Chapter 10

Methods to Investigate the Kinetic Profile of Cysteine Desulfurases

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Abstract

Biological iron-sulfur (Fe-S) clusters are essential protein prosthetic groups that promote a range of biochemical reactions. In vivo, these clusters are synthesized by specialized protein machineries involved in sulfur mobilization, cluster assembly, and cluster transfer to their target proteins. Cysteine desulfurases initiate the first step of sulfur activation and mobilization in cluster biosynthetic pathways. The reaction catalyzed by these enzymes involves the abstraction of sulfur from the amino acid L-cysteine, with concomitant formation of alanine. The presence and availability of a sulfur acceptor modulate the sulfur-transferase activity of this class of enzymes by altering their reaction profile and catalytic turnover rate. Herein, we describe two methods used to probe the reaction profile of cysteine desulfurases through quantification of alanine and sulfide production in these reactions.

Key words Fe-S clusters, Cysteine desulfurase, Alanine, Sulfide, Sulfur acceptor, Methylene blue, NDA

1 Introduction

Iron-sulfur clusters are among the most ubiquitous metal cofactors and are found in metalloenzymes that possess important redox, catalytic, and regulatory functions [1, 2]. These clusters mediate processes such as gene expression, electron transfer, and catalysis while also playing key roles in central metabolic pathways [3]. The most prevalent clusters exist as [2Fe-2S], [4Fe-4S], and [3Fe-4S] assemblies of iron ions and inorganic sulfide, and these cofactors are commonly bound to their protein partners via cysteine-thiolate ligands [1]. Each iron of a [2Fe-2S] cluster is typically coordinated by two cysteine thiolate ligands, while the [4Fe-4S] and [3Fe-4S] clusters are coordinated by one cysteine ligand. Thus, in all cases, the iron ion is in a tetrahedral coordination environment, enabling facile one-electron redox reactions [4].

It is well known that certain apo forms of cluster-containing proteins can be activated in vitro by simply adding sulfide and iron

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ions [5, 6]. Although this observation led to the initial assumption that Fe-S clusters can be spontaneously fused into their protein partners in vivo, this view was not compatible with the physiological toxicity of Fe and S at concentrations needed for in vitro cluster maturation. A more realistic scenario entails the delivery of Fe and S to apo-[Fe-S] proteins via specific carrier proteins known to shuttle Fe and S in nonhazardous forms [7]. The *Azotobacter vinelandii* Nif system, comprising NifS and NifU, was the first biosynthetic system to be identified, and is involved in the initial assembly and shuttling of Fe-S clusters destined for nitrogenase [8]. The identification of the Nif system led to the subsequent discovery of the Isc system, which is involved in the housekeeping synthesis of Fe-S clusters in *A. vinelandii* and many other bacterial species [9–11].

Fe-S cluster biosynthetic pathways have been identified in a wide range of species belonging to various ecological and phylogenetic groups [12–17]. All Fe-S cluster biosynthetic pathways studied thus far utilize a general three-step scheme that includes: a sulfur activation reaction, the preformation of Fe-S clusters, and the incorporation of these clusters into their final acceptor proteins. Hence, a model for the biological formation of Fe-S clusters minimally requires a sulfur-activating enzyme and a protein that serves as the assembly site of Fe-S clusters, more commonly known as the scaffold protein. Once synthesized, clusters are then transferred to their final targets, where they perform their biological functions [1, 18].

The first step of sulfur mobilization in the synthesis of Fe-S clusters is catalyzed by cysteine desulfurases (EC 2.8.1.7). These enzymes are also required for the initiation of several biosynthetic pathways in which sulfur-containing cofactors are synthesized [19, 20]. So far, all characterized cysteine desulfurases adopt homodimeric structures containing a pyridoxal 5′-phosphate (PLP) active site cofactor coordinated by a highly conserved lysine via a Schiff base [21]. These enzymes are known to catalyze the Cys:sulfur acceptor sulfurtransfer reactions via a double displacement mechanism [22, 23] (Fig. 1a).

The first half of a cysteine desulfurase-catalyzed reaction results in the formation of alanine and a persulfide intermediate in the active site of the enzyme, defined here as CD-SSH [20, 21]. In vitro, the rate of this half-reaction can be determined in the presence of reducing agents that reduce the enzyme's persulfide intermediate, releasing free sulfide and regenerating the enzyme for the next catalytic cycle (Fig. 1b). In the absence of a reducing agent and/or a sulfur acceptor, the enzyme reaction leads to the formation of polysulfides and the release of colloidal sulfur (S_8) (Fig. 1c). The reactivity of the persulfurated and polysulfurated (CD-SS-$S_{(1–7)}$H) forms is reduced, as evidenced by slower turnover rates of Ala formation under nonreducing conditions [21]. In vivo, the persulfide intermediate initiates the second half of the catalytic

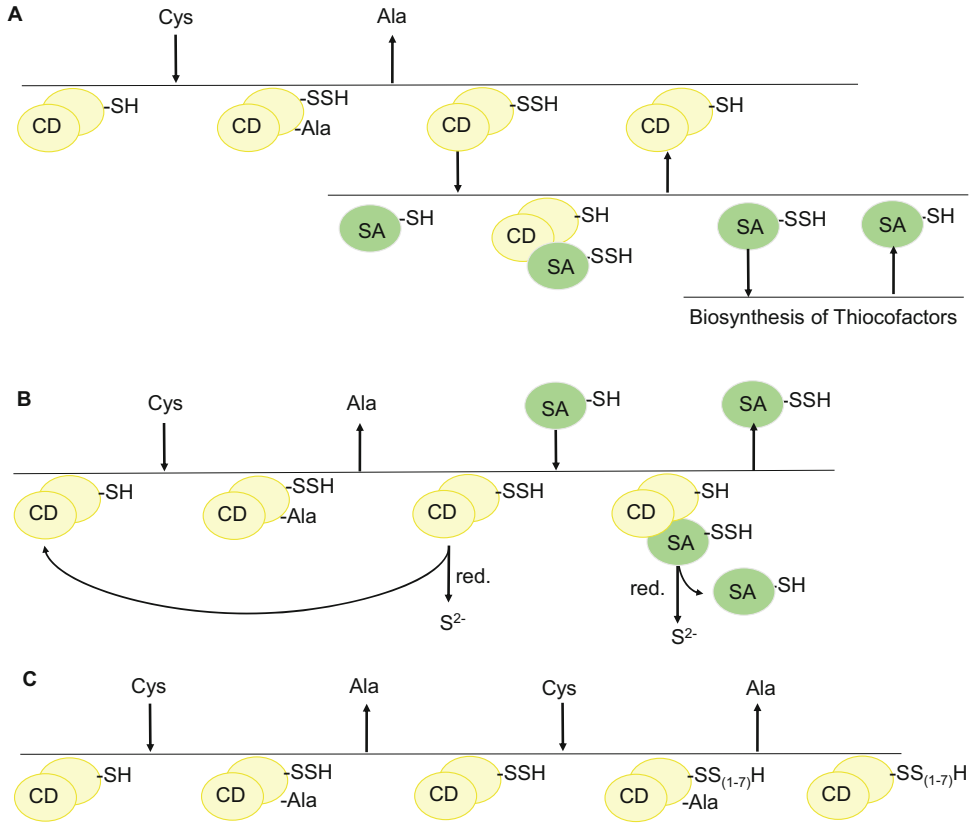

Fig. 1 Double displacement mechanism of Cys:sulfur acceptor sulfurtransferase reactions. (**a**) In vivo reaction. Persulfide sulfur is transferred from the cysteine desulfurase to the sulfur acceptor for the biosynthesis of thiocofactors. (**b**) Reducing/sulfur acceptor reaction. Persulfide sulfur is either transferred from the cysteine desulfurase to sulfur acceptor or directly reduced by a reductant, releasing sulfide. If sulfur transfer is achieved, the persulfide on the sulfur acceptor is also susceptible to reduction. (**c**) Nonreducing/no sulfur acceptor reaction. The cysteine desulfurase undergoes multiple turnovers leading to the formation of colloidal sulfur (S_8)

cycle, which includes the transfer of the persulfide sulfur to an acceptor molecule for the synthesis of Fe-S clusters or other S-containing cofactors [19, 24–26] (Fig. 1a). Thus, kinetic assays probing the complete reaction cycle include the cysteine desulfurase and corresponding sulfur acceptors.

Cysteine desulfurases are classified into two groups based on sequence similarity and tertiary structural motifs [27]. Members of the group I desulfurases, including *Escherichia coli* IscS and *A. vinelandii* NifS, contain a characteristic 12-amino acid residue sequence insertion following the active site cysteine [28]. These conserved moieties form a flexible, structural loop proposed to adopt distinct conformations during catalysis. Conversely, group II cysteine desulfurases, such as *E. coli* SufS and CsdA, lack this structural element and react through a conserved cysteine residue

located within a shorter and more structured loop [29]. This distinction is thought to result in greater steric hindrance and consequently require the involvement of specific sulfur acceptor for effective sulfur transfer. For example, the *Bacillus subtilis* SufS class II enzyme activity is enhanced up to 200-fold in the presence of its dedicated sulfur acceptor, SufU [23, 30]. Therefore, the distinct kinetic profiles of class I and class II enzymes are attributed to the functional and structural differences among members of this general class of enzymes.

In addition to sulfur acceptors modulating cysteine desulfurase activity, the presence and type of reducing agents can also affect the activity of cysteine desulfurases. The synthetic reducing agent dithiothreitol (DTT) can directly interact with the PLP cofactor, thereby decreasing the enzymes' effectiveness in sulfur abstraction and transfer. Furthermore, the persulfide bond on a cysteine desulfurase can be cleaved by artificial reducing agents. In some cases, this direct reaction interferes with the sulfur transfer step by excluding the involvement of sulfur acceptors, ultimately skewing the kinetic behavior of the cysteine desulfurase and distorting the assessment of their in vivo functionality [18, 23, 28, 31–43]. For instance, the enhancement of *B. subtilis* NifZ activity by its sulfur acceptor ThiI is only observed in the absence of a reducing agent [44]. Physiological reducing agents, such as the thioredoxin/thioredoxin reductase (Trx) system, display persulfide reductase activity and can participate in coupled reaction schemes with cysteine desulfurases and sulfur acceptors. For the *B. subtilis* SufS and YrvO cysteine desulfurases, the Trx system is effective in reducing persulfides that are formed within sulfur acceptors but not cysteine desulfurases [45]. The distinct reactivity of physiological and artificial reducing agents must be considered when analyzing the kinetic profile of reactions promoted by these enzymes.

The characterization of the mechanistic steps involved in the synthesis of Fe-S clusters can be quite challenging and generally requires multiple validation methods. This chapter describes experimental approaches used for the kinetic investigation of the first step of Fe-S cluster assembly reactions, which involves cysteine desulfurases. These methods include the detection of alanine, a byproduct in these reactions, and sulfide, a constituent of nascent clusters, and a product released upon reductive cleavage.

2 Materials

2.1 Assay Components

1. Cysteine desulfurase: 20 μM cysteine desulfurase purified in MOPS buffer (*see* **Notes 1** and **2**). Protein stock is stored at −80 °C in 100 μL aliquots.

2. Sulfur acceptor: 20 μM sulfur acceptor purified in MOPS buffer (*see* **Notes 1** and **2**). Protein stock is stored at −80 °C in 100 μL aliquots.
3. MOPS buffer: 50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 8.0. The buffer is prepared in deionized (DI) water, and the pH is adjusted with 10 M NaOH. This buffer should be degassed before use in assays that are coupled to the synthesis Fe-S clusters (*see* **Notes 1** and **2**).
4. L-Cysteine solution: 10 mM L-cysteine hydrochloride prepared fresh in MOPS buffer (*see* **Note 2**).
5. Fe solution: 20 mM ferrous ammonium sulfate. This solution is freshly prepared in DI water (*see* **Note 2**).
6. DTT solution: 0.5 M dithiothreitol in MOPS buffer, pH 8. This solution can be prepared and stored in 500 μL aliquots at −20 °C for up to a year.

2.2 Sulfide Quantification

1. NaOH solution: 12% sodium hydroxide in DI water.
2. ZnOAc solution: 10% zinc acetate in DI water.
3. DMPD solution: 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl.
4. Fe solution: 30 mM iron(III) chloride in 1.2 M HCl.
5. Sulfide standard solution: 1 mM sodium sulfide nonahydrate prepared fresh with DI water in a volumetric flask.
6. 25 μL and 200 μL gas-tight syringes.
7. Vials with rubber septa (for Subheading 3.2.1).
8. Plastic cuvette (for Subheading 3.2.1).
9. Spectrophotometer (for Subheading 3.2.1).
10. Parafilm (for Subheading 3.2.2).
11. 96-Well plate (for Subheading 3.2.2).
12. Microplate reader (for Subheading 3.2.2).

2.3 Alanine Quantification

1. TCA solution: 10% trichloroacetic acid in DI water.
2. KCN solution: 40 mM potassium cyanide in DI water. Prepare a fresh solution once every week and store it in the refrigerator.
3. NDA solution: 8 mM naphthalene-2,3-dicarboxaldehyde in DI water. Prepare a fresh solution once every week and store it in the refrigerator.
4. Alanine standard solution: 1 mM L-alanine in DI water.
5. Borate buffer: 300 mM sodium borate in DI water, pH 9.0. Adjust the pH with 10 M NaOH.

6. NDA mix: 0.1 mM NDA, 1 mM KCN in 100 mM sodium borate buffer, pH 9. The solution is prepared from stock solutions of each ingredient and mixed immediately before use.
7. HPLC grade water (for Subheading 3.3.1).
8. HPLC grade methanol (for Subheading 3.3.1).
9. HPLC coupled with a fluorescence detector (for Subheading 3.3.1).
10. HPLC vials (for Subheading 3.3.1).
11. C-18 column, 150 mm CL × 4.6 mm ID, 5 μM particle size (for Subheading 3.3.1).
12. Black 96-well plate with a clear bottom (for Subheading 3.3.2).
13. Microplate reader fluorimeter (for Subheading 3.3.2).

3 Methods

The sulfurtransferase reaction of cysteine desulfurases is initiated upon binding the substrate cysteine to the PLP cofactor. This event leads to the formation of an external Cys-PLP Schiff base (Fig. 2). The electronic arrangement of the Cys-PLP adduct allows for the nucleophilic attack of the active site cysteine residue onto the cysteine substrate, resulting in the cleavage of the carbon-sulfur bond of the substrate and the final formation of the enzyme persulfide intermediate (CD-SSH) and alanine [22, 46]. The activated sulfur is then transferred to a sulfur acceptor via a persulfide sulfur transfer reaction or directly to a nascent Fe-S cluster, completing the reaction cycle [18, 43]. The methods described below have been developed to characterize the kinetic profile of reactions involving cysteine desulfurases by quantifying alanine and sulfide produced in these reactions.

3.1 Cysteine Desulfurase Assay

3.1.1 Cysteine Desulfurase Assays Under Nonreducing Conditions

Assays performed under nonreducing conditions in the presence or absence of sulfur acceptor molecules lead to the formation of Ala and accumulation of protein persulfides and polysulfides [8]. These latter products can be quantified through the sulfide assay, when reaction time points are reduced prior to detection [38](Fig. 3). The kinetic profile of reactions performed in the presence of sulfur acceptors typically displays a biphasic behavior (Fig. 4). The first phase is marked by a fast rate of alanine formation, attributed to rapid catalytic turnover in the presence of available sulfur acceptor substrate. The length and amplitude of this phase are stoichiometrically proportional to the amount of sulfur acceptor substrate present in the reaction [18, 38, 44]. Under nonreducing conditions and in the absence of subsequent downstream pathway components, persulfurated sulfur acceptors do not engage in subsequent catalytic cycles or, to a lesser extent, do not participate

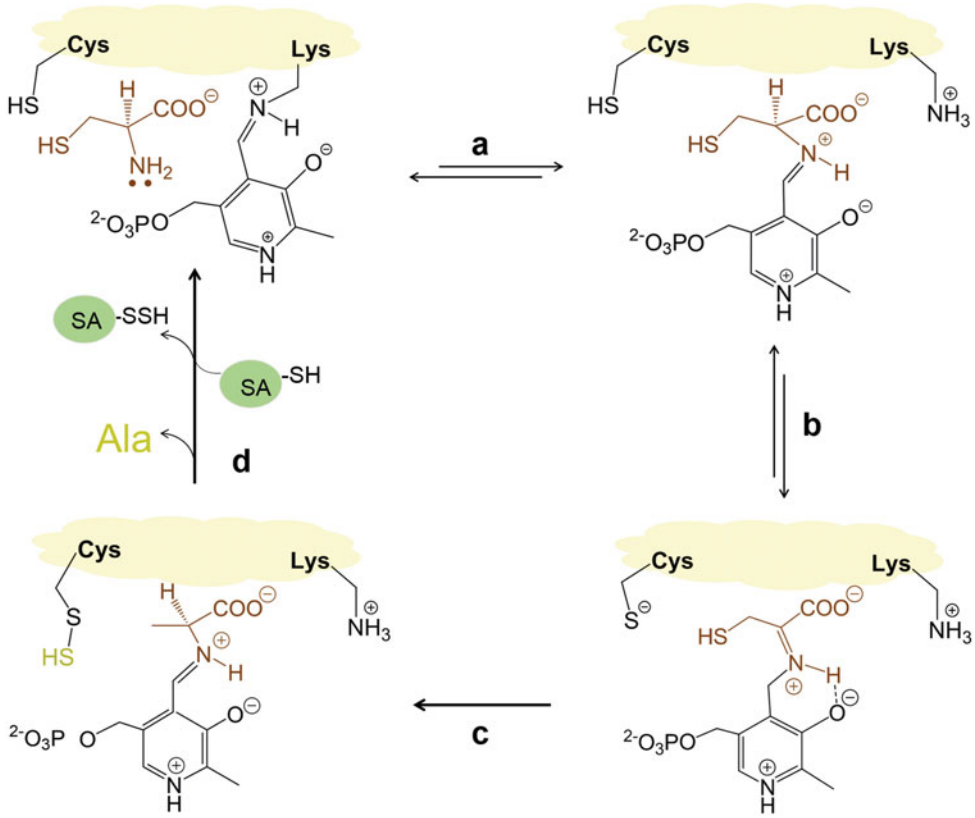

Fig. 2 Mechanism of cysteine desulfurase reaction. (**a**) The reaction proceeds with the binding of cysteine (substrate) to the PLP cofactor, forming the cys-PLP intermediate. (**b**) The formation of the enzyme persulfide intermediate is jumpstarted via deprotonation of the enzyme cys-thiol. (**c**) Deprotonated enzyme cys-thiol abstracts sulfur from cysteine via a nucleophilic attack. (**d**) Alanine is released, and the enzyme is regenerated for another catalytic cycle

with the same efficiency. At this point, the reaction transitions to a slow catalytic profile, as observed in the second phase of the reaction. The slow steady-state turnover presents similar rates of reactions performed without sulfur acceptors. The typical nonreducing reaction is performed as follows:

1. Mix 0.2 μM of cysteine desulfurase and 2 μM of the sulfur acceptor in MOPS buffer, and start the reaction by adding 0.5 mM of cysteine (*see* **Note 3**).
2. In determining biphasic profiles, take a range of time points to calculate the rates of both phases. Consider taking time points at 0.5, 1, 1.5, 2, 3, 6, 9, and 12 min. For an accurate rate determination, utilize the slope of at least three-time points in each phase.
3. For sulfide quantification, at each time point, take 800 μL of reaction aliquot and mix with 25 μL of NaOH solution to stop

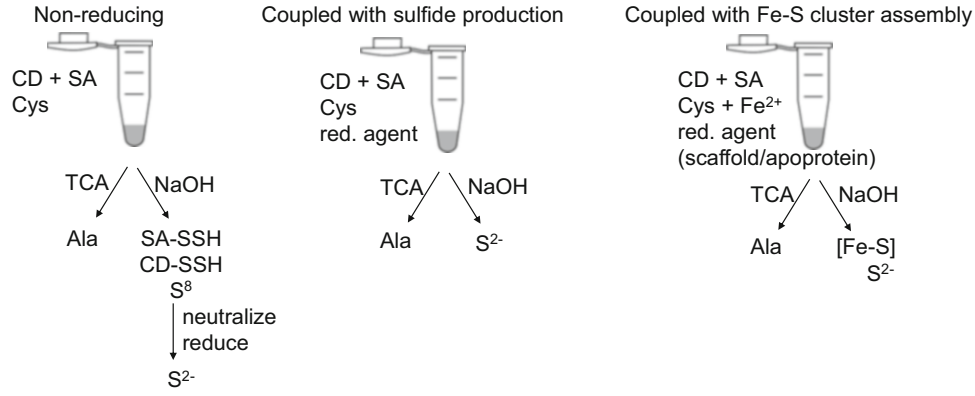

Fig. 3 Cysteine desulfurase assays. Nonreducing reactions involving cysteine desulfurase (CD), sulfur acceptor (SA), and the substrate cysteine (Cys) lead to the production of Ala, protein persulfides (SA-SSH and CD-SSH), and polysulfide (S_8). For alanine quantification using the Ala-NDA detection method, reaction time points are quenched with TCA. For sulfide quantification using the methylene blue detection method, reaction time points are quenched with NaOH and subsequently neutralized with Tris and incubated with a reducing agent to liberate S^{2-}. Reactions coupled to sulfide production are performed in the presence of a reducing agent generating sulfide during the course of the reaction. Reactions coupled to Fe-S cluster assembly are performed in the presence of Fe^{2+} and, when relevant, assay reactions include the presence of a scaffold and the final apo-protein. Fe-S clusters, free and/or associated, and S^{2-} generated in these reactions can be directly quantified through the methylene blue method

the reaction. Then readjust the pH by adding 200 μL of 1 M Tris buffer pH 8.0. Reduce persulfides and polysulfides by adding 3 μL of 1 M DTT solution prior to sulfide quantification (*see* Subheading 3.2 for detection method).

4. For Ala quantification, at each time point, take a 100 μL aliquot of the reaction and mix with 20 μL TCA solution to stop the reaction and proceed with Ala quantification (*see* Subheading 3.3 for detection method).
5. When characterizing biphasic reaction profiles, repeat **steps 1–4** using different equivalents of sulfur acceptors (e.g., 20 and 40 equivalents of sulfur acceptor in relation to cysteine desulfurase concentration).

3.1.2 Cysteine Desulfurase Assays Coupled with Sulfide Production

Assays performed in the presence of reducing agents lead to the direct formation of sulfides in solution (Fig. 3). Reducing agents are able to react with persulfides formed within the cysteine desulfurase and sulfur acceptors. This reaction setup is distinct from the setup of assays performed under nonreducing conditions, as it leads to regeneration of the sulfur acceptor and, in some cases, the cysteine desulfurase [21]. Under reducing conditions, persulfides formed within the cysteine desulfurase and sulfur acceptor are released into the solution in the form of sulfide [41, 42, 44]. It is important to consider that the involvement of some sulfur acceptor molecules may be obscured in reactions performed in the presence

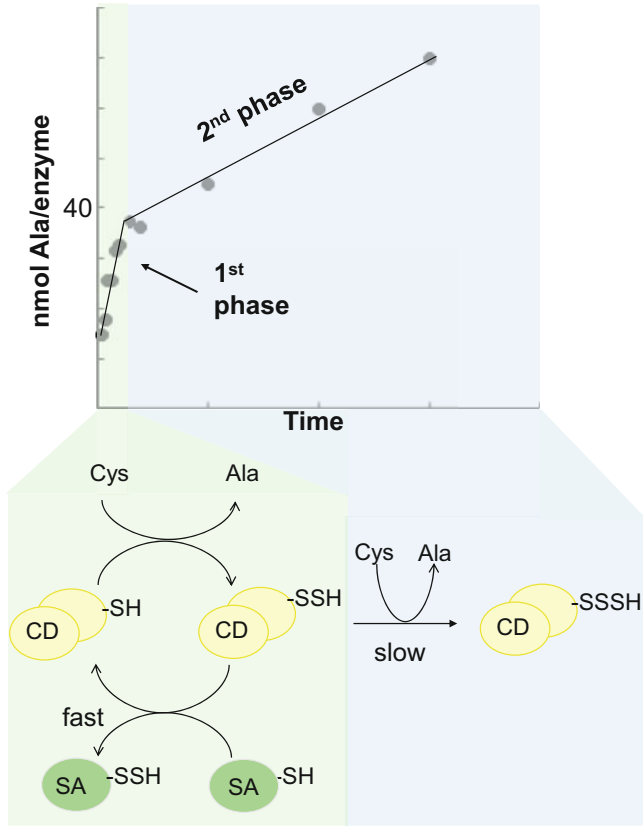

Fig. 4 Biphasic cysteine desulfurase reaction profile in the presence of a sulfur acceptor under nonreducing conditions. A rapid formation of alanine is observed in the presence of a sulfur acceptor (first phase; shaded in green). This burst is caused by a swift catalytic turnover of the cysteine desulfurase and is proportional to the equivalents of sulfur acceptor used in the reaction. After all the sulfur acceptor is persulfurated, the cysteine desulfurase slowly forms polysulfides and, ultimately, colloidal sulfur (second phase; shaded in blue). This data was obtained from the kinetic analysis of E. coli CsdA:CsdE:40CsdL sulfur transfer reaction

of artificial reductants. These agents may directly interact with the enzyme, effectively bypassing the sulfur transfer event of the second half of the reaction. Reducing reactions are performed following the standard protocol:

1. Mix 0.2 μM of cysteine desulfurase, 2 μM of the sulfur acceptor in MOPS buffer in the presence of 2 mM of DTT, and start the reaction by adding 0.5 mM of cysteine (*see* **Notes 3** and **4**).
2. Take a range of time points for an accurate rate determination similar to Subheading 3.1.1, **step 2**.
3. For sulfide quantification, at each time point, take 800 μL aliquot and mix with 25 μL of NaOH solution to stop the

reaction and proceed directly with sulfide quantification (*see* Subheading 3.2 for detection method).

4. For Ala quantification, at each time point, take a 100 μL aliquot and mix with 20 μL of TCA solution to stop the reaction and proceed with Ala quantification (*see* Subheading 3.3 for detection method).

3.1.3 Sulfur Mobilization Assays Coupled to the Fe-S Cluster Assembly

Cysteine desulfurase assays coupled to cluster assembly reactions are conducted under anaerobic conditions as Fe-S clusters are easily degraded upon oxygen exposure. The synthesis of Fe-S clusters onto a scaffold protein requires a source of sulfur (provided by a cysteine desulfurase), iron, and electrons [26, 42, 43]. In addition to promoting the assembly of Fe-S species, scaffold proteins are able to accumulate persulfides and polysulfides, which need to be reduced before quantification of the total sulfide mobilized. Conversely, sulfur associated with Fe-S clusters, also known as acid-labile sulfur, along with free sulfides, can be directly detected via the methylene blue method [47]. In cluster assembly reactions where the scaffold protein is not completely apo, the acid-labile sulfur from the cluster must be quantified and taken into account when analyzing data. Sulfur transfer reactions coupled to the synthesis of Fe-S clusters follow this typical protocol:

1. Set up all reaction components in the anaerobic glove box. Transfer the buffer, water, pipette tips, vials, and other glassware the day before the experiments. Prepare the cysteine and iron solutions freshly inside the glove box on the day of the experiment. Transfer the proteins from the freezer directly into the glove box (*see* **Note 2**).
2. Set up an Fe-S cluster assembly reaction by mixing 0.1 μM of cysteine desulfurase, 2 μM of sulfur acceptor, 20 μM of Fe solution in MOPS buffer in the presence of 2 mM of DTT, and start the reaction by adding 0.5 mM of cysteine (*see* **Note 5**).
3. Take a range of time points for an accurate rate determination while samples remain in the glove box (*see* **Note 6**).
4. For sulfide quantification, at each time point, take 800 μL aliquot and mix with 25 μL of NaOH solution to stop the reaction and proceed directly with sulfide quantification (*see* Subheading 3.2 for detection method).
5. For Ala quantification, at each time point, take a 100 μL aliquot and mix with 20 μL of TCA solution to stop the reaction and proceed with Ala quantification (*see* Subheading 3.3 for detection method).

3.2 Sulfide Detection Method

Sulfide is produced upon reducing persulfides or acid treatment of Fe-S clusters. It can be quantified in colorimetric assays that couple

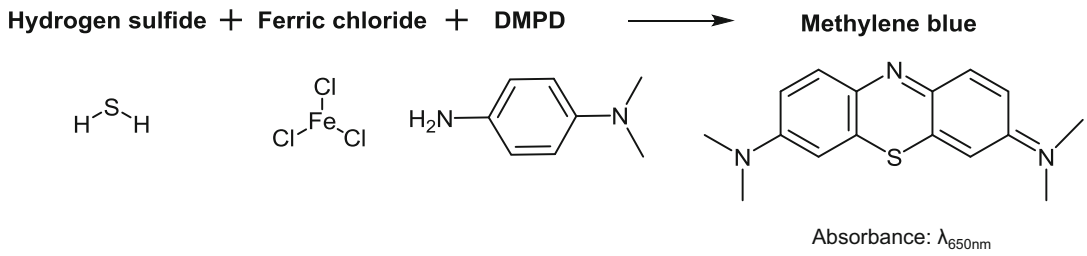

Fig. 5 Methylene blue reaction. Sulfide liberated during the acid treatment of Fe-S clusters or reduction of persulfide bound to cysteine desulfurase/sulfur acceptor can be detected via methylene blue formation

sulfide release to the formation of methylene blue [48] (Fig. 5). In reactions performed in the presence of reducing agents, persulfides formed within cysteine desulfurases and sulfur acceptors are directly reduced, releasing free sulfide. In reactions performed in the absence of reducing agents, mobilized sulfur in the form of persulfide and polysulfide can be quantified by stopping reaction time points and reducing these samples prior to quantification of total mobilized sulfur (Fig. 3). The rates of sulfide production are calculated by the slopes of at least three reaction time points. Methylene blue formed in sulfur transfer reactions can be detected in a standard spectrophotometer (Subheading 3.2.1) or plate reader (Subheading 3.2.2).

3.2.1 *Cuvette Assay*

1. Select each reaction time point containing 800 μL aliquot and 25 μL of NaOH, and add 50 μL of ZnOAc. Close the vials with a stopper (*see* **Note 7**).
2. Once all the time points have been collected, develop the reaction. Using a syringe, add 100 μL of DMPD and 100 μL of $FeCl_3$ to each vial and invert a few times.
3. Allow the reaction to proceed for 20 min to complete formation of methylene blue. Centrifuge individual reaction times points if the reaction is cloudy and read the absorbance at 650 nm with a spectrophotometer (*see* **Note 8**).
4. Prepare a standard curve of sodium sulfide under the same conditions to quantify the amount of sulfide produced in the reaction at each time point. A linear standard curve ranges from 5 to 100 nmol of sulfide (*see* **Note 9**).

3.2.2 *Plate Assay*

The volume of reagents, especially proteins, required for the cuvette assay is quite large and not always feasible. This detection method can be adapted to smaller reaction volumes in a clear 96-well plate.

1. Using a 96-well plate, stop each 200 μL reaction time point with 6.25 μL of the NaOH solution and 12.5 μL of the ZnOAc solution.

2. Once all time points are collected, seal the wells containing reaction time points with parafilm (*see* **Note 7**).
3. Using a syringe, add 25 µL of DMPD and 25 µL of $FeCl_3$, and reseal with parafilm after adding each reagent.
4. Carefully mix the reactions and allow methylene blue to form for 20 min.
5. If the sample is cloudy, centrifuge each time point for 5 min and pipette 200 µL of developed reaction into another clean, adjacent well. Determine the absorbance at 650 nm using a microplate reader (*see* **Note 8**).
6. Prepare a standard curve of sodium sulfide under the same conditions and use it as a reference. A linear standard curve ranges from 2 to 50 nmol of sulfide (*see* **Note 9**).

3.3 Alanine-NDA Detection Method

The reaction profile of cysteine desulfurases can be probed by quantifying the rate of alanine production determined via an NDA-derivatization reaction that forms a fluorescent product [23, 30, 38, 49, 50](Fig. 6). This detection method is not affected by the presence of reducing agents. Unlike the sulfide assay, this assay can be performed under nonreducing conditions or used to develop reactions performed with any reducing agent, including TCEP. Reaction time points are quenched with TCA and subsequently reacted with cyanide and NDA under basic conditions. Under these conditions, the primary amine of alanine reacts with NDA to form a fluorescent 1-cyano-2-alkyl-benz[f]isoindole adduct (*see* **Note 10**). This detection method is typically employed with HPLC separation of NDA-adducts coupled to fluorescence detection (Subheading 3.3.1). This method is very sensitive; as low as 0.1 pmol of alanine can be detected in a sample read.

3.3.1 NDA Assay with HPLC-Fluorescence Detection

1. Prepare reaction time points containing 100 µL of reaction aliquot and stop with 20 µL of 10% TCA, as described in Subheading 3.1 (*see* **Note 9**).

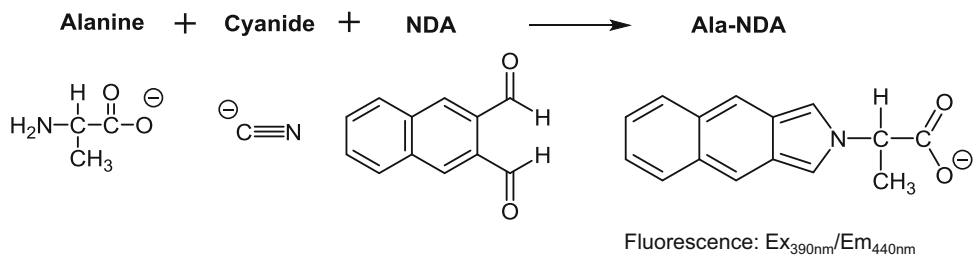

Fig. 6 Ala-NDA derivatization reaction. The alanine produced in the first step of a cysteine desulfurase-catalyzed reaction can be quantified through fluorescence detection of Ala-NDA adduct

2. Once all reaction time points are quenched, spin tubes for 5 min and transfer 100 μL of supernatant into HPLC vials (*see* **Note 1**).
3. Add 1 mL of freshly prepared NDA mix (0.1 mM NDA and 1 mM KCN in 100 mM borate buffer pH 9) and incubate vials in the dark for 1 h (*see* **Notes 11–13**).
4. Inject 10 μL of each sample onto a C-18 column connected to an HPLC coupled to a fluorescence detector (Ex_{390nm}/Em_{440nm}) at a flow rate of 0.5 mL/min. Run a gradient of 0–100% B over 12 min using 5% water/MeOH (Solvent B) and 5% MeOH/water (Solvent A). The Ala-NDA adduct elutes at 8 min.
5. Prepare a calibration curve of alanine under the same conditions to quantify the integrated fluorescence peak area of reaction time points. A linear standard curve ranges from 1 to 50 pmol of Ala-NDA adduct injected in each HPLC run.

3.3.2 NDA Assay with Direct Fluorescence Detection

The alanine vial assay protocol can be adapted for direct quantification using a black 96-well plate and plate reader fluorimeter. In this method layout, the background signal is significantly higher; as a consequence, this method is about ten times less sensitive than the HPLC method (Subheading 3.2.1). For direct fluorescence detection, the standard curve and the sample must be derivatized and read simultaneously as the fluorescence levels change with time of incubation.

1. Prepare reaction time points containing 50 μL of reaction aliquot stopped with 5 μL of 10% TCA and mix gently by pipetting up and down.
2. Once all reaction time points are completed, set up a standard curve of alanine under the same conditions (*see* **Note 14**).
3. Add 200 μL of freshly prepared NDA mix to each sample and standard to allow the Ala-NDA to form.
4. Allow the reaction to develop in the dark for an hour before measuring the fluorescence at Ex_{390nm}/Em_{440nm} in a microplate fluorescence reader.

4 Notes

1. For the detection of alanine, it is important for enzymes to be isolated in a buffer that does not contain a primary amine since these functional groups also react with NDA thereby interfering with the derivatization reaction. For the detection of sulfide, other buffers can be used, including Tris-HCl, phosphate, bicine, and HEPES. An important variant is the pH of reaction

assays. The reaction profile is dependent on pH with a defined pK_a and varies with the enzyme of study. The maximum activity of the *B. subtilis* SufS, YrvO, and NifZ is achieved at pH 8.

2. Experiments involving sulfur transfer reactions coupled to the assembly of Fe-S clusters require conditions devoid of oxygen. In these experiments, proteins are purified anaerobically. The frozen aliquots may be thawed under argon or nitrogen bubbling gas or directly transferred to the anaerobic chamber for Fe-S synthesis assays. The buffers should be degassed for at least 30 min while stirring with alternate sparging of nitrogen or argon gas cycles. Iron and cysteine solutions should be prepared inside the glove box on the day of the experiment.
3. For the method described, the activity of the *B. subtilis* SufS containing 10 equivalents of SufU in the presence of 2 mM DTT and 0.5 mM cysteine at pH 8 is 13 min^{-1} (280 nmol/min/mg). However, different cysteine desulfurases display distinct catalytic rates varying from 0.01 to 100 min^{-1}. Their turnover rate is dependent on the presence and concentration of sulfur acceptors, type of reducing agent, pH, and concentration of cysteine. Thus, the amount of enzyme used in the assay needs to take into consideration the activity of the enzyme and the sensitivity of the detection method used.
4. Reagents such as buffer, DTT, iron, and glutathione interfere with methylene blue quantification. Tris (2-carboxyethyl) phosphine (TCEP) reduces methylene blue and cannot be used as a reducing agent in sulfide assays. Physiological reducing agents, such as the thioredoxin/thioredoxin reductase system, provide a controlled physiological reductant system for effective reduction of persulfides.
5. The quantity of each reagent can be adjusted based on the reactivity of the cysteine desulfurase. To limit the formation of free Fe-S species, limit the concentration of Fe to no more than 10 molar equivalents of the scaffold present in the reaction.
6. For anaerobic assays, reactions can be developed outside the anaerobic chamber.
7. The $FeCl_3$ and DPMD solutions are acidic and lead to the formation of hydrogen sulfide gas. Thus, the formation of methylene blue in reaction time points must be performed in closed reaction containers or wells sealed with parafilm to prevent loss of this analyte.
8. Quenching the reaction with NaOH solution along with developing reactants leads to protein precipitation. For assays performed with higher protein concentrations, the sample may become cloudy, interfering with absorbance readings. In

those cases, centrifuge the samples for 5 min and use the supernatant for the absorbance measurements.

9. Prepare the standard curve with the same buffer, reducing agent, and the same cysteine concentration used in the assay. These components affect the absorbance of methylene blue.
10. NDA generally reacts with primary amines. Thus, buffers that have primary amines, such as Tris, should be avoided. Though cysteine has a primary amine, reaction with NDA results in an unstable adduct with lower fluorescence intensity.
11. Centrifuge reaction time points and transfer the supernatant into the vial before developing to prevent clogging the HPLC column.
12. NDA and KCN solutions can be prepared once every week, but the NDA mix should be prepared right before the derivatization reaction.
13. The fluorescence of the Ala-NDA adduct decays after 4 h, and hence, reaction time points must be developed accordingly. While several assays can be carried simultaneously, reaction with the NDA mix can be spaced based on the HPLC running time.
14. When performing assays using different concentrations of Cys, complete Ala standard curve using solutions containing the same concentration of Cys used in the assays.

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