamide gel [10% (w/v) polyacrylamide] of different stages of the TdcG purification is shown. Lane M, molecular mass markers; lane 1, an aliquot of BL21(DE3) pET100-G crude extract (100 μg of protein); lane 2, an aliquot of the flow-through from the HiTrap Ni^{2+}-chelating column (100 μg of protein); lane 3, 5 μg of purified His-tagged TdcG. Polypeptides were visualized after staining the gel with Coomassie Brilliant Blue R-250.

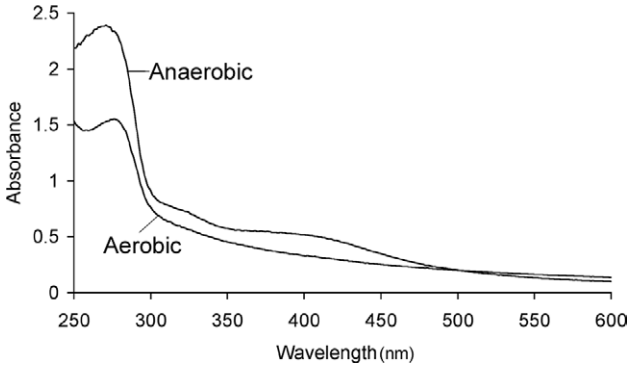

Fig. 2. UV–visible spectra of aerobically and anaerobically purified His-tagged TdcG. The concentration of the aerobic and anaerobic samples of TdcG was 18.8 μM.

8.7 ± 0.3 and 0.65 mol sulfide per mol of anaerobically and aerobically purified TdcG, respectively. Taken together, these findings indicate that anaerobically purified TdcG probably has 2 $[4Fe–4S]^{2+}$ clusters per dimer, while in the aerobically purified enzyme the clusters are degraded.

3.2. Kinetics of TdcG

Anaerobically purified TdcG had a specific activity of 307 U mg of protein^{-1} (5.1 μkat mg^{-1}) with 40 mM L-serine as substrate. Determination of initial rates over a L-serine concentration range of 1–50 mM revealed that the enzyme had a high activity (Fig. 3A) and fitting the data to a Lineweaver–Burk plot (Fig. 3B) yielded an apparent K_M of 4.8 mM. The K_M for

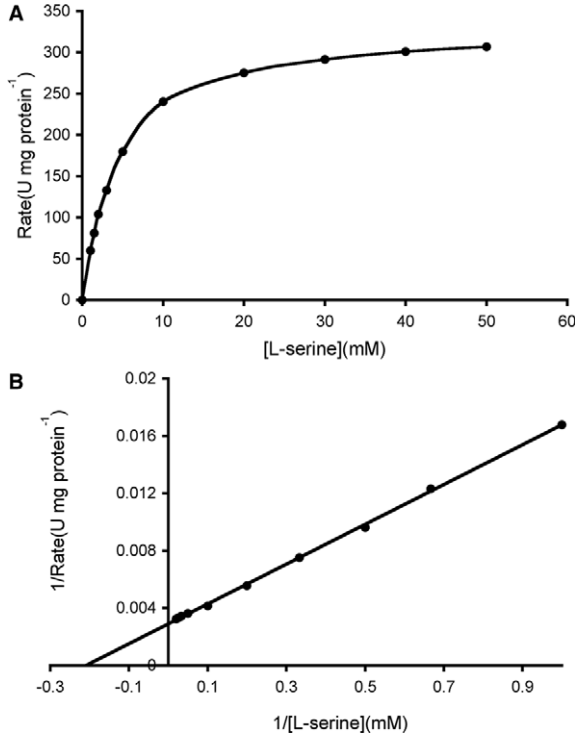

Fig. 3. Activity of TdcG as a function of L-serine concentration. Assays were performed as described in Section 2. The initial rate as a function of substrate concentration is shown in (A) and the Lineweaver–Burk plot of the data in (A) is shown in (B). The kinetic constants were determined to be: $K_M = 4.8$ mM and $V_{max} = 344.8$ U mg of protein^{-1}.

TdcG is in the range of that determined for L-serine dehydratase isoenzyme 1 from *E. coli* (K_M 2.67 mM) [5]. The k_{cat} of TdcG with L-serine was determined to be 544 s^{-1} (272 s^{-1} per monomer). We were unable to detect any activity of TdcG with 50 mM L-threonine as substrate (<0.01 units mg^{-1}).

3.3. TdcG enzyme activity is dependent on an intact iron–sulfur cluster

We noted that aerobically purified TdcG could not be isolated with an intact [4Fe–4S] cluster (Fig. 2) and this enzyme preparation was also devoid of enzyme activity. Enzymically active TdcG was placed in an anaerobic cuvette and the UV–visible spectrum of the enzyme and its activity were recorded. The sealed cuvette was opened and the protein solution was mixed in the presence of air. Loss of enzyme activity followed an exponential decay curve with an estimated half-life of 20 min. Complete loss of enzyme activity was observed after 120 min exposure to air. This loss of enzyme activity correlated with the loss of the broad absorption shoulder at 400 nm (data not shown). The inactivated enzyme had an optical spectrum in the 400–420 nm region that was indistinguishable from the aerobically purified enzyme (see Fig. 2).

Based on these findings, we suggest that, in analogy to the heterodimeric L-serine dehydratase from *P. asaccharolyticus* and the homodimeric *C. jejuni* and *E. coli* enzymes, the $[4Fe–4S]^{2+}$ of homodimeric TdcG is essential for catalytic activity and probably interacts directly with the substrate L-serine. Our ability to isolate TdcG in a highly active form, and with a near intact $[4Fe–4S]^{2+}$ cluster, provides an excellent basis for further spectroscopic and structure-function analyses of the enzyme.

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