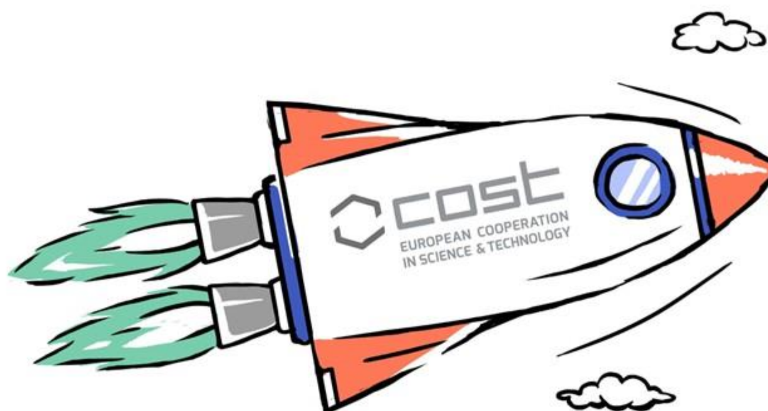

COST Action CA21115

Report date: November 2024

WG4. Mechanistic Studies of FeS Chemistry

General description: This report describes the protocols developed and used by Action members for overproduction of various FeS enzymes to study their mechanism of action

Period: Nov 2022 – Nov 2024

Section 1. General protocol used for expression and purification of iron-sulfur proteins

Over expression and purification of Fe-S cluster proteins.
Plasmids are generally purchased from Genscript. C-terminally His-tagged versions are often useful for ease of purification and for native mass spectrometry measurements. The His-tag does not interfere with the assembly of the cluster. Proteins are over-produced in *E. coli* BL21DE3 (New England Biolabs Inc). Briefly, bacterial cultures are grown at 37 °C, in LB broth supplemented with the appropriate antibiotic, until $A_{600\ nm}$ reaches 0.6 - 0.8. Cultures are then cold shocked on ice for 18 min. Protein overproduction is initiated by the addition of 10 μM isopropyl β-D-thiogalactopyranoside and cultures incubated at 30 °C. After 50 min, cultures are supplemented with 200 μM ferric ammonium citrate, 50 μM L-methionine and incubated for a further 3.5 hr. Cells are harvested by centrifugation and stored at -80 °C until needed.

Protein purification and handling is carried out under strictly anaerobic conditions (O_2 ≤ 2 ppm) in anaerobic glove box (MBraun or Belle Technology) unless otherwise stated. Briefly, cells are resuspended in anaerobic buffer and disrupted by sonication outside of the glovebox. The cell lysate is clarified by centrifugation in sealed anaerobic tubes outside of the glovebox, 40,000 × g for 45 min at 1 °C. The supernatant is loaded on to a HiTrap Ni^{2+} chelating column (2 × 5 ml; Cytiva) previously equilibrated with buffer, and washed with 5% (v/v) of an equivalent buffer containing 200 mM imidazole. Bound proteins are eluted using a linear gradient from 5% to 50% (v/v) of the imidazole-containing buffer. Fractions containing the Fe-S protein are pooled and loaded directly onto a HiTrap Heparin HP column (2 × 1 ml; Cytiva, washed and eluted with buffer containing 2 M NaCl. Fe-S protein-containing fractions are pooled and stored in an anaerobic freezer until needed. The [4Fe-4S] cluster-containing protein concentration is determined using an appropriate extinction coefficient and apo-protein concentration determined using a colorimetric method (eg that of Smith (Pierce).

In vitro reconstitution of Fe-S proteins, including isotope labelling.
Sample preparation

1. Protein solution(s) to be analyzed, at the highest purity possible, or associated equipment for the in-house preparation of high purity protein samples [1,2].
2. Stable isotopes; Iron-57 (≥ 95%) and sulfur-34 (≥ 98%)(Cambridge Isotope Laboratories, Inc).
3. L-cysteine, ferrous ammonium sulfate, DL-dithiothreitol.
4. Ammonium acetate (≥ 98%).
5. Ammonium hydroxide solution (≥99%).
6. Glacial acetic acid (≥99%).
7. Zeba^{TM} Spin desalting column, 7K MWCO (Thermo Scientific).
8. PD-10 desalting columns, with Sephadex G-25 resin, 5K MWCO (Cytiva).
9. PD MiniTrap G-25, 5K MWCO (Cytiva).
10. LC-MS grade water, ≤ 50 ppb common metal ions, 0.2 μm filtered.
11. LC-MS grade formic acid, ≤ 0.5 ppm common metal ions.
12. LC-MS grade acetonitrile (≥99%), ≤ 100 ppb common metal ions.
13. A suitable reversed phased column.
14. Microcentrifuge.

Protocol

While it is usually advantageous to combine protein expression with *in vivo* iron-sulfur cluster assembly, *in vitro* reconstitutions can be used where this is not possible. Below is a brief protocol for *in vitro* reconstitution and the preparation of isotopically labeled Fe-S clusters.

1. Transfer previously prepared apo-protein (see [2]) to an anaerobic cabinet and equilibrate.
2. Prepare the following solutions: Solution-1, 50 mM (12.8 mg/mL) L-cysteine, 125 mM (41.7 mg/mL) dithiothreitol. Solution-2, 20 mM (78.5 mg/10 mL) ferrous ammonium sulfate.
3. Add an appropriate aliquot of solution-1 to the apo-protein to give a final concentration of 1 mM L-cysteine and 2.5 mM dithiothreitol. For ^{34}S-labeled clusters, use ^{34}S-L-cysteine or ^{34}S-sulfide (for detailed methodology see [3]).
4. Add an appropriate volume of solution-2 to the apo-protein. For [4Fe-4S] clusters add seven-fold excess of Fe^{2+} ions over the apo-protein. For ^{57}Fe-labeled clusters, use ^{57}Fe dissolved in HCl (for detailed methodology see [3]).
5. To initiate iron-sulfur cluster reconstitution, add purified NifS [4] to a final concentration of 225 nM. Seal the reaction mixture and incubate between 25 and 37 °C for up to 5 hours.
6. Pass the reconstitution mixture through a 0.2 μm syringe filter to remove any particulate matter.
7. Separate the reconstituted protein from low molecular weight reactants using one of two methods. The first, which is ideal for small scale preparations up to 2.5 mL, involves rapid gel filtration using a PD10 column (Cytiva). The second method, which is ideal for larger preparations up to 25 mL, involves the use of an ion exchange column to simultaneously clean and concentrate the reconstituted protein (see [3] for further details). The reconstituted protein is eluted in buffer containing ~1 M NaCl.

Refs

[1] Freibert SA, Weiler BD, Bill E, Pierik AJ, Muhlenhoff U, Lill R (2018) Biochemical reconstitution and spectroscopic analysis of iron-sulfur proteins. Meth Enzymol 599:197-226

[2] Crack JC, Green J, Thomson AJ, Le Brun NE (2014) Techniques for the production, isolation, and analysis of iron-sulfur proteins. Methods Mol Biol 1122:33-48

[3] Crack JC, Stewart MYY, Le Brun NE (2019) Generation of ^{34}S-substituted protein-bound [4Fe-4S] clusters using ^{34}S-L-cysteine. Biol Meth Prot 4:byp015

[4] Zheng L, White RH, Cash VL, Jack RF, Dean DR (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci U S A 90:2754-2758

Section 2. Protocol for purification of NadA

All steps under anaerobic conditions

Buffer preparation:

1. 100 mL lysis buffer 100 mM Tris-HCl, pH 8, 50 mM NaCl.

2. 250 mL Wash buffer 100 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM imidazole

3. 50 mL Elution buffer 100 mM Tris-HCl, pH 8, 50 mM NaCl, 200 mM imidazole

4. PD25 buffer 100 mM Tris-HCl, pH 8, 50 mM NaCl.

Preparation:

1. Thaw all the cells collected inside the glove box at 17 °C using a vortex.

2. Add 1 mL of lysozyme 1mg/mL and 1 mM PMSF to each 10 ml lysed cells, then add lysis buffer to the mixture to 50 mL. Mix well by vortexing.

3. After 45 min incubation inside the glove box, three subsequent freeze-thaw cycles are performed: fast freezing of the cells in liquid nitrogen (outside the glove box) and slow thawing at 17°C (inside the glove box).

4. Transfer in ultracentrifuge tubes.

5. Ultracentrifuge: 40000 rpm, 4^0C, 60 mins.

Purification:

1. Bring back the ultracentrifuge tubes to glovebox. Collect the supernatant very carefully using a pipet.

2. Wash the resin with wash buffer (10 x the volume of the Ni2+ resin).

3. Load the soluble protein extracts onto the Nickel-Nitrilotriacetic Acid /Ni-NTA column (Qiagen, 5 mL).

4. Wash the resin with wash buffer (10 x the volume of the Ni2+ resin). Keep the flow-through

5. Elution of the protein with elution buffer (2x the volume of the resin)

6. Remove imidazole by loading the eluted protein onto a PD25 column using PD25 buffer.

7. Concentrate using Amicon device (cut-off 10 kDa)

8. Freeze the protein with liquid nitrogen and store at -80°C.

Section 3. Protocol for Purification of holo-CISD3 for *in vitro* studies

The CISD3 ORF without the mitochondrial targeting signal peptide (amino acids 37–127) was inserted into a pET28a(+) plasmid. The plasmid was used to transform *E. coli* BL21 (DE3) GOLD competent cells. Cell growth was performed at 37 °C in either LB, for nonlabeled protein, or in M9 minimal media supplemented with 1.2 g/L of $(^{15}NH_4)_2SO_4$, 3 g/L of glucose, 4 ml of Q solution (metal mix solution: 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM of each of $CoCl_2$, $CuCl_2$, $NiCl_2$, Na_2MnO_4, Na_2SeO_3, and H_3BO_3). A total of 500 µM Mohr's Salt was added when the cell culture reached an A_{600} of 0.6. At an A_{600} of 0.8 to 1, the temperature was lowered to 18 °C and the protein overexpression was induced with 0.1 mM IPTG. Cells were incubated at 18 °C overnight and centrifuged.

Cells were resuspended in 80 ml of 20 mM Tris–HCl pH 7.5 buffer and lysed by adding CelLytic Reagent (0.8 g/1 L culture) (Merck). After centrifugation, the supernatant was loaded on a 5 ml HiTrap SP FF cationic exchange column. The column was washed with a linear NaCl gradient (0–500 mM) until CISD3 protein solution eluted with 20 mM Tris–HCl pH 7.5300 mM NaCl. The fractions containing the protein were collected, concentrated, and injected in Superdex 200 Increase 16/60 75 pg previously equilibrated with Tris–HCl 50 mM NaCl 150 mM pH 7.5 buffer. All the buffers used for purification were rendered oxygen-depleted by introducing a nitrogen flux into the solution. The iron content of the reduced CISD3, determined following a procedure reported in the articles (78–81) is consistent with an 85% metallated protein (∼1.70 $[Fe_2S_2]^{2+}$ clusters per CISD3 protein).

Publication: Biochemical and cellular characterization of the CISD3 protein: Molecular bases of cluster release and destabilizing effects of nitric oxide,

Journal of Biological Chemistry 100, 105745

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