Identification of persulfide-binding and disulfide-forming cysteine residues in the NifS-like domain of the molybdenum cofactor sulfurase ABA3 by cysteine-scanning mutagenesis

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The Moco (molybdenum cofactor) sulfurase ABA3 from Arabidopsis thaliana catalyses the sulfuration of the Moco of aldehyde oxidase and xanthine oxidoreductase, which represents the final activation step of these enzymes. ABA3 consists of an N-terminal NifS-like domain that exhibits L-cysteine desulfurase activity and a C-terminal domain that binds sulfurated Moco. The strictly conserved Cys430 in the NifS-like domain binds a persulfide intermediate, which is abstracted from the substrate L-cysteine and finally needs to be transferred to the Moco of aldehyde oxidase and xanthine oxidoreductase. In addition to Cys430, another eight cysteine residues are located in the NifS-like domain, with two of them being highly conserved among Moco sulfurase proteins and, at the same time, being in close proximity to Cys430. By determination of the number of surface-exposed cysteine residues and the number of persulfide-binding cysteine residues in combination with the sequential substitution of each of the nine cysteine residues, a second persulfide-binding cysteine residue, Cys506, was identified. Furthermore, the active-site Cys430 was found to be located on top of a loop structure, formed by the two flanking residues Cys428 and Cys455, which are likely to form an intramolecular disulfide bridge. These findings are confirmed by a structural model of the NifS-like domain, which indicates that Cys428 and Cys455 are within disulfide bond distance and that a persulfide transfer from Cys430 to Cys506 is indeed possible.

Key words: ABA3, active-site loop, Arabidopsis thaliana, molybdenum cofactor sulfurase, persulfide.

INTRODUCTION

Molybdenum enzymes catalyse diverse key reactions in the global cycles of carbon, nitrogen and sulfur [1,2]. With the exception of bacterial nitrogenase, all molybdenum enzymes contain the so-called Moco (molybdenum cofactor) in which the molybdenum is co-ordinated by the dithiolene group of a molybdopterin backbone. As determined by the co-ordination chemistry of the molybdenum ligands, eukaryotic molybdenum enzymes were previously divided into two families; enzymes of the sulfite oxidase family are characterized by a Moco, whose molybdenum additionally ligates two oxo ligands and a protein-derived cysteiny1 sulfurl, whereas enzymes of the xanthine oxidase family bind a Moco, whose molybdenum ligates one oxo ligand, a hydroxy group and a terminal sulfo. In higher eukaryotes, sulfite oxidase family are characterized by a Moco, whose molybdenum additionally ligates two oxo ligands and a protein-derived cysteiny1 sulfurl, whereas enzymes of the xanthine oxidase family bind a Moco, whose molybdenum ligates one oxo ligand, a hydroxy group and a terminal sulfo. In higher eukaryotes, sulfite oxidase family are characterized by a Moco, whose molybdenum additionally ligates two oxo ligands and a protein-derived cysteiny1 sulfurl, whereas enzymes of the xanthine oxidase family bind a Moco, whose molybdenum ligates one oxo ligand, a hydroxy group and a terminal sulfo.
number of conserved cysteine residues, it is tempting to speculate that the persulfide, whose generation is dependent at least on Cys\(^{428}\) \cite{9}, is transmitted within the ABA3 protein from the initial persulfide-binding cysteine residue to one or several other cysteines before reaching its final destination, the C-terminally bound Moco. Such a chain of at least two persulfide-binding cysteine residues has been shown previously for other proteins that mobilize sulfur and deliver it to processes such as iron– sulfur cluster biogenesis \cite{13}. It was thus an aim of the present study to investigate the role of cysteine residues in the N-terminal cysteine desulfurase domain of ABA3, with particular focus on the characterization of the active site with its conserved Cys\(^{428}\), Cys\(^{430}\) and Cys\(^{435}\) and the identification of other cysteine residues that are possibly involved in intramolecular persulfide transport.

**EXPERIMENTAL**

**Expression and purification of recombinant ABA3-NifS**

Standard expression of ABA3-NifS and its cysteine variants was performed using the pQE80 plasmid (Qiagen) in freshly transformed Escherichia coli DL41 cells essentially as described previously \cite{9}. Cells were grown aerobically in LB (Luria–Bertani) medium in the presence of 50 \(\mu\)g/ml ampicillin at 22 \(^\circ\)C to a \(\text{OD}_{600}\) of 1.0 before induction. Expression of the respective protein was induced with 50 \(\mu\)M isopropyl \(\beta\)-D-thiogalactopyranoside and 0.17 \(\mu\)g of pyridoxin per 1 litre of expression culture was added to support synthesis of the PLP cofactor. After culturing for 20 h at 24 \(^\circ\)C, cells were harvested by centrifugation at 11000 \(g\) and 4 \(^\circ\)C for 5 min and stored at –70 \(^\circ\)C until use. Cell lysis was achieved by passing through a French pressure cell followed by sonication for 5 min on ice. After centrifugation at 45000 \(g\) for 45 min at 4 \(^\circ\)C, His\(_6\)-tagged proteins were purified on a Ni\(^{2+}\)-nitriiotriacetate superflow matrix (Qiagen) under native conditions at 4 \(^\circ\)C in buffers of pH 9.3 according to the manufacturer’s instructions and eluted in elution buffer (50 mM sodium phosphate, pH 9.3, containing 300 mM NaCl, 250 mM imidazole and 10% glycerol). After purification by affinity chromatography, the recombinant NifS-like domain of ABA3 was characterized by an average specific cysteine desulfurase activity of 8.7 \(\pm\) 1.1 mol · min\(^{-1}\) · mol\(^{-1}\) and a PLP saturation of 100%.  

**Determination of protein concentrations**

Concentrations of total soluble protein were determined either by using Roti\(^\text{®}-\text{Quant solution (Roth)}\) as described in \cite{14} or by using the absorbance of proteins at 280 nm and the molar absorption coefficient of 57800 M\(^{-1}\) · cm\(^{-1}\) for ABA3-NifS as determined by the ProtParam program (http://br.expasy.org).

**Determination of the PLP content of ABA3-NifS proteins**

To determine the content of protein-bound PLP, the PLP-specific absorption at 420 nm was measured in preparations of ABA3-NifS and its variants and compared with a PLP standard curve (0–350 \(\mu\)M PLP)

**Standard SDS/PAGE**

SDS/PAGE was carried out as described in \cite{15}, using a 5% stacking gel and 12% separating gels. Staining of electrophoresed proteins was performed in the presence of Coomassie Brilliant Blue R250 (Serva). Molecular mass standards and protein samples were pretreated with 2-mercaptoethanol for 5 min at 95 \(^\circ\)C prior to loading on to the gel.

**Molecular mass determination**

After purification of the recombinant proteins by affinity chromatography, size-exclusion chromatography was performed using an ÄKTA Basic system and an analytical Superdex\(^\text{TM}200-10/30\) column (GE Healthcare) to determine the molecular mass of the native proteins. The column was equilibrated in PBS prior to separation of 200 \(\mu\)g of the respective ABA3-NifS variant at a flow rate of 0.4 ml/min. The molecular mass was determined using a calibration curve obtained from the retention times of standard proteins (aldolase, 161 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; and RNase A, 13.7 kDa). Each time, \(V_o\) was determined with Blue Dextran 2000 (2000 kDa).

**Preparation of persulfide-free and persulfide-loaded proteins**

Potentially bound persulfides were released from ABA3-NifS by treatment with 5 mM DTT (dithiothreitol) for 2 h at 4 \(^\circ\)C in 0.1 M Tris/HCl buffer, pH 9.3. After incubation, ABA3-NifS was rebuffered to 0.1 M Tris/HCl buffer, pH 9.3, on Sephadex G-50 Nick columns (GE Healthcare), and small amounts of denatured protein were removed by centrifugation at 22000 \(g\) and 4 \(^\circ\)C for 15 min. Preparation of persulfide-saturated ABA3-NifS was achieved by incubation of ABA3-NifS in 0.1 M Tris/HCl buffer, pH 9.3, with 0.5 mM L-cysteine for 30–60 min at room temperature (23 \(^\circ\)C). Subsequently, excess L-cysteine was removed by rebuffering the protein sample to 0.1 M Tris/HCl buffer, pH 9.3, on Sephadex G-50 Nick columns.

**Determination of cysteine desulfurase activity**

Cysteine desulfurase proteins release L-alanine during the decomposition of L-cysteine. In the presence of NAD\(^{+}\), L-alanine is subsequently converted into pyruvate by alanine dehydrogenase with concomitant formation of NADH. NADH is thus generated linearly from L-alanine and therefore from L-cysteine. For a standard determination of cysteine desulfurase activity, 15 \(\mu\)g of the respective ABA3-NifS protein was used in a total volume of 360 \(\mu\)l of 0.1 M Tris/HCl, pH 9.3, containing 25 mM DTT. The reaction was started by addition of 40 \(\mu\)l of a 10 mM L-cysteine solution (final concentration 1 mM) and the reaction mixture was incubated with shaking for 30 min at 37 \(^\circ\)C. The reaction was stopped by heat shock at 72 \(^\circ\)C for 10 min and samples were subsequently stored on ice. Reference samples of identical composition were stopped immediately after addition of the substrate L-cysteine. For quantification of L-alanine in the reference samples or as formed during decomposition of L-cysteine by ABA3-NifS, 100 \(\mu\)l of a 1:1 mixture (50 \(\mu\)l of each) of 0.2 unit of alanine dehydrogenase from Bacillus subtilis (Sigma) and 0.1 M NAD\(^{+}\) in 0.1 M Tris/HCl, pH 9.3, was added to the reaction mixture and incubated for 80 min at 37 \(^\circ\)C. After incubation, the reaction mixture was centrifuged at 11000 \(g\) for 5 min to spin down the precipitates that eventually formed. The supernatant was used to measure the absorbance of NADH at 340 nm. To minimize calculation mistakes due to the absorbance of possible NADH contaminations, the absorbance of the reference samples was subtracted from that of the incubated samples. A standard curve was obtained from samples containing different concentrations of L-alanine (0, 0.05, 0.1, 0.15, 0.2 and 0.25 mM) in 400 \(\mu\)l of 0.1 M Tris/HCl, pH 9.3. By using this
improved assay, the determination of background activities, as had been required in our Methylene Blue assay described previously [9], was fully avoided.

**Analysis of protein-bound persulfides by the 1,5-I-AEDANS (N-iodoacetyl-N′-(5-sulfo-1-naphthyl)-ethylendiamine) gel assay**

The basic principle of the identification of protein-bound persulfides by using 1,5-I-AEDANS has been described previously by Zheng et al. [5]. For identification of persulfides bound to ABA3-NifS, 20–30 μg of the respective protein, either persulfide-loaded (i.e. L-cysteine-treated) or persulfide-free (i.e. DTT-treated) in 13.5 μl of 0.1 M Tris/HCl, pH 9.3, were supplemented with 0.5 μl of a 0.5 mM 1,5-I-AEDANS solution and incubated for 1–2 h at 4°C. It is noteworthy that the reaction mixture must not contain molecules that are known to react with 1,5-I-AEDANS (e.g. DTT, 2-mercaptoethanol, L-cysteine or imidazole respectively). After incubation, unbound 1,5-I-AEDANS was allowed to react with L-cysteine (1 μl of a 4 mM stock solution) for 30 min at room temperature to prevent reaction of 1,5-I-AEDANS in subsequent reaction steps. To release possibly bound persulfides as 1,5-I-AEDANS–persulfide conjugates from the protein, 1 μl of a 100 mM DTT solution was added to the reaction mixture. Subsequently, the reaction mixture was supplemented with 2 μl of 10× native loading buffer (2 M sucrose and 1% Bromophenol Blue) and electrophoresed for 45 min at 200 V on 12% SDS/polyacrylamide gels (adjusted to pH 9.5 with 4 M NaOH to improve separation of the different 1,5-I-AEDANS reaction products). By exposure to UV light, 1,5-I-AEDANS reaction products such as 1,5-I-AEDANS-labelled persulfides, 1,5-I-AEDANS-labelled proteins and excess 1,5-I-AEDANS bound by L-cysteine were visualized. After exposure to UV light, the same gel was stained with Coomassie Brilliant Blue to allow comparison of protein amounts.

**Determination of surface-exposed cysteines by treatment with N-EM (N-ethylmaleimide)**

Like 1,5-I-AEDANS, N-EM is a thiol-specific agent that alkylates accessible protein thiols in the neutral pH range, which was used in the present study to irreversibly prevent persulfide binding to surface-exposed cysteines. Accordingly, persulfide-free proteins in 50 mM sodium phosphate buffer, pH 7.0, were used as starting material. For each sample, 1–3 nmol of the respective ABA3-NifS protein was incubated in a total volume of 40 μl of 50 mM sodium phosphate buffer, pH 7.0, overnight at 22°C in the presence of precisely defined amounts of N-EM (0–10-fold molar excess of N-EM from a 0.3 M stock solution in ethanol). After overnight incubation and pelleting of precipitated protein by centrifugation (at 21000 g), the desired amount of protein was used either for determination of cysteine desulfurase activity or for 1,5-I-AEDANS gel assays.

**Quantification of persulfides by cyanide treatment**

For quantification of persulfides bound by ABA3-NifS, each sample contained 8 nmol of the respective persulfide-saturated protein in 150 μl of 0.1 M Tris/acetate, pH 8.6 (due to the low sensitivity of the assay, the expected yield of persulfide should exceed 35 μM). After the addition of 15 μl of a 0.5 M potassium cyanide solution, the reaction mixtures were incubated overnight at 22°C and subsequently centrifuged at 4°C at 21000 g to remove precipitated proteins. Formed thiocyanate was separated from the remaining protein with a 10 kDa MWCO (molecular-mass cut-off) Vivaspin concentrator (Sartorius) by centrifugation at 11000 g for 30 min. A 110 μl volume of the flow-through was mixed with 110 μl of a solution consisting of 10 g of Fe(NO₃)₃·9H₂O and 20 ml of 65% HNO₃ per 150 ml and was incubated at room temperature for 1 min, during which time the reddish iron–cyanate complex was formed. Finally, samples were centrifuged at 21000 g for 2 min and the clear supernatant was used to measure the absorbance of the iron–cyanate complex at 460 nm. Comparison of the absorbances of protein samples with a thiocyanate standard curve enabled quantification of protein-bound persulfides.

**Preparation of ABA3-NifS for MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight)-MS analysis**

For identification of surface-exposed cysteines by MALDI–TOF-MS analysis, 75 μg of the respective persulfide-free ABA3-NifS variant in 54 μl of 0.1 M sodium phosphate buffer containing 200 mM NaCl, pH 8.0, was treated with 13.5 μl of a 50 mM 4-vinylpyridine solution and incubated for 1 h at 4°C. Unbound 4-vinylpyridine was allowed to react with 7.5 μl of a 1 M solution of L-cysteine for 30 min at room temperature. A 15 μl portion of each sample was supplemented with 5 μl of SDS loading buffer (50% glycerol, 3.5% SDS, 15% 2-mercaptoethanol and 0.02% Bromphenol Blue), electrophoresed by SDS/PAGE (12% gels) and stained with Coomassie Brilliant Blue to visualize the proteins of interest. Subsequently, the proteins of interest were excised from the gel and stored at −20°C prior to tryptic digestion, extraction and MALDI–TOF-MS analysis.

**ITC (isothermal titration calorimetry)**

All ITC experiments were performed using a VP-ITC isothermal titration calorimeter (MicroCal) at 23°C and 5 μcal/s. The proteins used were dialysed overnight at 4°C against 0.1 M sodium phosphate buffer, pH 9.3, and a 0.5 mM L-cysteine solution was prepared in identical buffer. Both L-cysteine and proteins were degassed for 15 min immediately before the experiment. Each experiment contained 20 μM of the respective ABA3-NifS protein and was performed with 50 injections of L-cysteine (20×2 μl, 20×5 μl and 10×10 μl) at intervals of 240 s.

**RESULTS**

Identification of surface-exposed cysteines in ABA3-NifS

Our previous work on ABA3-NifS has demonstrated that at least Cys430 is crucial for the decomposition of the substrate L-cysteine and for formation of a persulfide intermediate [9]. However, since this persulfide has to be transmitted to the Moco bound to the C-terminal domain of ABA3, other cysteine residues may be involved in the intramolecular sulfur transfer as well. It was therefore an aim of the present study to determine the number of cysteine residues in ABA3-NifS that are exposed on the surface of the protein and which could thus serve as additional persulfide-binding residues. For this purpose, the thiol-specific alkylating agent N-EM was used to bind to surface-exposed cysteine thiols of ABA3-NifS, which consequently caused the inability of the respective cysteines to bind a persulfide. In fact, addition of a 2-fold molar excess of N-EM to purified ABA3-NifS resulted in the total loss of cysteine desulfurase activity, indicating that two surface-exposed cysteine residues of ABA3-NifS were required for full activity (Figure 1). This was confirmed by another experiment in which an excess of
the thiol-specific alkylating and fluorescing agent 1,5-I-AEDANS was added to the N-EM-treated protein (see Supplementary Figure S1A at http://www.BiochemJ.org/bj/441/bj4410823add.htm). In samples treated with ≥2.0 mol of N-EM per mol of ABA3-NifS, all surface-exposed cysteines were blocked as indicated by the inability of 1,5-I-AEDANS to bind to ABA3-NifS.

Identification of persulfide-binding cysteines in ABA3-NifS

To study the persulfide-binding capacity of ABA3-NifS, an assay system was developed on the basis of the fact that 1,5-I-AEDANS does not discriminate between surface-exposed cysteine thiols (Cys–SH) and persulfides bound to such surface-exposed cysteines (Cys–S–SH). Upon treatment with reducing agents such as DTT, however, the cysteine–persulfide bond is cleaved and the persulfide-bound 1,5-I-AEDANS (1,5-I-AEDANS–SH) is released from the protein. In contrast, 1,5-I-AEDANS cannot be released from the protein as it is bound directly to a cysteine residue. The subsequent separation of these samples on denaturing polyacrylamide gels followed by UV-light exposure allows the identification of 1,5-I-AEDANS-specific fluorescence derived either from protein-bound 1,5-I-AEDANS or from persulfide-bound 1,5-I-AEDANS. As shown in Figure 2 (left-hand panel, left-hand lane), the 1,5-I-AEDANS-specific fluorescence remained bound to ABA3-NifS when the protein was pretreated with DTT and desalted to ensure that it was free from persulfides. In contrast, the 1,5-I-AEDANS-specific fluorescence was removed from ABA3-NifS when the protein was allowed to decompose its substrate L-cysteine and to generate persulfides (Figure 2, left-hand panel, right-hand lane). Although these observations, at first sight, only confirm what has been shown previously, namely that ABA3-NifS principally is able to bind a persulfide [9], the combination of 1,5-I-AEDANS treatment and electrophoretic separation additionally demonstrates that not only a part, but all, of the 1,5-I-AEDANS-specific fluorescence has been removed from the protein. This result therefore demonstrates that each of the two surface-exposed cysteine residues of ABA3-NifS (Figure 1) was loaded with persulfide prior to 1,5-I-AEDANS treatment and reductive cleavage of the 1,5-I-AEDANS–persulfide conjugates. In support of this, removal of ABA3-NifS-bound persulfides by cyanide treatment likewise identified 2 mol of persulfide per mol of ABA3-NifS (Supplementary Figure S1B).

Analysis of the conserved triple-cysteine motif of ABA3-NifS

Although ABA3-NifS possesses nine cysteines, some other cysteine desulfurase proteins, such as Sulfolobus solfataricus NifS, possess only one cysteine residue [16]. This cysteine, which corresponds to Cys430 in ABA3-NifS, is strictly conserved among all bacterial and eukaryotic cysteine desulfurases. Besides Cys430, however, ABA3-NifS possesses another eight cysteines with three of them being highly conserved among Moco sulf saturases, but not being present in other cysteine desulfurases (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410823add.htm). Interestingly, the two conserved cysteine residues at positions 428 and 435 are in close proximity to the putative active-site Cys430.

To study the importance of the conserved triple-cysteine motif of ABA3-NifS for activity and persulfide transfer, each of the three cysteine residues was replaced with alanine, yielding the variants C428A, C430A and C435A. In addition, all possible combinations of double mutants (C428/430A, C428/435A and C430/435A) as well as the common triple mutant (C428/430/435A) were generated. After expression and purification, all of these ABA3-NifS variants showed a structural integrity identical with wild-type ABA3-NifS with respect to yield, purity (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410823add.htm) and PLP content. Yet, in the presence of L-cysteine as substrate, only the control protein and the variants C435A and C428/435A exhibited wild-type-like activity, whereas all other variants were inactive (see Supplementary Figure S4A at http://www.BiochemJ.org/bj/441/bj4410823add.htm). When the variants were co-incubated with L-cysteine to allow persulfide formation, the addition of 1,5-I-AEDANS and the subsequent electrophoretic separation visualized persulfides likewise only at the control protein and the C435A and C428/435A variants (Supplementary Figure S4B). Since exclusively the substitution
Cys435 resulted in fully active protein, whereby an essential and C428/430/435A, the combined substitution of Cys428 and in inactive proteins in the case of the variants C428A, C428/430A indicates the new appearance of a third surface-exposed cysteine. Results are means 3 mol of N-EM per mol of protein are required to abolish the activity of this variant, which was confirmed by the cyanide-based release of 3 mol of persulfide per mol of ABA3-NifS/C435A (results not shown).

Like the active site of the NifS-like domain of the Moco sulfurase ABA3-NifS variants C428A and C428/430A and C428/430/435A required only 1 mol of N-EM per mol of protein to eliminate 1,5-I-AEDANS-specific fluorescence (Supplementary Figures S6A, S6C and S6E). In most experiments, the variant C430A likewise required 1 mol of N-EM to abolish binding of 1,5-I-AEDANS. In some experiments, however, approximately 1.5 mol of N-EM per mol of protein was required to block 1,5-I-AEDANS-specific fluorescence of the C430A variant (Supplementary Figure S6B), indicating that the single mutation of Cys430 causes slight conformational changes. Summarizing the results of these experiments (Table 1), the following statements can be made. (i) Except for the C430/435A variant, substitution of Cys430 resulted in only one surface-exposed cysteine, thereby confirming the importance of Cys430. (ii) The C435A variant provided a third surface-exposed cysteine residue, demonstrating that neither Cys435 nor the newly exposed cysteine were exposed on the surface of ABA3-NifS under normal conditions. In contrast, the C430/435A variant resembled a wild-type situation and presented two surface-exposed cysteines, which must be ascribed to the loss of Cys430 and the simultaneous exposure of a new cysteine to the surface caused by the Cys435 mutation. (iii) A mutation in Cys428 caused loss of one surface-exposed cysteine in the C428A variant, but in combination with mutations in Cys430 (variants C428/430A and C428/430/435A) it did not cause the complete loss of surface-exposed cysteines. Rather, the combined mutation of Cys428 and Cys435 did not cause any changes in the number of surface-exposed cysteines, indicating that Cys428 is not the second surface-exposed cysteine in ABA3-NifS, but is the newly generated surface-exposed cysteine in the C435A variant.

### Analysis of the remaining cysteine residues of ABA3-NifS

To identify the unknown second persulfide-binding cysteine, each of the remaining six cysteine residues of ABA3-NifS was replaced with alanine, yielding the variants C101A, C151A, C206A, C250A, C377A and C456A. In addition, double

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**Table 1** Summary of results obtained for the ABA3-NifS variants of the triple-cysteine motif

<table>
<thead>
<tr>
<th>ABA3-NifS variant</th>
<th>Number of surface-exposed cysteine residues</th>
<th>Cysteine desulfurase activity (mol of alanine/min per mol of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>8.72 ± 1.06</td>
</tr>
<tr>
<td>C428A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C430A</td>
<td>1–2</td>
<td>0</td>
</tr>
<tr>
<td>C435A</td>
<td>3</td>
<td>9.64 ± 1.28</td>
</tr>
<tr>
<td>C428/430A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C428/435A</td>
<td>2</td>
<td>9.56 ± 0.82</td>
</tr>
<tr>
<td>C430/435A</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>C428/430/435A</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Figure 3** Quantification of surface-exposed cysteine residues of the active ABA3-NifS variants C435A and C428/435A

The ABA3-NifS variants C428/435A (A) and C435A (B) were treated with different concentrations of N-EM to provoke the inhibition of cysteine desulfurase activity. In the case of the C428/435A variant (A), the activity is completely inhibited by 2 mol of N-EM per mol of protein, indicating the existence of two surface-exposed cysteine residues. In the case of the C435A variant (B), 3 mol of N-EM per mol of protein are required to abolish the activity of this variant, which indicates the new appearance of a third surface-exposed cysteine. Results are means ± S.D. for three independent experiments.
mutants of these variants were generated containing the C430A substitution to eliminate the initial persulfide. As the variants C206A and C206/430A appeared to be unstable, they were replaced with the variants C206S and C206S/C430A respectively. The basic characterization by CD spectroscopy and size-exclusion chromatography showed that all variants had the same overall structure (see Supplementary Figure S7 at http://www.BiochemJ.org/bj/441/bj4410823add.htm) and were dimers (see Supplementary Table S1 at http://www.BiochemJ.org/bj/441/bj4410823add.htm), like wild-type ABA3-NifS. Moreover, all newly generated ABA3-NifS variants exhibited wild-type-like activity, with the exception of the C206S variant, whose activity was reduced to 18% (see Supplementary Figure S8A at http://www.BiochemJ.org/bj/441/bj4410823add.htm). In contrast, the simultaneous substitution of Cys101 and 4-vinylpyridine to label surface-exposed cysteine of wild-type ABA3-NifS, except for those of the triple-cysteine motif (Cys428, Cys430 and Cys435) was found with exactly one 4-vinylpyridine label, which may indicate that this residue is not directly exposed on the surface of the protein, but is near to it. 

Identification of Cys206 as the second surface-exposed cysteine residue in ABA3-NifS

MALDI–TOF-MS technology was used to identify the unknown surface-exposed cysteine residue of wild-type ABA-NifS. For this purpose, the protein was initially treated with DTT to remove putatively bound persulfides. After removal of DTT and persulfides, ABA3-NifS was co-incubated with the thiol-specific alkylating agent 4-vinylpyridine to label surface-exposed cysteines. Free 4-vinylpyridine was subsequently removed by an excess of L-cysteine prior to denaturing and electrophoresis of the protein on SDS/polyacrylamide gels. The resulting protein bands were excised from the gel, and unlabelled cysteine residues were reduced with DTT and irreversibly alkylated by iodoacetamide to C-carboxamido-methylcysteine. After tryptic digestion, ABA3-NifS-derived peptides were purified and finally subjected to MALDI–TOF-MS analysis. Since each cysteine residue of ABA3-NifS, except for those of the triple-cysteine motif, is separated from other cysteines by trypsin-cleavage sites, 4-vinylpyridine-labelled peptides directly allow the identification of surface-exposed cysteines. In fact, in five independent experiments, the peptide carrying the triple-cysteine motif of Cys206, Cys377 and Cys456 was found with exactly one 4-vinylpyridine label (results summarized in Table 2). Obviously, only one of the three cysteine residues is accessible to 4-vinylpyridine, and the previous experiments suggest that this is Cys456. In support of this, the variant C428/435A, which retained only Cys430 of the triple-cysteine motif, likewise allowed the identification of one 4-vinylpyridine label at this specific peptide, which undoubtedly identifies Cys456 as the surface-exposed cysteine. Interestingly, analysis of the ABA3-NifS variant C430/435A likewise identified one 4-vinylpyridine label at this peptide, which at first appears confusing when considering the identification of Cys377 as the only surface-exposed cysteine of this peptide. However, previous experiments (Figure 3B, Supplementary Figure S5B and Supplementary Figures S6A and S6D) already indicated that Cys377, which is the remaining cysteine residue in the ‘triple-cysteine peptide’ of the C430/435A variant, represents a new surface-exposed cysteine in the absence of Cys435. The peptide carrying Cys206, the mutation of which appeared to be crucial for protein stability, was found to be 4-vinylpyridine-labelled in four out of five experiments, thereby allowing the conclusion that Cys206 represents the second surface-exposed cysteine residue of ABA3-NifS. Peptides that contain Cys101, Cys377 and Cys456 respectively have not been found to be 4-vinylpyridine-labelled in any of the experiments, thereby precluding the possibility of these cysteines being surface-exposed. Only the Cys206-carrying peptide was found twice with a 4-vinylpyridine label, which may indicate that this residue is not directly exposed on the surface of the protein, but is near to it.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of MALDI–TOF-MS analysis of 4-vinylpyridine-labelled ABA3-NifS</th>
</tr>
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<tbody>
<tr>
<td>ABA3-NifS cysteine residue</td>
<td>Results of MALDI–TOF-MS analysis</td>
</tr>
<tr>
<td>Cys101</td>
<td>No label identified</td>
</tr>
<tr>
<td>Cys151</td>
<td>No label identified</td>
</tr>
<tr>
<td>Cys206</td>
<td>Labelled in four of five experiments</td>
</tr>
<tr>
<td>Cys377</td>
<td>No label identified</td>
</tr>
<tr>
<td>Cys430</td>
<td>Labelled in one of one experiment (i.e. surface-exposed in the C430/435A variant)</td>
</tr>
<tr>
<td>Cys435</td>
<td>Labelled in one of one experiment (i.e. surface-exposed in the C428/435A variant)</td>
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<tr>
<td>Cys456</td>
<td>Labelled in two of five experiments</td>
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Cys430 is essential for substrate binding and persulfide formation in the active site of ABA3-NifS

ITC was used to study the interaction between ABA3-NifS and its substrate L-cysteine with regard to the importance of Cys430 for substrate- and persulfide-binding. The ITC technology basically relies on the generation or absorption of heat occurring when two molecules bind to each other. As a consequence, the heat generated or consumed provides a thermodynamic profile of the investigated molecular interaction. An initial observation of the interaction between ABA3-NifS and L-cysteine was that heat is generated only when the ABA3-NifS monomer/L-cysteine ratio does not exceed 1:1. This suggests that the heat released results from the binding of L-cysteine to the protein rather than from the decomposition of L-cysteine for persulfide formation. The latter event would be represented by heat generation up to a ratio of 2 mol of L-cysteine per mol of ABA3-NifS monomer on the basis of the finding that each monomer binds two persulfides derived from two molecules of L-cysteine. Moreover, heat is released when L-cysteine is added to persulfide-free ABA3-NifS (Figure 4A), but not when added to persulfide-saturated ABA3-NifS (Figure 4B), suggesting that
L-cysteine-binding to ABA3-NifS requires the competence of the protein to generate and bind new persulfides. This is supported on the one hand by the finding that DTT treatment of persulfide-saturated ABA3-NifS restored the ability of the protein to bind L-cysteine (represented by the reconstitution of heat generation; Figure 4C), and on the other hand by the observation that binding of L-cysteine to the protein did not occur when the C430A variant of ABA3-NifS was used (represented by the absence of thermodynamic changes; Figure 5). In particular, the latter experiment demonstrates that Cys\(^{430}\) is crucial for binding and/or proper positioning of the substrate L-cysteine and most likely represents the initial persulfide-binding site in the ABA3-NifS protein.

**DISCUSSION**

Cysteine desulfurase proteins, including the *A. thaliana* Moco sulfurase ABA3 ([9] and the present study), share a strictly conserved cysteine residue in the active site, whose mutation or inhibition by thiol-specific agents dramatically affects cysteine desulfurase activity. It has been demonstrated, initially for *A. vinelandii* NifS [8] and later also for other NifS-like cysteine desulfurases of different origins (summarized in [17]), that this cysteine residue is essential for binding the persulfide intermediate, which is the ultimate sulfur source for various cellular processes such as iron–sulfur cluster biogenesis. In fact, some cysteine desulfurases, such as *S. solfataricus* NifS [16], possess only this particular cysteine residue, whereas the majority of cysteine desulfurase proteins, including Moco sulfurases, harbour significantly more cysteines. With a total of nine cysteines, the N-terminal domain of the Moco sulfurase ABA3 possesses another two conserved cysteine residues, Cys\(^{428}\) and Cys\(^{435}\), in very close proximity to the strictly conserved Cys\(^{430}\), thus forming a triple-cysteine motif that is conserved among all Moco sulfurases (with the only exception of HxB from *Aspergillus nidulans*). Mutagenesis of each of these cysteine residues and the combined mutation of two or all three cysteines respectively allowed the investigation of the resulting ABA3-NifS variants with respect to cysteine desulfurase activity and surface-exposed cysteines. According to the findings of these investigations (Table 1), a model of the active site of ABA3-NifS was created that takes each single experiment with these variants into consideration without allowing inconsistencies between the results of these experiments (Figure 6). All results indicate that Cys\(^{430}\) represents the central cysteine residue in the active site of ABA3-NifS, as its mutation resulted in the complete loss of activity and the ability to bind a persulfide intermediate, as was demonstrated previously for the respective mutations in *A. vinelandii* NifS [8], and *E. coli* CSD (cysteine sulfinate desulfinase) and SufS [18]. Moreover, ITC analysis likewise confirmed the importance of Cys\(^{430}\), as the C430A variant was unable to bind the L-cysteine substrate (Figure 5), which suggests that Cys\(^{430}\) is required not only for catalysis, but also for binding and positioning of the substrate within the active site. Cys\(^{430}\) must therefore be considered the first cysteine in the intramolecular sulfur transport chain of ABA3. In the model, the Cys\(^{430}\), flanking residues Cys\(^{428}\) and Cys\(^{435}\) form a disulfide bridge that allows formation of a loop with the active-site Cys\(^{430}\) on top (Figure 6A). Although lacking disulfide-bridge-forming cysteine residues, a flexible loop directed towards the PLP cofactor and the substrate-binding pocket has been shown also for other cysteine desulfurases [17,19,20] and may thus represent a general feature of cysteine-desulfurating proteins, including Moco sulfurases. The model also considers the relationship between Cys\(^{430}\) and the two disulfide-bridge-forming cysteine residues, as mutation of Cys\(^{430}\) (Figure 6C) indeed abolishes the activity of ABA3-NifS, but does not affect the disulfide bridge between Cys\(^{428}\) and Cys\(^{435}\). In contrast, mutation of Cys\(^{428}\) destroys the disulfide bridge between Cys\(^{428}\) and Cys\(^{435}\) with simultaneous formation of an alternative bridge between Cys\(^{435}\) and Cys\(^{430}\) (Figure 6B). This is supported by the observation that the C428A variant of ABA3-NifS lost cysteine desulfurase activity and

![Figure 4 ITC analysis of substrate-binding to ABA3-NifS](image-url)

A 20 μM concentration of persulfide-free (A), persulfide-saturated (B) or DTT-treated persulfide-saturated (C) ABA3-NifS in 2.3 ml of 0.1 M sodium phosphate buffer, pH 9.3, was supplemented with a 0.5 mM solution of L-cysteine in 50 sequential steps (20 steps of 2 μM, 20 steps of 5 μM and 10 steps of 10 μM). (D) 1,5-I-AEDANS PAGE of the proteins used in (A), (B) and (C). The left-hand panel shows 1,5-I-AEDANS fluorescence after exposure to UV light, and the right-hand panel reveals Coomassie Brilliant Blue staining of the proteins used.
possesses only one residual 1,5-I-AEDANS-accessible cysteine residue that appeared unable to accept a persulfide. When the disulfide bridge in ABA3-NifS was destroyed by mutagenesis of Cys435 (Figure 6D), an additional, hitherto not-surface-exposed, cysteine residue became accessible to N-EM and 1,5-I-AEDANS, and cyanide treatment showed that the variant C435A accepts a third persulfide, which must be ascribed to this newly exposed residue. On the basis of MALDI–TOF–MS analysis of the C430/435A variant, which revealed that the only remaining cysteine in the triple-cysteine motif of this variant is labelled by 4-vinylpyridine, and the results of the C428/435A variant, in which exclusively Cys430 is labelled by 4-vinylpyridine (Table 2), this residue is most likely to be Cys428. The model gains further support by analysis of the C428/430A variant (Figure 6E), in which activity as well as disulfide-bridge formation are fully abolished, and by the C428/435A variant, which is incapable of forming the disulfide bridge, but retains the persulfide-binding capacity of Cys430, thus being fully active (Figure 6F). Similar to the C428/435A variant, the C430/435A variant (Figure 6G) is characterized by destruction of the disulfide bridge and preservation of two surface-exposed cysteines. In the latter variant, however, the mutation of Cys430 eliminated the surface-exposed and persulfide-binding active-site cysteine on the one hand, whereas the mutation of Cys435 uncovered Cys428 as an alternative persulfide-binding residue on the other hand. Since the alternatively exposed Cys428 is unable to serve as an active-site cysteine residue, the C430/435A variant did not exhibit cysteine desulfurase activity. The model is completed by the results obtained from the triple mutant C428/430/435A (Figure 6H), which is unable to form a disulfide bridge and does not possess an active-site cysteine residue, and thus is unable to generate persulfides and to exhibit activity. Interestingly, the model of the active-site loop of ABA3-NifS suggests that the intramolecular disulfide bridge is not essential for cysteine desulfurase activity, at least in vitro, as revealed by the variants C435A and C428/435A.
which both do not form disulfide bridges, but retain wild-type-like activities. Whether or not the disulfide bond is retained and required for cysteine desulfurase and Moco sulfuration activity of ABA3 in vivo remains to be shown in future work.

ABA3-NifS was found to possess two surface-exposed cysteine residues with persulfide-binding capacity (Figure 2B), one of them being identified as the active-site Cys430. As each of the two Cys430-flanking cysteines could be excluded as the second persulfide-binding residue, all of the remaining cysteine residues of ABA3-NifS were replaced with alanine or serine. In the case that the unknown second cysteine was substituted, a reduction in the number of surface-exposed cysteines to one per ABA3-NifS monomer with a concomitant reduction in cysteine desulfurase activity could be expected. Furthermore, in the case of the ABA3-NifS variant that carries the C430A substitution in addition to the substitution of the unknown cysteine, a complete loss of surface-exposed cysteines was proposed. However, with respect to activity, dimerization and the number of surface-exposed cysteines, all of the investigated single-mutated variants behaved like wild-type ABA3-NifS, whereas all double mutants in the C430A background presented the characteristics of the C430A variant (Supplementary Table S1 and Supplementary Figures S8–S10). Only those variants that carried the C206S substitution differed from the control protein in that the C206S variant presented only 18% activity and in that both variants (C206S and the C206S/C430A), tended to precipitate upon N-EM or 1,5-I-AEDANS treatment. A precise quantification of surface-exposed cysteines of these ABA3-NifS variants was therefore precluded.

Nevertheless, fitting well to the expected reduction in cysteine desulfurase activity, MALDI–TOF analysis identified Cys206 as surface-exposed in four of five experiments. Together with the observation that substitution of none of the other remaining cysteines affected the number of surface-exposed cysteines or cysteine desulfurase activity, these results suggest that Cys206 indeed represents the second surface-exposed and persulfide-binding cysteine of ABA3-NifS besides Cys430.

When comparing sequences of the functionally described Moco sulfurases from A. thaliana [6], tomato [21], human [22], mouse (S. Rump, R.R. Mendel and F. Bittner, unpublished work), cattle [23], fruitfly [10], silkworm [24] and A. nidulans [10], different degrees of conservation are displayed for the various cysteine residues (Supplementary Figure S2). Only two cysteine residues are strictly conserved among the N-terminal domains of these Moco sulfurases, represented by the active-site Cys430 and Cys456 of ABA3. Interestingly, no effect could be observed after substitution of Cys456 with regard to PLP saturation, the number of surface-exposed cysteines, cysteine desulfurase activity, dimerization and stability. It was thus impossible to ascribe a specific function to Cys456 and, accordingly, an involvement in the intramolecular persulfide transfer in ABA3 must be excluded. The Cys430-flanking residues Cys428 and Cys435 are conserved among most, but not all, Moco sulfurases. The HxB protein from A. nidulans lacks both cysteine residues, which supports a restricted need of Moco sulfurases for these cysteines as likewise suggested by the C428/435A variant of ABA3-NifS, which maintained full activity. Remarkably, Cys206, which has been identified as the second surface-exposed cysteine residue in ABA3-NifS, is strictly conserved only among the Moco sulfurases from plants and fruitfly, although corresponding cysteines are present in all other Moco sulfurases either three residues upstream (as in the case of mammalian Moco sulfurases) or 11–12 residues downstream of Cys206 in ABA3 (as in the case of silkworm and A. nidulans Moco sulfurases respectively). The ubiquitous presence of Cys206 and corresponding cysteines in other Moco sulfurases and the fact that Cys206 is exposed on the surface of ABA3-NifS therefore indicates a conserved function of this residue, probably in accepting a persulfide from the active-site Cys430 and its further transfer to the C-terminal domain of ABA3. To verify the hypothesis of a possible persulfide transfer between Cys430 and Cys206, as well as the deduced disulfide bond between Cys428 and Cys435, a theoretical structure of ABA3-NifS was calculated on the basis of the existing structure of Synechocystis SufS [25]. With an estimated accuracy of approximately 70%, this structure reveals the position of each cysteine residue in the ABA3-NifS protein (except Cys456, Figure 7A), thereby providing a possibility to compare the results obtained in the present study with the structural information of the investigated protein.
indicated by the theoretical structure, Cys\textsuperscript{101}, Cys\textsuperscript{151} and Cys\textsuperscript{377} are components of β-sheets, whereas Cys\textsuperscript{250} is deeply buried in the protein core. For these residues, neither the theoretical structure nor the results of the present study could predict an involvement in catalysis or in intramolecular sulfur transfer. Although the position of Cys\textsuperscript{436} could not be resolved in this structure, a functional relevance of this residue was likewise excluded on the basis of the findings of the present study. In contrast, for those cysteines whose replacement with alanine caused dramatic changes in activity, the number of surface-exposed cysteines and persulfide-binding capacity, the structural model confirms their functional importance: as presumed from the biochemical data, Cys\textsuperscript{430} is located on top of a loop near the PLP cofactor with its thiol group directed to the protein’s surface (Figure 7B). Cys\textsuperscript{428} and Cys\textsuperscript{435} are in close proximity to each other and may indeed form a disulfide bridge, which keeps the active-site loop with Cys\textsuperscript{430} in an optimal position during the PLP-dependent abstraction of sulfur from the l-cysteine substrate. It is tempting to speculate that this loop is required to provide a certain degree of mobility to Cys\textsuperscript{430} for transferring its persulfide to Cys\textsuperscript{336}. In fact, both residues appear to be close enough to allow persulfide transfer among them, in particular when movement of Cys\textsuperscript{336} is enabled by the Cys\textsuperscript{428}Cys\textsuperscript{435}-stabilized loop.

AUTHOR CONTRIBUTION
Markus Lehrke performed experiments and participated in writing the paper. Steffen Rump performed experiments and structure calculation. Torsten Heidenreich performed experiments. Jörg Wissig performed MALDI–TOF-MS analysis. Ralf Mendel financed the project, provided intellectual input and proofread the paper prior to submission. Florian Bittner financed the project, provided the idea for this work, supervised the experiments and wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Identification of persulfide-binding and disulfide-forming cysteine residues in the NifS-like domain of the molybdenum cofactor sulfurase ABA3 by cysteine-scanning mutagenesis

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Figure S1 Quantification of surface-exposed cysteines and bound persulfides at ABA3-NiIS

(A) 1,5-I-AEDANS-based quantification of surface-exposed cysteines. ABA3-NiIS was pretreated with the indicated concentrations of N-EM prior to co-incubation with 1,5-I-AEDANS and electrophoresis on 12% denaturing polyacrylamide gels. Exposure to UV light identifies that 1,5-I-AEDANS has no access to ABA3-NiIS when 1 mol of protein was pretreated with 2 mol of N-EM (upper gel), thereby confirming the existence of two surface-exposed cysteine residues at ABA3-NiIS. After exposure to UV light, the gel was stained with Coomassie Brilliant Blue to show equal loading of lanes (lower gel). (B) Quantification of ABA3-NiIS-bound persulfides by cyanolysis. Persulfides were released from ABA3-NiIS by cyanide treatment and quantified as thiocyanate (n = 4). The PLP saturation of ABA3-NiIS was determined on the basis of the PLP-specific absorbance at 420 nm and served as an internal reference for the amount of ABA3-NiIS. Results are means ± S.D.
Figure S2  Sequence comparison of eukaryotic Moco sulfurase NifS-like domains

Sequences of the following proteins and organisms were used: Arabidopsis, Arabidopsis thaliana ABA3; tomato, Solanum lycopersicum FLACCA; human, Homo sapiens HMCS; mouse, Mus musculus MOCOS; cattle, Bos taurus MCSU; fruitfly, Drosophila melanogaster MAROON-LIKE; silkworm, Bombyx mori OG; Aspergillus, Aspergillus nidulans HxB; IscS, Escherichia coli IscS; NifS, Azotobacter vinelandii NifS. Sequences of the bacterial cysteine desulfurases IscS and NifS were used to demonstrate conservation of the active-site cysteine. The sequence alignment was generated using ClustalW and Boxshade 3.21.
The active site of the NiFe-like domain of the Moco sulfurase ABA3

Figure S3 Polyacrylamide control gel of purified ABA3-NiFe variants of the triple-cysteine motif

Each lane was loaded with 12 μg of the respective ABA3-NiFe variant purified by affinity chromatography. After electrophoresis on a 12 % denaturing polyacrylamide gel, the gel was stained with Coomassie Brilliant Blue. Molecular mass in kDa is shown on the left-hand side. WT, wild-type.

Figure S4 Persulfide-binding capacities of Cys428, Cys430 and Cys435 variants

(A) Cysteine desulfurase activity and (B) persulfide formation capacity of ABA3-NiFe variants with deficiencies in the triple-cysteine motif (control: wild-type ABA3-NiFe). After treatment with L-cysteine as substrate for persulfide formation and subsequent gel electrophoresis, exposure to UV light identified persulfide formation capacity only for the ABA3-NiFe control and the variants C435A and C428/435A (upper panel in B). Low-intensity bands with identical and slightly lower mobility of 1,5-I-AEDANS/persulfide conjugates correspond to unbound 1,5-I-AEDANS. Both types of band can clearly be distinguished when exposed to UV light as 1,5-I-AEDANS/persulfide bands have a cyan colour, whereas free 1,5-I-AEDANS appears lime green (results not shown). After exposure to UV light, the gel was stained with Coomassie Brilliant Blue to show equal loading of lanes (lower panel in B). Results in (A) are means ± S.D. for four independent experiments.
Figure S5 1,5-I-AEDANS-based quantification of surface-exposed cysteines of the active ABA3-NifS variants C435A and C428/435A
The ABA3-NifS variants C428/435A (A) and C435A (B) were treated with different concentrations of N-EM to provoke the inhibition of 1,5-I-AEDANS binding. In the case of the C428/435A variant (A), the existence of two surface-exposed cysteine residues is confirmed by the complete inhibition by 1,5-I-AEDANS binding to the variant when the protein was pretreated with \( \geq 2 \) mol of N-EM (upper gel). In the case of the C435A variant (B), \( \geq 3 \) mol of N-EM are required to abolish binding of 1,5-I-AEDANS to the variant protein (upper gel), which indicates the appearance of a third surface-exposed cysteine. Coomassie Brilliant Blue staining of the gels in (A) and (B) was performed to show equal loading of lanes (lower gels).

Figure S6 Quantification of surface-exposed cysteines of inactive ABA3-NifS variants
The ABA3-NifS variants C428A (A), C430A (B), C428/430A (C), C430/435A (D) and C428/430/435A (E) and an ABA3-NifS control protein (F) were pretreated with the indicated concentrations of N-EM prior to co-incubation with 1,5-I-AEDANS and electrophoresis on 12% denaturing polyacrylamide gels. Exposure to UV light demonstrates that the variant C428A harbours one, the C430A variant one or two, the C428/430A one, the C430/435A variant two, the triple mutant C428/430/435A one and the control two surface-exposed cysteines (upper gels). After exposure to UV light, each gel was stained with Coomassie Brilliant Blue to show equal loading of lanes (lower gels).
Figure S7  CD spectra of ABA3-NifS and cysteine variants

WT, wild-type.

Figure S8  Cysteine desulfurase activity of the remaining ABA3-NifS cysteine variants

(A) Activities of the variants with single mutations in Cys101, Cys151, Cys206, Cys250, Cys377 and Cys456. (B) Activities of the variants with simultaneous mutations in Cys101, Cys151, Cys206, Cys250, Cys377 or Cys456, and Cys430 (control: wild-type ABA3-NifS). Results in (A) are means ± S.D. for at least three independent experiments.
Figure S9  Quantification of surface-exposed cysteines of the remaining ABA3-NiFS cysteine variants

(A) N-EM-dependent inhibition of cysteine desulfurase activity. (B) N-EM-dependent inhibition of 1,5-I-AEDANS binding to the respective ABA3-NiFS variant (upper gels). Coomassie Brilliant Blue staining of the gels from (A) was performed to show equal loading of lanes (lower gels).
The active site of the NiFS-like domain of the Moco sulfurase ABA3

Figure S10 Quantification of surface-exposed cysteines of the ABA3-NiFS double mutants C101/430A, C151/430A, C250/430A, C377/430A and C456/430A

Upper gels: N-EM-dependent inhibition of 1,5-I-AEDANS binding to the respective ABA3-NiFS variant. Lower gels: Coomassie Brilliant Blue staining of the upper gels was performed to show equal loading of lanes.

Table S1 Determination of the molecular mass of ABA3-NiFS cysteine variants

The molecular mass of ABA3-NiFS proteins was determined by size-exclusion chromatography using a calibration curve with standard proteins.

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