Lehrke, M., Rump, S., Heidenreich, T., Wissing, J., Mendel, R.R., Bittner, F.

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

(2012) Biochemical Journal, 441 (3), pp. 823-832.

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

Markus LEHRKE*, Steffen RUMP*, Torsten HEIDENREICH*, Josef WISSING†, Ralf R. MENDEL*[1], Florian BITTNER*

* Department of Plant Biology, Braunschweig University of Technology, Humboldtstrasse 1, 38023 Braunschweig, Germany; † Helmholtz Center for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany

Short Title: The active site of the NifS-like domain of the Moco sulfurase ABA3

The molybdenum cofactor sulfurase ABA3 from *Arabidopsis thaliana* catalyzes the sulfuration of the molybdenum cofactor 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 molybdenum cofactor. The strictly conserved cysteine430 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 molybdenum cofactor of aldehyde oxidase and xanthine oxidoreductase. In addition to cysteine430, another eight cysteine residues are located in the NifS-like domain, with two of them being highly conserved among molybdenum cofactor sulfurase proteins and at the same time being in close proximity to cysteine430. By determination of the number of surface-exposed cysteine residues and the number of persulfide-binding cysteines in combination with the sequential substitution of each of the nine cysteines, a second persulfide-binding cysteine residue, cysteine206, was identified. Furthermore, the active-site cysteine430 was found to be located on top of a loop structure, formed by the two flanking cysteines428 and cysteine435, 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 cysteine428 and cysteine435 are in disulfide bond distance and that a persulfide transfer from cysteine430 to cysteine206 is indeed possible.

Key words: ABA3, Arabidopsis thaliana, Moco sulfurase, active site loop, persulfide, MOSC

INTRODUCTION

Molybdenum enyzmes catalyze diverse key reactions in the global cycles of carbon, nitrogen, and sulfur [1, 2]. With the exception of bacterial nitrogenase, all molybdenum enyzmes contain the so-called molybdenum cofactor (Moco) in which the molybdenum is coordinated by the dithiolene group of a molybdopterin backbone. According to the coordination 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 cysteinyl sulfur, while enzymes of the xanthine oxidase family bind a Moco, whose molybdenum ligates one oxo-ligand, a hydroxyl group, and a terminal sulfur. In higher eukaryotes, sulfite oxidase and nitrate reductase are members of the sulfite oxidase family, whereas aldehyde oxidase and xanthine oxidoreductase belong to the xanthine oxidase family of molybdenum enzymes. With regard to the terminal sulfur ligand, the enzymes of the xanthine oxidase family are unique in that this particular ligand needs to be delivered in a specific enzymatic reaction [3]. The enzyme catalyzing this sulfuration step is referred to as Moco sulfurase and first insights into its mode of action were obtained upon studies of the recombinant protein from *Arabidopsis thaliana* [4]. ABA3

Abbreviations used: 1,5-I-AEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylene diamine; ITC, isothermal titration calorimetry; Moco, molybdenum cofactor; N-EM, *N*-ethylmaleimide; PLP, pyridoxal-5'-phosphate; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

[1] To whom correspondence should be addressed (email r.mendel@tu-bs.de); Humboldtstrasse 1, 38023 Braunschweig, Germany; phone: +49-531-3915870; fax: +49-531-3918128.

can be divided into two domains, an N-terminal domain that is referred to as ABA3-NifS according to its sequence similarities to cysteine desulfurase proteins such as the NifS protein from *Azotobacter vinelandii* [5,6], and a C-terminal domain, which reveals similarities only to the recently identified mARC or MOSC domain proteins, respectively [7,8]. As typical for cysteine desulfurases, the ABA3-NifS domain has been demonstrated to bind a pyridoxal-5'-phosphate (PLP) cofactor, which is essentially required for cysteine desulfurase activity [9]. Moreover, it possesses a conserved cysteine residue (cysteine430) that accepts the sulfur, which is abstracted from the substrate L-cysteine during cysteine desulfuration. Thus, the N-terminal domain alone is sufficient to exhibit cysteine desulfurase activity and to bind the mobilized sulfur in form of a persulfide. While the C-terminal domain of ABA3 was initially believed to be involved in recognition of the target enzymes aldehyde oxidase and xanthine oxidoreductase [6, 10], it has later been shown to be required for binding of Moco [11]. Remarkably, the ABA3-bound Moco was found in sulfurated and unsulfurated form, suggesting that the C-terminal domain functions as platform onto which the Moco is bound and provided with sulfur delivered by the N-terminal domain to generate sulfurated Moco. Since in the absence of ABA3 aldehyde oxidase and xanthine oxidoreductase have desulfo-Moco bound in their active sites [11, 12], it is still an open question whether only the sulfur of ABA3-bound Moco is transferred to the Moco of aldehyde oxidase and xanthine oxidoreductase, or whether the entire sulfurated Moco of ABA3 replaces the desulfo-Moco in aldehyde oxidase and xanthine oxidoreductase.

An intriguing fact is that each domain of ABA3 harbors nine cysteines with some of them being highly conserved among Moco sulfurases. In the N-terminal domain, cysteine428, cysteine430, cysteine435, and cysteine456 are conserved, whereas cysteine542, cysteine755 and cysteine758 are conserved in the C-terminal domain. Due to the high number of conserved cysteine residues it is tempting to speculate that the persulfide, whose generation is dependent at least on cysteine430 [9], is transmitted within the ABA3 protein from the initial persulfide-binding cysteine to one or several other cysteines before reaching its final destination, the C-terminally bound Moco. Such chain of at least two persulfide-binding cysteines has been shown earlier for other proteins which mobilize sulfur and deliver it to processes such as iron-sulfur cluster biogenesis [13]. It was thus an aim of this work to study the role of cysteines in the N-terminal cysteine desulfurase domain of ABA3 with particular focus on the characterization of the active site with its conserved cysteines cysteine428, cysteine430, and cysteine435, and the identification of other cysteine residues that are possibly involved in the intramolecular persulfide transport.

EXPERIMENTAL

Expression and purification of recombinant ABA3-NifS

Standard expression of ABA3-NifS and its cysteine variants was performed from the pQE80 plasmid (Qiagen, Hilden, Germany) in freshly transformed *Escherichia coli* DL41 cells basically as described ealier [9]. Cells were grown aerobically in LB medium in the presence of 50 µg/mL ampicillin at 22 °C to an A_{600} = 1.0 before induction. Expression of the respective protein was induced with 50 µM isopropyl-β-D-thiogalactopyranoside and 0.17 g pyridoxin/L expression culture was added to support synthesis of the PLP cofactor. After culturing for 20 h at 24°C, cells were harvested by centrifugation (11,000 x g, 4 °C, 5 min) and stored at –70 °C until use. Cell lysis was achieved by passaging through a French pressure cell followed by sonication for 5 min on ice. After centrifugation at 45.000 x g for 45 min at 4 °C, His_6-tagged proteins were purified on a nickel-nitrilotriacetic acid superflow matrix (Qiagen) under native conditions at 4 °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 sodium chloride, 250 mM imidazole, and 10 % glycerol). After purification by affinity chromatography, the recombinant NifS-like domain of ABA3 is characterized by an average specific cysteine desulfurase activity of 8.7 ± 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 use of Roti Quant solution (Roth, Karlsruhe, Germany) according to [14] or by using the absorbance of proteins at 280 nm and the molar extinction coefficient of 57,800 M^{-1} cm^{-1} for ABA3-NifS as determined by the program "ProtParam" (http://br.expasy.org).

Determination of the pyridoxal-5'-phosphate 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 to a PLP standard curve (0 - 350 µM PLP)

Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to [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, Heidelberg, Germany). Molecular mass standards and protein samples were pretreated with β-mercaptoethanol for 5 min at 95 °C prior to loading onto the gel.

Molecular mass determination

After purification of the recombinant proteins by affinity chromatography, size exclusion chromatography was performed with an Äkta Basic system and an analytical Superdex 200-HR10/30 column (Amersham Pharmacia Biotech, Freiburg, Germany) to determine the molecular mass of the native proteins. The column was equilibrated in 1 x phosphate buffered saline prior to separation of 200 µg 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, ribonuclease A 13.7 kDa). Each time v0 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 dithiothreitol for 2 h at 4 °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 (Amersham Pharmacia Biotech) and small amounts of denatured protein were removed by centrifugation at 22,000 x g and 4 °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. 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 to pyruvate by alanine dehydrogenase with concomitant formation of NADH. NADH is thus linearly generated from L-alanine, and therewith from L-cysteine. For a standard determination of cysteine desulfurase activity, 15 µg of the respective ABA3-NifS protein was used in total volume of 360 µL 0.1 M Tris/HCl, pH 9.3, containing 25 mM dithiothreitol. The reaction was started by addition of 40 µL of a 10 mM L-cysteine solution (final concentration 1 mM), and incubated with shaking for 30 min at 37 °C. The reaction was stopped by a heat shock at 72 °C for 10 min and 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, respectively, 100 µL of a 1:1 mixture (50 µL each) of 0.2 units alanine dehydrogenase from *Bacillus subtilis* (Sigma, Taufkirchen, Germany) and 0.1 M NAD^+ in 0.1 M Tris/HCl, pH 9.3, were added to the reaction and incubated for 80 min at 37 °C. After incubation, the reaction was centrifuged at 11,000 x g for 5 min to spin down eventually occuring precipitates. 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 substracted from 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, 0.25 mM) in 400 µL 0.1 M Tris/HCl, pH 9.3. By this improved assay

determination of background activities as occured in our formerly used "methylene blue assay" [9] is fully avoided.

Analysis of protein-bound persulfides by the 1,5-I-AEDANS gel assay

The basic principle of the identification of protein-bound persulfides by use of *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylene diamine (1,5-I-AEDANS) has been described earlier by Zheng and coworkers [8]. 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. dithiothreitol-treated) in 13.5 µL 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 must not contain molecules that are known to react with 1,5-I-AEDANS (e.g. dithiothreitol, β-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) within 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 conjugate from the protein, 1 µL of a 100 mM dithiothreitol solution was added to the reaction mix. Subsequently, the reaction was supplemented with 2 µL 10x native loading buffer (2 M sucrose, 1 % bromophenol blue) and electrophoresed for 45 min at 200 V on 12 % SDS-PA 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-labeled persulfides, 1,5-I-AEDANS-labeled 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 treatement with N-ethylmaleimide

Like 1,5-I-AEDANS, *N*-ethylmaleimide (N-EM) is a thiol-specific agent that alkylates accessible protein thiols in the neutral pH range, which is used in this work 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 50 mM sodium phosphate buffer, pH 7.0 overnight at 22 °C in the presence of precisely defined amounts of N-EM (0- to 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, 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 0.1 M Tris acetate, pH 8.6 (due to low sensitivity of the assay, the expected yield of persulfide should exceed 35 µM). After addition of 15 µL of a 0.5 M potassium cyanide solution, the reactions were incubated overnight at 22 °C and subsequently centrifuged at 4 °C to remove precipitated proteins. Formed thiocyanate was separated from remaining protein with a 10 kDa MWCO vivaspin concentrator (Sartorius, Göttingen, Germany) by centrifugation at 11,000 x g. 110 µL of the flow-through were mixed with 110 µL of a solution consisting of 10 g $Fe(NO_3)_3$ x $9H_2O$ and 20 mL of 65 % HNO_3 per 150 mL and incubated at room temperature for 1 min, in which the reddish iron/cyanate complex is formed. Finally, samples were centrifuged at 21,000 x 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 analysis

For identification of surface-exposed cysteines by MALDI-TOF analysis, 75 µg of the respective persulfide-free ABA3-NifS variant in 54 µL 0.1 M sodium phosphate buffer containg 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. 15 µL of each sample was supplemented with 5 µL of SDS loading buffer (50 % glycerol, 3.5 % SDS, 15 % β-mercaptoethanol, 0.02 % bromophenol blue), electrophoresed on 12 % SDS polyacrylamide 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 analysis.

Isothermal titration calorimetry (ITC)

All ITC experiments were performed by using the VP-ITC Isothermal Titration Calorimeter from MicroCal (Northampton, MA, USA) at 23 °C and 5 µcal/sec. The used proteins were dialysed overnight at 4 °C to 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 degased for 15 min right before the experiment. Each experiment contained 20 µM of the respective ABA3-NifS protein and was performed with 50 injections of L-cysteine (20x 2 µL, 20x 5 µL, 10x 10 µL) with intervals of 240 sec.

RESULTS

Identification of surface-exposed cysteines in ABA3-NifS

Our previous work on ABA3-NifS has demonstrated that at least cysteine430 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 this study to determine the number of cysteine residues in ABA3-NifS, which 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 causes the inability of the respective cysteines to bind a persulfide. In fact, addition of a two-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 are required for full activity (Fig. 1). This is 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 (Suppl. Fig. 1A). In samples treated with 2.0 or more moles 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 based on 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 like dithiothreitol 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 in case it is bound directly to a protein cysteine. 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 panel, left lane), the 1,5-I-AEDANS-specific fluorescence remained bound to ABA3-NifS when the protein was pretreated with dithiothreitol and desalted to ensure that it is 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 (Fig. 2, left panel, right lane). While these observations at a first view only confirm what has been shown earlier, 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 cysteines of ABA3-NifS (Fig. 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 moles persulfide per mol ABA3-NifS (Suppl. Fig. 1B).

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

While ABA3-NifS possesses nine cysteines, some other cysteine desulfurase proteins like *Sulfolobus solfataricus* NifS possess only one cysteine residue [16]. This cysteine, which corresponds to cysteine430 in ABA3-NifS, is strictly conserved among all bacterial and eukaryotic cysteine desulfurases. Besides cysteine430 however, ABA3-NifS possesses another eight cysteines with three of them being highly conserved among Moco sulfurases but not being present in other cysteine desulfurases (Suppl. Fig. 2). Interestingly, with their locations at positions 428 and 435, these two conserved cysteines are in close proximity to the putative active site cysteine430.

To study the importance of the conserved triple cysteine motif of ABA3-NifS for activity and persulfide transfer, each of the three cysteines was substituted by alanine, yielding the variants C428A, C430A, and C435A. In addition, all possible combinations of double mutants (C428/430A, C428/435A, C430/435A) as well as the common triple mutant (C428/430/435A) were generated. After expression and purification, all these ABA3-NifS variants showed a structural integrity identical to wildtype ABA3-NifS with respect to yield, purity (Suppl. Fig. 3) and PLP content. Yet, in the presence of L-cysteine as substrate, only the control protein and the variants C435A and C428/435A exhibited wildtype-like activity while all other variants were inactive (Suppl. Fig. 4A). 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 (Suppl. Fig. 4B). Since exclusively the substitution of cysteine430 in all combinations resulted in a complete loss of activity, this residue could thereby be confirmed as the essential active site cysteine, which is limiting for initial persulfide formation. Although the substitution of cysteine428 likewise resulted in inactive proteins in case of the variants C428A, C428/430A and C428/430/435A, the combined substitution of cysteines428 and 435 resulted in fully active protein, whereby an essential function of cysteine428 was excluded.

To analyze whether cysteine428 or cysteine435, respectively, represents the second persulfide-binding cysteine besides cysteine430 in ABA3-NifS, the number of surface-exposed cysteines was determined in the active variants C435A and C428/435A by use of N-EM and subsequent 1,5-I-AEDANS treatment. As shown for the ABA3-NifS control protein, the activity of the variant C428/435A was completely inhibited by 2 moles N-EM per mol protein (Fig. 3A), which is consistent with the absence of 1,5-I-AEDANS-specific fluorescence after pretreatment of the protein with 2 moles N-EM per mol protein (Suppl. Fig. 5A). Surprisingly, the complete inhibition of activity of the C435A variant required 3 moles N-EM per mol protein (Fig. 3B), which is confirmed by the absence of 1,5-I-AEDANS-specific fluorescence after pretreatment of the protein with 3 moles N-EM per mol protein (Suppl. Fig. 5B). Obviously, the mutation of the C435A variant caused the exposure of a third cysteine on the surface, which is further confirmed by the cyanide-based release of 3 moles persulfide per mol ABA3-NifS/C435A (data not shown).

Like the active cysteine variants, also the inactive variants were analyzed for surface-exposed cysteines by use of N-EM and 1,5-I-AEDANS treatment (Suppl. Fig. 6). Among those, only the variant C430/435A and the control protein presented two surface-exposed cysteines (Suppl. Fig. 6D and F). In contrast, the variants C428A, C428/430A, and C428/430/435A required only 1 mol N-EM per mol protein to eliminate 1,5-I-AEDANS-specific fluorescence (Suppl. Fig. 6A, C, and E). In most experiments the variant C430A likewise required 1 mol N-EM to abolish binding of 1,5-I-AEDANS. In some experiments however, about 1.5 mol N-EM per mol protein was required to block 1,5-I-AEDANS-specific fluorescence of the C430A variant (Suppl. Fig. 6B), indicating the single mutation of cysteine430 causes slight conformational changes. Summarizing the data of these experiments (Tab. 1), the following statements can be made: (i) Except for the C430/435A variant, substitution of cysteine430 resulted in only one surface-exposed cysteine, thereby confirming the importance of cysteine430. (ii) The C435A variant provides a third surface-exposed cysteine, demonstrating that neither cysteine435 nor the newly exposed cysteine are exposed on the surface of ABA3-NifS under normal conditions. In contrast, the C430/435A variant resembles a wildtype situation and presents two

surface-exposed cysteines, which must be ascribed to the loss of cysteine430 and the simultaneous exposition of a new cysteine to the surface caused by the cysteine435 mutation. (iii) A mutation in cysteine428 causes loss of one surface-exposed cysteine in the C428A variant, but in combination with mutations in cysteine430 (variants C428/430A and C428/430/435A) it did not cause the complete loss of surface-exposed cysteines. Rather, the combined mutation of cysteine428 and cysteine435 did not cause any changes in the number of surface-exposed cysteines, indicating cysteine428 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 substituted by alanine, yielding the variants C101A, C151A, C206A, C250A, C377A, and C456A. In addition, double mutants of these variants were generated containing the cysteine430-to-alanine substitution to eliminate the initial persulfide. As the variants C206A and C206/430A appeared to be instable, they were replaced by the variants C206S and C206S/C430A, respectively. The basic characterization by circular dichroism spectroscopy and size exclusion chromatography showed that all variants had the same overall structure (Suppl. Fig. 7) and were dimers (Suppl. Tab. 1) like wildtype ABA3-NifS. Moreover, all newly generated ABA3-NifS variants exhibited wildtype-like activity, with the exception of the C206S variant, whose activity was reduced to 18% (Suppl. Fig. 8A). In contrast, the simultaneous substitution of cysteine430 lead to a complete loss of activity in all double mutants (Suppl. Fig. 8B) as found for all previously investigated C430A variants. For further characterization of the active variants C101A, C151A, C206S, C250A, C377A, and C456A, the number of surface-exposed cysteines was determined by using N-EM and 1,5-I-AEDANS treatments (Suppl. Fig. 9). It must be noted here that the C206S variant could not be investigated in these experiments as it strongly tended to precipitate when subjected to N-EM treatment. Yet, all other variants presented two surface-exposed cysteines as indicated by the requirement of 2 moles N-EM per mol protein to abbrogate cysteine desulfurase activity and binding of 1,5-I-AEDANS. Since these variants behaved like wildtype ABA3-NifS, it must be excluded that any of the mutated cysteines represents the unknown second surface-exposed cysteine. This is confirmed by the observation that the inactive double mutants C101/430A, C151/430A, C250/430A, C377/430A, and C456/430A presented one residual cysteine on their surface (Suppl. Fig. 10), which must be ascribed to the loss of cysteine430. Like the C206S variant, also the C206S/C430A variant could not be analyzed in these experiments.

Identification of cysteine206 as the second surface-exposed cysteine in ABA3-NifS

MALDI-TOF technology was used to identify the unknown surface-exposed cysteine of wildtype ABA-NifS. For this purpose, the protein was initially treated with dithiothreitol to remove putatively bound persulfides. After removal of dithiothreitol 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 unlabeled cysteine residues were reduced with dithiothreitol and irreversibly alkylated by iodoacetamide to S-carboxyamido methylcysteine. After tryptic digestion, ABA3-NifS-derived peptides were purified and finally subjected to MALDI-TOF analysis. Since each cysteine of ABA3-NifS, except the triple cysteine motif, is separated from other cysteines by trypsin cleavage sites, 4-vinylpyridine-labeled peptides directly allow the identification of surface-exposed cysteines. In fact, in five independent experiments the peptide carrying the triple cysteine motif with the cysteines428, 430, and 435, was found with exactly one 4-vinylpyridine label (data summarized in Tab. 2). Obviously, only one of the three cysteines is accessible to 4-vinylpyridine, and the previous experiments suggest that this cysteine is represented by cysteine430. In support of this, the variant C428/435A, which just retained cysteine430 of the triple cysteine motif, likewise allowed the identification of one 4-vinylpyridine label at this specific peptide, which undoubtfully identifies cysteine430 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 cysteine430 as the only surface-exposed cysteine of this peptide. However, previous experiments (Fig. 3B, Suppl. Fig. 5B and Suppl. Fig. 6A+D) already indicated that cysteine428, which is the remaining cysteine in the "triple cysteine peptide" of the C430/435A variant, represents a new surface-exposed cysteine in the absence of cysteine435. The peptide carrying cysteine206, whose mutation appeared to be crucial for protein stability, was found 4-vinylpyridine-labeled in four out of five experiments, thereby allowing to conclude that cysteine206 represents the second surface-exposed cysteine of ABA3-NifS. Peptides that contain the residues cysteine101, cysteine151, cysteine250, or cysteine377, respectively, have not been found 4-vinylpyridine-labeled in any of the experiments, thereby precluding the possibility of these cysteines being surface-exposed. Just the cysteine456-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 near to it.

Cysteine430 is essential for substrate binding and persulfide formation in the active site of ABA3-NifS

Isothermal titration calorimetry (ITC) was used to study the interaction between ABA3-NifS and its substrate L-cysteine with regard to the importance of cysteine430 for substrate- and persulfide-binding. The ITC technology basically relies on the generation or absorption of heat occuring when two molecules bind to each other. As a consequence, the generated or consumed heat 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 released heat 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 moles L-cysteine per mol ABA3-NifS monomer based on 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 (Fig. 4A) but not when added to persulfide-saturated ABA3-NifS (Fig. 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 dithiothreitol treatment of persulfide-saturated ABA3-NifS restored the ability of the protein to bind L-cysteine (represented by reconstitution of heat generation; Fig. 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; Fig. 5). In particular the latter experiment demonstrates that cysteine430 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, this work], 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 by 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 like *S. solfataricus* NifS [16] possess only this particular cysteine residue whereas the majority of cysteine desulfurase proteins including Moco sulfurases harbor significantly more cysteines. With a total of nine cysteines, the N-terminal domain of the Moco sulfurase ABA3 possesses another two conserved cysteines, cysteine428 and cysteine435, in very close proximity to the strictly conserved cysteine430, 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 cysteines 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 (Tab. 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 (Fig. 6). All results indicate that cysteine430 represents the central cysteine 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 earlier for the respective mutations in *A. vinelandii* NifS [8], *E. coli* CSD and SufS [18]. Moreover, ITC analysis likewise confirmed the importance of cysteine430 as the C430A variant was unable to bind the L-cysteine substrate (Fig. 5), which suggests that cysteine430 is not only required for catalysis but also for binding and positioning of the substrate within the active site. Cysteine430 must therefore be considered the first cysteine in the intramolecular sulfur transport chain of ABA3. In the model, the cysteine430-flanking residues cysteine428 and cysteine435 form a disulfide bridge that allows formation of a loop with the active site cysteine430 on top (Fig. 6A). Although lacking disulfide bridge-forming cysteines, 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 relation between cysteine430 and the two disulfide bridge-forming cysteines as a mutation in cysteine430 (Fig. 6C) indeed abolishes the activity of ABA3-NifS but did not affect the disulfide bridge between cysteine428 and cysteine435. In contrast, a mutation in cysteine428 destroys the disulfide bridge between cysteine428 and cysteine435 with simultaneous formation of an alternative bridge between cysteine435 and cysteine430 (Fig. 6B). This is supported by the observation that the C428A variant of ABA3-NifS lost cysteine desulfurase activity and possesses only one residual 1,5-I-AEDANS-accessible cysteine that appeared unable to accept a persulfide. When the disulfide bridge in ABA3-NifS was destroyed by mutagenesis of cysteine435 (Fig. 6D), an additional, hitherto not surface-exposed cysteine 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. Based on MALDI-TOF analysis of the C430/435A variant, which revealed that the only remaining cysteine in the triple-cysteine motif of this variant is labeled by 4-vinylpyridine, and on the results of the C428/435A variant, in which exclusively cysteine430 is labeled by 4-vinylpyridine (Tab. 2), this residue is most likely represented by cysteine428. The model gains further support by analysis of the C428/430A variant (Fig. 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 persulfide-binding capacity of cysteine430, thus being fully active (Fig. 6F). Similar to the C428/435A variant, the C430/435A variant (Fig. 6G) is characterized by destruction of the disulfide bridge and preservation of two surface-exposed cysteines. In the latter variant however, the mutation of cysteine430 eliminated the surface-exposed and persulfide-binding active site cysteine on the one hand, whereas the mutation in cysteine435 uncovered cysteine428 as alternative persulfide-binding residue on the other hand. Since the alternatively exposed cysteine428 is unable to serve as active site cysteine, 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 (Fig. 6H), which is unable to form a disulfide bridge and does not possess an active site cysteine 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 wildtype-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 cysteines with persulfide-binding capacity (Fig. 2B), one of them being identified as the active site cysteine430. As each of the two cysteine430-flanking cysteines could be excluded as second persulfide-binding residue, all remaining cysteines of ABA3-NifS were exchanged for alanine or serine, respectively. In case the unknown second cysteine was substituted, a reduction of the number of surface-exposed cysteines to one per ABA3-NifS monomer with concomitant reduction of cysteine desulfurase activity could be expected. Furthermore, in 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 singularly mutated variants behaved like wildtype ABA3-NifS whereas all double mutants in the C430A background presented the characteristics of the C430A variant (Suppl. Tab. 1, Suppl. Fig. 8, 9, 10). Only those variants that carried the cyteine206-to-serine substitution differed from the control

protein in that the C206S variant presented only 18 % activity and in that both, the C206S and the C206S/C430A variant, 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 of cysteine desulfurase activity, MALDI-TOF analysis identified cysteine206 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 actvity, respectively, these results suggest that cysteine206 indeed represents the second surface-exposed and persulfide-binding cysteine of ABA3-NifS besides cysteine430.

When comparing sequences of the functionally described Moco sulfurases from *A. thaliana* [6], tomato [21], human [22], mouse [Rump, S., Mendel, R. R., Bittner. F., unpublished], cattle [23], fruitfly [10], silkworm [24] and *A. nidulans* [10], different degrees of conservation are displayed for the various cysteine residues (Suppl. Fig. 2). Only two cysteines are strictly conserved among the N-terminal domains of these Moco sulfurases, represented by the active site cysteine430 and cysteine456 of ABA3. Interestingly, no effect could be observed after substitution of cysteine456 with regard to PLP saturation, the number of surface-exposed cysteines, cysteine desulfurase activity, dimerization, and stability, respectively. It was thus impossible to ascribe a specific function to cysteine456 and accordingly, an involvement in the intramolecular persulfide transfer in ABA3 must be excluded. The cysteine430-flanking residues cysteine428 and cysteine435 are conserved among most, but not all Moco sulfurases. The HxB protein from *A. nidulans* lacks both cysteines, which supports an restricted need of Moco sulfurases for these cysteines as likewise suggested by the C428/435A variant of ABA3-NifS, which maintained full activity. Remarkably, cysteine206, which has been identified as the second surface-exposed cysteine in ABA3-NifS, is strictly conserved only among the Moco sulfurases from plants and fruit fly, though corresponding cysteines are present in all other Moco sulfurases either three residues upstream (as in case of mammalian Moco sulfurases) or 11-12 residues downstream from cysteine206 in ABA3 (as in case of silkworm and *A. nidulans* Moco sulfurases, respectively). The ubiquitous presence of cysteine206 and corresponding cysteines in other Moco sulfurases and the fact that cysteine206 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 cysteine430 and its further transfer to the C-terminal domain of ABA3. To verify the hypothesis of a possible persulfide transfer between cysteine430 and cysteine206 as well as the deduced disulfide bond between cysteine428 and cysteine435, a theoretical structure of ABA3-NifS was calculated based on 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 cysteine456; Fig. 7A), thereby providing a possibility to compare the results obtained in this study with the structural information of the investigated protein. As indicated by the theoretical structure, the cysteines at positions 101, 151, and 377 are components of β-sheets, whereas cysteine250 is deeply buried in the protein core. For these residues, neither the theoretical structure nor the results of our investigations could predict an involvement in catalysis or in intramolecular sulfur transfer. Although the position of cysteine456 could not be resolved in this structure, a functional relevance of this residue was likewise excluded based on the findings of this study. In contrast, for those cysteines, whose substitution by 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, cysteine430 is located on top of a loop near to the PLP cofactor with its thiol group directed to the protein's surface (Fig. 7B). Cysteine428 and cysteine435 are in close proximity to each other and may indeed form a disulfide bridge, which keeps the active site loop with cysteine430 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 cysteine430 for transferring its persulfide to cysteine206. In fact, both residues appear to be close enough to allow persulfide transfer among them, in particular when movement of cysteine430 is enabled by the cysteine428/cysteine435-stabilized loop.

ACKNOWLEDGEMENTS

We thank our students Alexander Gust, Christian Ruppelt and Sebastian Miethe for their assistance.

FUNDINGS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG grant Me 1266/21-1 to RRM and FB).

REFERENCES

1. Hille, R. (1996) The mononuclear molybdenum enzymes. Chem. Rev. **96**, 2757-2816
2. Hille, R, Nishino, T. and Bittner, F. (2011) Molybdenum enzymes in higher organisms. Coord. Chem. Rev. **255**, 1179-1205
3. Wahl, R. C., Warner, C. K., Finnerty, V. and Rajagopalan, K. V. (1982) Evidence for the inorganic nature of the cyanolyzable sulfur of molybdenum hydroxylases. J. Biol. Chem. **257,** 3958-3962
4. Bittner, F., Oreb, M. and Mendel, R. R. (2001) ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. J. Biol. Chem. **276**, 40381-40384
5. Zheng, L., White, R. H., Cash, V. L., Jack, R. F. and Dean, D. R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc. Natl. Acad. Sci. U S A **90,** 2754-2758
6. Zheng, L., White, R.H., Cash, V.L. and Dean, D.R. (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the *nifS* gene product. Biochemistry **33**, 4714-4720
7. Havemeyer, A., Bittner, F., Wollers, S., Kunze, T., Mendel, R. R. and Clement, B. (2006) Identification of the Missing Component in the Mitochondrial Benzamidoxime Prodrug-converting System as a Novel Molybdenum Enzyme. J. Biol. Chem. **281**, 34796-34802
8. Wahl, B., Reichmann, D., Niks, D., Biester, H., Krompholz, N., Havemeyer, A., Clement, B., Meßerschmidt, T., Rothkegel, M., Hille, R., Mendel, R.R. and Bittner, F. (2010) Biochemical and spectroscopic characterization of the human mitochondrial Amidoxime Reducing Components hmARC-1 and hmARC-2 suggest the existence of a third molybdenum enzyme family in eukaryots.J. Biol. Chem. **285**, 37847-37859
9. Heidenreich, T., Wollers, S., Mendel, R. R. and Bittner, F. (2005) Characterization of the NifS-like domain of ABA3 from *Arabidopsis thaliana* provides insight into the mechanism of molybdenum cofactor sulfuration. J. Biol. Chem. **280**, 4213-4218
10. Amrani, L., Primus, J., Glatigny, A., Arcangeli, L., Scazzocchio, C. and Finnerty, V. (2000) Comparison of the sequences of the *Aspergillus nidulans hxB* and *Drosophila melanogaster ma-l* genes with *nifS* from *Azotobacter vinelandii* suggests a mechanism for the insertion of the terminal sulphur atom in the molybdopterin cofactor. Mol. Microbiol. **38,** 114-125
11. Wollers, S., Heidenreich, T., Zarepour, M., Zachmann, D., Kraft, C., Zhao, Y., Mendel, R. R. and Bittner, F. (2008) Binding of sulfurated molybdenum cofactor to the C-terminal domain of ABA3 from Arabidopsis thaliana provides insight into the mechanism of molybdenum cofactor sulfuration. J. Biol. Chem. **283**, 9642-9650
12. Schwartz, S. H., Leon-Kloosterziel, K. M., Koornneef, M. and Zeevaart, J. A. (1997) Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. Plant Physiol**. 114,** 161-166
13. Kessler, D. (2006) Enzymatic activation of sulfur for incorporation into biomolecules in

prokaryotes. FEMS Microbiol. Rev. **30**, 825-840
14. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgramme quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-54
15. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685
16. She, Q., Singh, R. K., Confalonieri, F., Zivanovic, Y., Allard, G., Awayez, M. J., Chan-Weiher, C. C., Clausen, I. G., Curtis, B. A., De Moors, A., Erauso, G., Fletcher, C., Gordon, P. M., Heikamp-de Jong, I., Jeffries, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. P., Redder, P., Schenk, M. E., Theriault, C., Tolstrup, N., Charlebois, R. L., Doolittle, W. F., Duguet, M., Gaasterland, T., Garrett, R. A., Ragan, M. A., Sensen, C. W. and Van der Oost, J. (2001) The complete genome of the crenarchaeon Sulfolobus solfataricus P2. Proc. Natl. Acad. Sci. U. S. A. **98**, 7835-7840
17. Mihara, H. and Esaki, N. (2002) Bacterial cysteine desulfurases: their function and mechanisms. Appl. Microbiol. Biotechnol. **60**, 12-23
18. Mihara, H., Kurihara, T., Yoshimura, T. and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: Mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. J. Biochem. **127**, 559-567
19. Kaiser. J. T., Clausen, T., Bourenkow, G. P., Bartunik, H. D., Steinbacher, S. and Huber, R. (2000) Crystal structure of a NifS-like protein from *Thermotoga maritima*: Implications for iron sulphur cluster assembly. J. Mol. Biol. **297**, 451-464
20. Cupp-Vickery, J. R., Urbina, H. and Vickery, L. E. (2003) Crystal structure of IscS, a cysteine desulfurase from *Escherichia coli*. J. Mol. Biol. **330**, 1049-1059
21. Sagi, M., Scazzocchio, C. and Fluhr, R. (2002) The absence of molybdenum cofactor sulfuration is the primary cause of the flacca phenotype in tomato plants. Plant J. **31**, 305-317
22. Ichida, K., Matsumura, T., Sakuma, R., Hosoya, T. and Nishino, T. (2001) Mutation of human molybdenum cofactor sulfurase gene is responsible for classical xanthinuria type II. Biochem. Biophys. Res. Commun. **282,** 1194-1200
23. Watanabe, T., Ihara, N., Itoh, T., Fujita, T. and Sugimoto, Y. (2000) Deletion mutation in *Drosophila ma-l* homologous, putative molybdopterin cofactor sulfurase gene is associated with bovine xanthinuria type II. J. Biol. Chem. **275,** 21789-21792
24. Komoto, N., Sezutsu, H., Yukuhiro, K., Banno, Y. and Fujii, H. (2003) Mutations of the silkworm molybdenum cofactor sulfurase gene, og, cause translucent larval skin. Insect Biochem. Mol. Biol. **33**, 417-427
25. Tirupati, B., Vey, J. L., Drennan, C. L. and Bollinger, J. M. Jr. (2004) Kinetic and structural characterization of Slr0077/SufS, the essential cysteine desulfurase from *Synechocystis sp*. Biochemistry **43**, 12210-12219

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

ABA3-NifS variant	Number of surface-exposed cysteines	Cysteine-desulfurase activity [mol alanine mol protein^{-1} min^{-1}]
control	2	8.72 ± 1.06
C428A	1	0
C430A	1-2	0
C435A	3	9.64 ± 1.28
C428/430A	1	0
C428/435A	2	9.56 ± 0.82
C430/435A	2	0
C428/430/435A	1	0

Table 2 Summary of MALDI-TOF analysis of 4-vinylpyridine-labeled ABA3-NifS.

ABA3-NifS cysteine residue	results in MALDI-TOF analysis
Cysteine 101	no label identified
Cysteine 151	no label identified
Cysteine 206	labeled in 4 of 5 experiments
Cysteine 250	no label identified
Cysteine 377	no label identified
Triple-cysteine motif (Cysteines 428, 430 and 435)	1 cysteine labeled in 5 of 5 experiments
Cysteine 428 in the C430/435A variant	labeled in 1 of 1 experiments (i.e. surface exposed in the C430/435A variant)
Cysteine 430 in the C428/435A variant	labeled in 1 of 1 experiments (i.e. surface exposed in the C428/435A variant)
Cysteine 456	labeled in 2 of 5 experiments

FIGURE LEGENDS

Fig. 1: Quantification of surface-exposed cysteines at ABA3-NifS. Cysteine desulfurase activity of ABA3-NifS before and after co-incubation with different concentrations of the thiol-specific alkylating agent *N*-ethylmaleimide (n=8). The activity is completely inhibited by 2 moles *N*-ethylmaleimide per mol ABA3-NifS, suggesting the existance of two surface-exposed cysteine residues.

Fig. 2: 1,5-I-AEDANS-based identification of ABA3-NifS-bound persulfides. Each lane contained 30 µg ABA3-NifS co-incubated with 1,5-I-AEDANS and electrophoresed on a denaturing polyacrylamide gel. After electrophoresis, the gel was exposed to UV light (left gel) and subjected to Coomassie Brilliant Blue staining for proving equal loading of lanes (right gel). DTT treatment of ABA3-NifS resulted in persulfide-free ABA3-NifS, which allows 1,5-I-AEDANS to bind directly to all surface-exposed cysteines of the protein (upper bright spot in left gel). Cysteine-treated ABA3-NifS is not labeled with 1,5-I-AEDANS (lower bright spot in left gel), indicating that all surface-exposed cysteines of the protein were fully saturated with persulfides that were released as persulfide-1,5-I-AEDANS conjugate.

Fig. 3: 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*-ethylmaleimide to provoke the inhibition of cysteine desulfurase activity. In case of the C428/435A variant (A), the activity is completely inhibited by 2 moles *N*-ethylmaleimide per mol protein (graph), indicating the existance of two surface-exposed cysteine residues. In case of the C435A variant (B), 3 moles *N*-ethylmaleimide per mol protein are required to abolish the activity of this variant, which indicates the new appearance of a third surface-exposed cysteine.

Fig. 4: ITC analysis of substrate-binding to ABA3-NifS. 20 µM persulfide-free (**A**), persulfide-saturated (**B**) and DTT-treated persulfide-saturated ABA3-NifS (**C**) in 2.3 mL were supplemented with a 0.5 mM solution of L-cysteine in 50 sequential steps (20 steps of 2 µL, 20 steps of 5 µL, and 10 steps of 10 µL). (**D**) 1,5-I-AEDANS polyacrylamide gel of the proteins used in (A), (B), and (C). Left panel reveals 1,5-I-AEDANS fluorescence after exposure to UV light, right panel reveals Coomassie Brilliant Blue staining of the used proteins.

Fig. 5: ITC analysis of substrate-binding to the ABA3-NifS variant C430A. 20 µM of the ABA3-NifS variant C430A in 2.3 mL were supplemented with a 0.5 mM solution of L-cysteine in 50 sequential steps (20 steps of 2 µL, 20 steps of 5 µL, and 10 steps of 10 µL). No release of heat can be observed, indicating that the substrate L-cysteine cannot bind the the C430A protein.

Fig. 6: Model of the active site of ABA3-NifS. The model depicts cysteine430 as the active site cysteine of ABA3, which is involved in substrate desulfuration and initial persulfide binding. In the wildtype protein (**A**), cysteine428 and cysteine435 form a disulfide bridge (Cys428-S-S-Cys435) with cysteine430 on top of it. Cysteine430 and another cysteine, most likely respresented by cysteine206, are exposed on the surface of the ABA3 protein and are capable of binding a persulfide that is delivered by cysteine430 to cysteine206. The model is based on the analysis of the ABA3-NifS variants C428A (**B**), C430A (**C**), C435A (**D**), C428/430A (**E**), C428/435A (**F**), C430/435A (**G**), C428/430/435A (**H**), whose specific features are indicated (substituted residues are marked in italics and by a star, surface-exposed persulfide-binding cysteines are indicated by "-S-SH").

Fig. 7: Theoretical structure of ABA3-NifS. (**A**) Overall structure of one monomer of the N-terminal domain of the Moco sulfurase ABA3. (**B**) Enlarged section of the active site of ABA3 with the ring structure of the PLP cofactor indicated on the upper right side. The sulfur atoms of cysteines are depicted as yellow balls with the position of the respective cysteine indicated. Cysteine430 forms the active site together with the PLP cofactor and is positioned on top of a loop, which is hold together by a putative disulfide bridge between cysteine428 and cysteine435. Cysteine206 is placed in the background close to cysteine430.

Figure 1

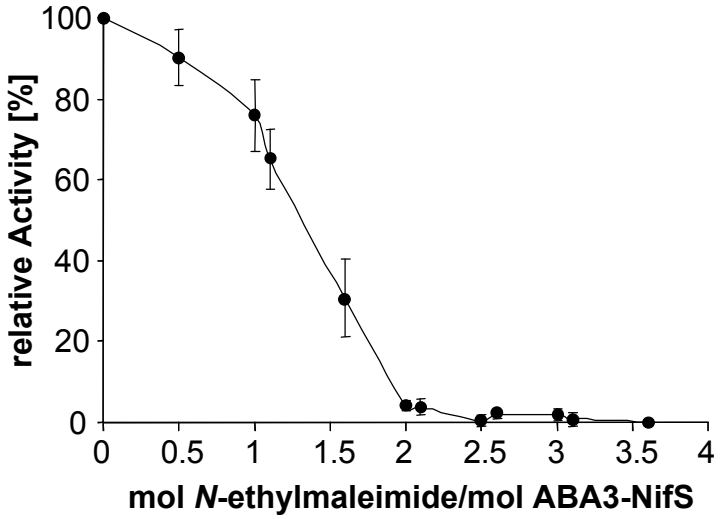


Fig. 1: Quantification of surface-exposed cysteines at ABA3-NifS. Cysteine desulfurase activity of ABA3-NifS before and after co-incubation with different concentrations of the thiol-specific alkylating agent *N*-ethylmaleimide (n=8). The activity is completely inhibited by 2 moles *N*-ethylmaleimide per mol ABA3-NifS, suggesting the existance of two surface-exposed cysteine residues.

Figure 2

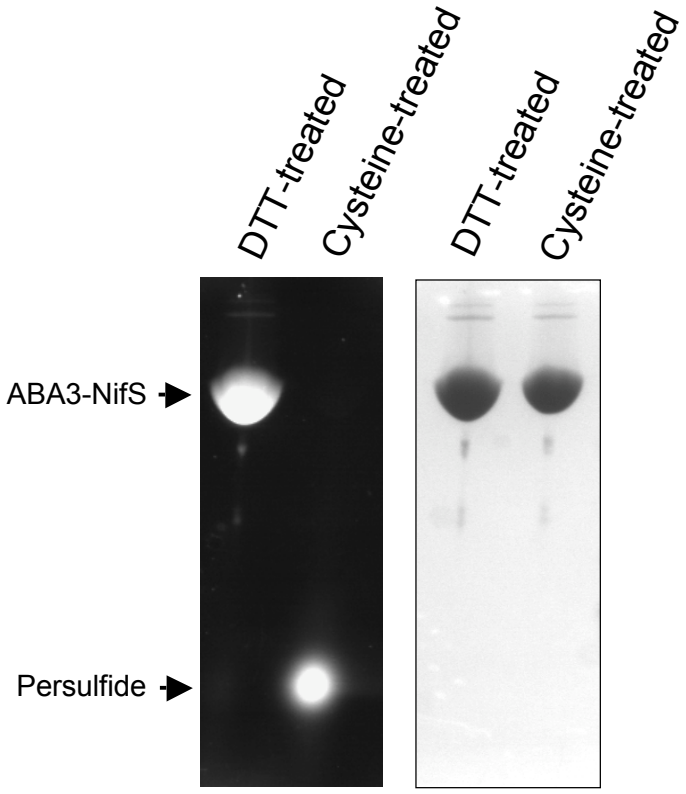


Fig. 2: 1,5-I-AEDANS-based identification of ABA3-NifS-bound persulfides. Each lane contained 30 µg ABA3-NifS co-incubated with 1,5-I-AEDANS and electrophoresed on a denaturing polyacrylamide gel. After electrophoresis, the gel was exposed to UV light (left gel) and subjected to Coomassie Brilliant Blue staining for proving equal loading of lanes (right gel). DTT treatment of ABA3-NifS resulted in persulfide-free ABA3-NifS, which allows 1,5-I-AEDANS to bind directly to all surface-exposed cysteines of the protein (upper bright spot in left gel). Cysteine-treated ABA3-NifS is not labeled with 1,5-I-AEDANS (lower bright spot in left gel), indicating that all surface-exposed cysteines of the protein were fully saturated with persulfides that were released as persulfide-1,5-I-AEDANS conjugate.

Figure 3

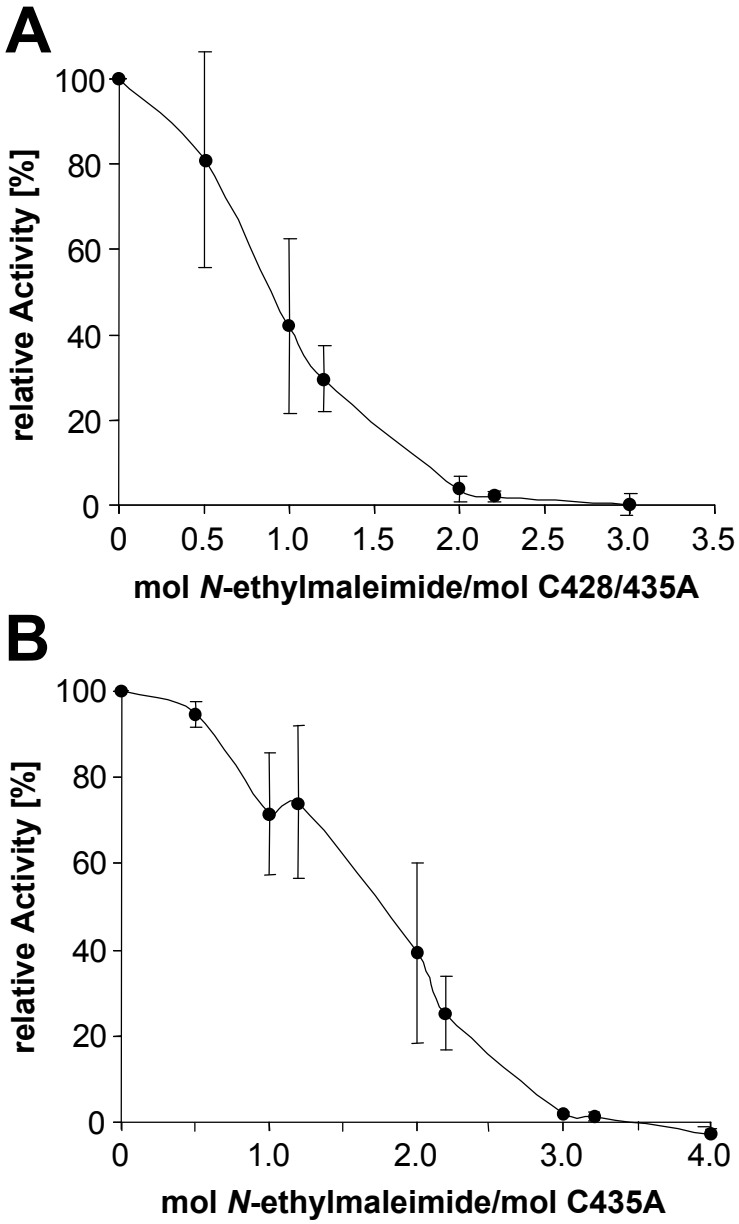


Fig. 3: Quantification of surface-exposed cysteines of the active ABA3-NifS variants C435A and C428/435A. The ABA3-NifS variants C428/435A (**A**) and C435A (**B**) were treated with different concentrations of *N*-ethylmaleimide to provoke the inhibition of cysteine desulfurase activity. In case of the C428/435A variant (A), the activity is completely inhibited by 2 moles *N*-ethylmaleimide per mol protein (graph), indicating the existance of two surface-exposed cysteine residues. In case of the C435A variant (B), 3 moles *N*-ethylmaleimide per mol protein are required to abolish the activity of this variant, which indicates the new appearance of a third surface-exposed cysteine.

Figure 4

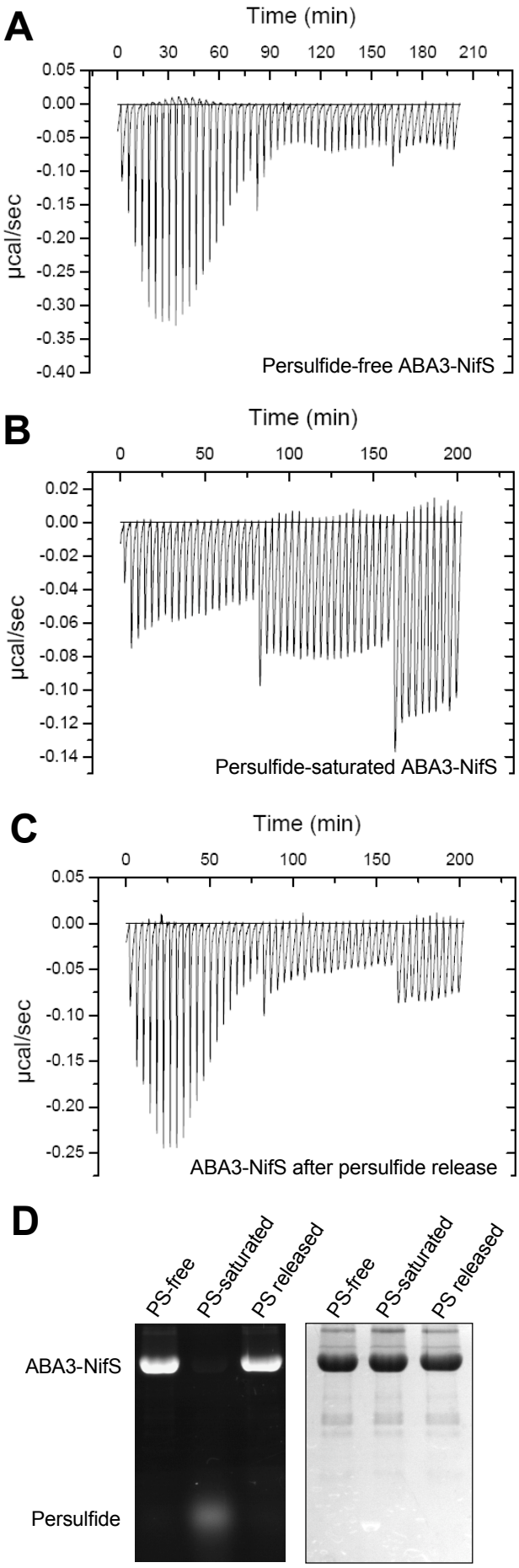

A
Time (min)
0 30 60 90 120 150 180 210
0.05 0.00 -0.05 -0.10 -0.15 -0.20 -0.25 -0.30 -0.35 -0.40
µcal/sec
Persulfide-free ABA3-NifS
B
Time (min)
0 50 100 150 200
0.02 0.00 -0.02 -0.04 -0.06 -0.08 -0.10 -0.12 -0.14
µcal/sec
Persulfide-saturated ABA3-NifS
C
Time (min)
0 50 100 150 200
0.05 0.00 -0.05 -0.10 -0.15 -0.20 -0.25
µcal/sec
ABA3-NifS after persulfide release
D
PS-free
PS-saturated
PS released
PS-free
PS-saturated
PS released
ABA3-NifS
Persulfide

Fig. 4: ITC analysis of substrate-binding to ABA3-NifS. 20 µM persulfide-free (**A**), persulfide-saturated (**B**) and DTT-treated persulfide-saturated ABA3-NifS (**C**) in 2.3 mL were supplemented with a 0.5 mM solution of L-cysteine in 50 sequential steps (20 steps of 2 µL, 20 steps of 5 µL, and 10 steps of 10 µL). (**D**) 1,5-I-AEDANS polyacrylamide gel of the proteins used in (A), (B), and (C). Left panel reveals 1,5-I-AEDANS fluorescence after exposure to UV light, right panel reveals Coomassie Brilliant Blue staining of the used proteins.

Figure 5

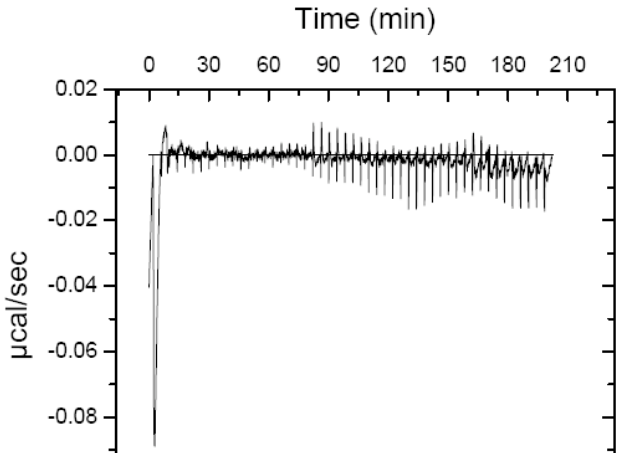


Fig. 5: ITC analysis of substrate-binding to the ABA3-NifS variant C430A. 20 µM of the ABA3-NifS variant C430A in 2.3 mL were supplemented with a 0.5 mM solution of L-cysteine in 50 sequential steps (20 steps of 2 µL, 20 steps of 5 µL, and 10 steps of 10 µL). No release of heat can be observed, indicating that the substrate L-cysteine cannot bind the the C430A protein.

Figure 6

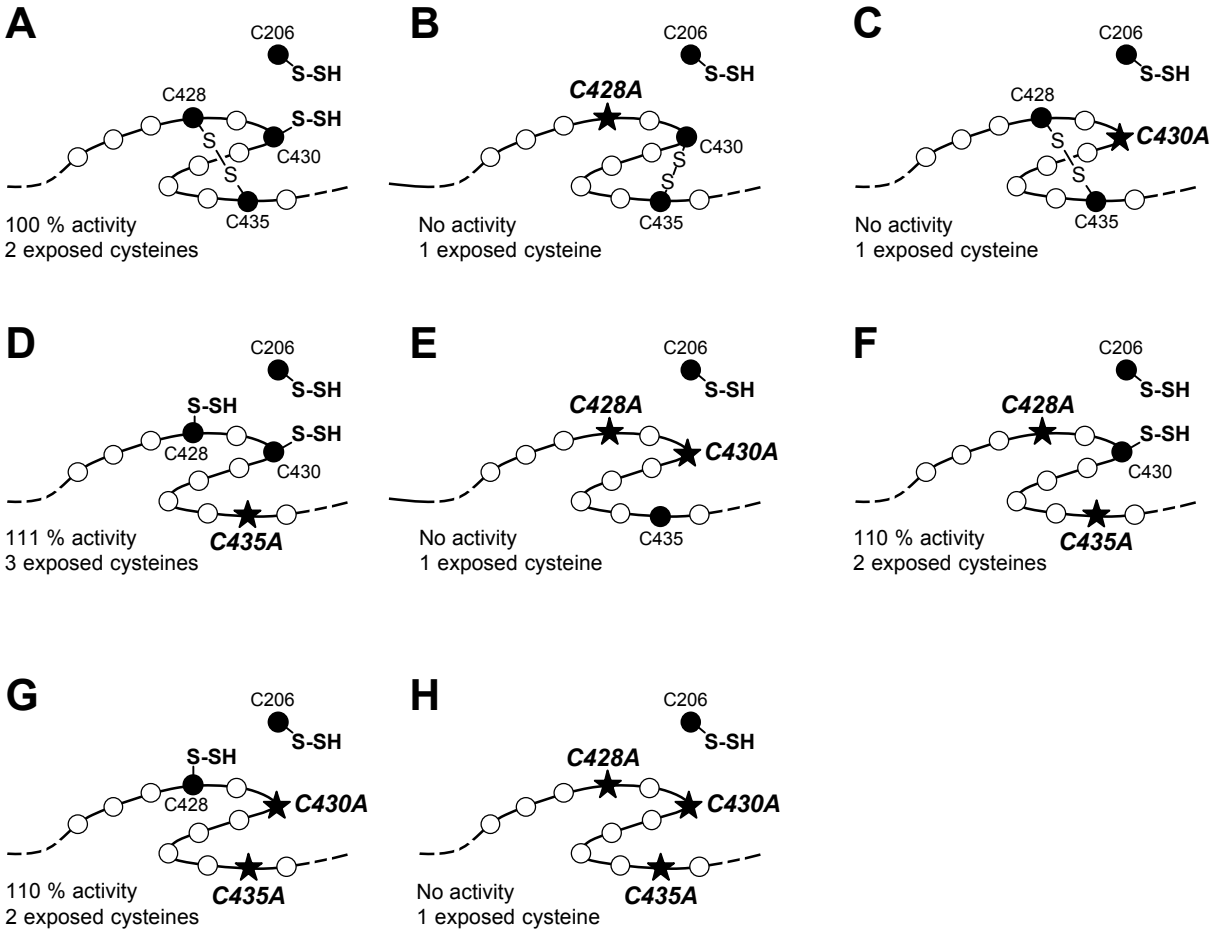


Fig. 6: Model of the active site of ABA3-NifS. The model depicts cysteine430 as the active site cysteine of ABA3, which is involved in substrate desulfuration and initial persulfide binding. In the wildtype protein (**A**), cysteine428 and cysteine435 form a disulfide bridge (Cys428-S-S-Cys435) with cysteine430 on top of it. Cysteine430 and another cysteine, most likely respresented by cysteine206, are exposed on the surface of the ABA3 protein and are capable of binding a persulfide that is delivered by cysteine430 to cysteine206. The model is based on the analysis of the ABA3-NifS variants C428A (**B**), C430A (**C**), C435A (**D**), C428/430A (**E**), C428/435A (**F**), C430/435A (**G**), C428/430/435A (**H**), whose specific features are indicated (substituted residues are marked in italics and by a star, surface-exposed persulfide-binding cysteines are indicated by "-S-SH").

Figure 7

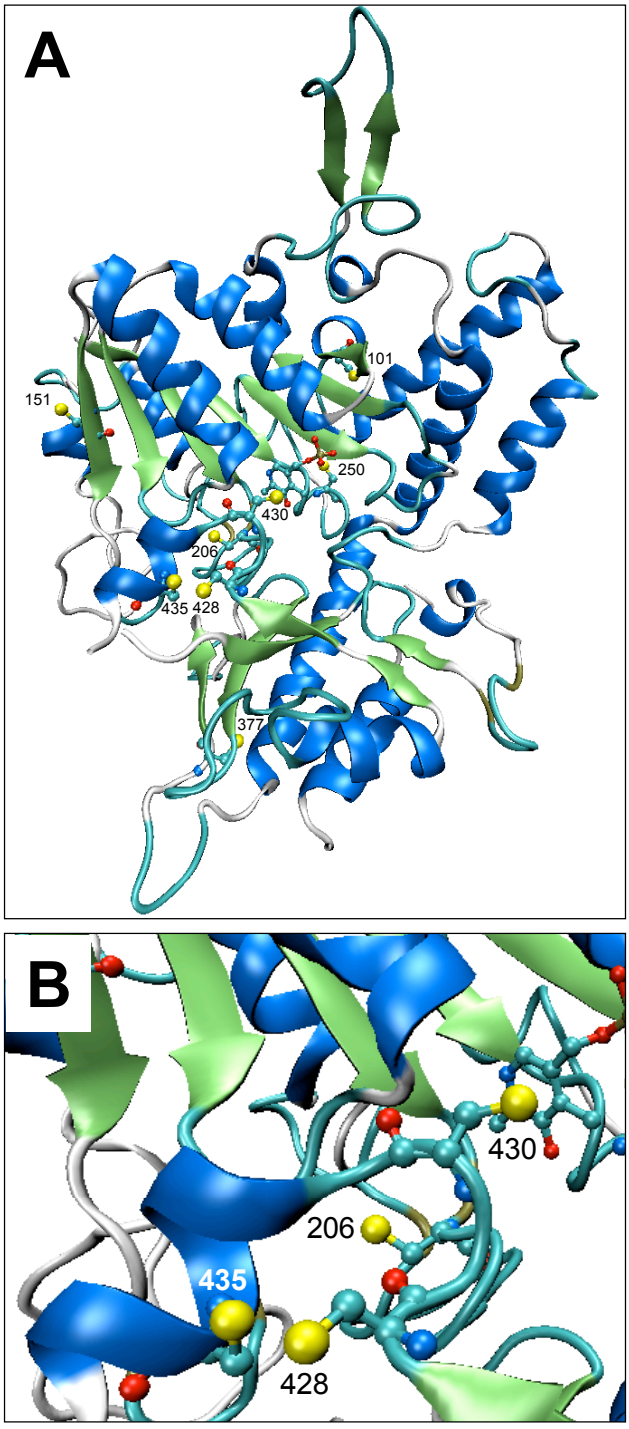
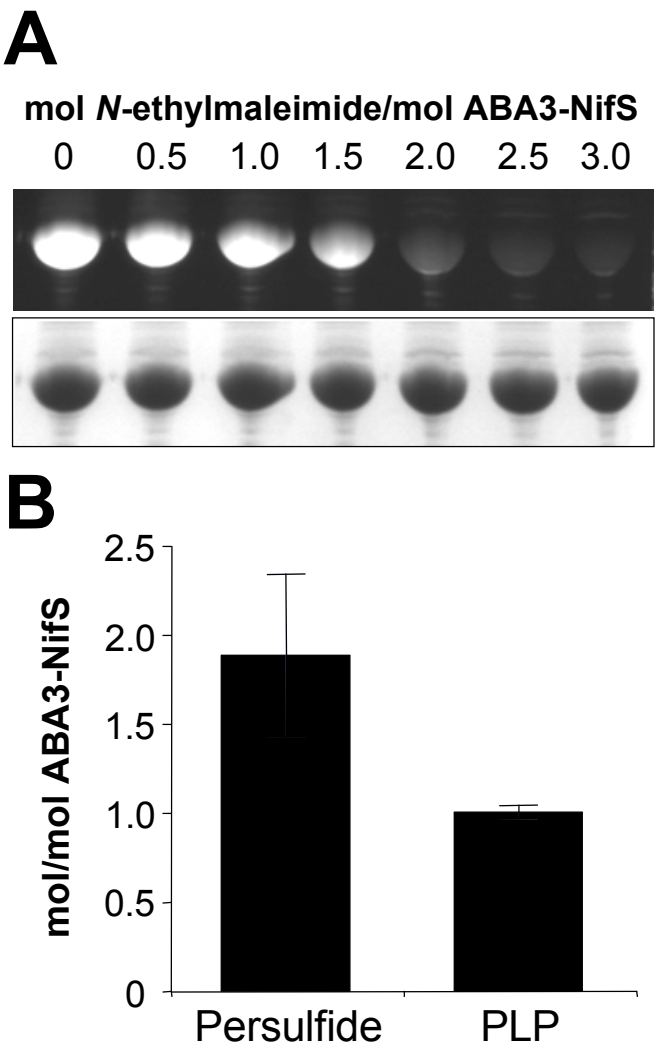


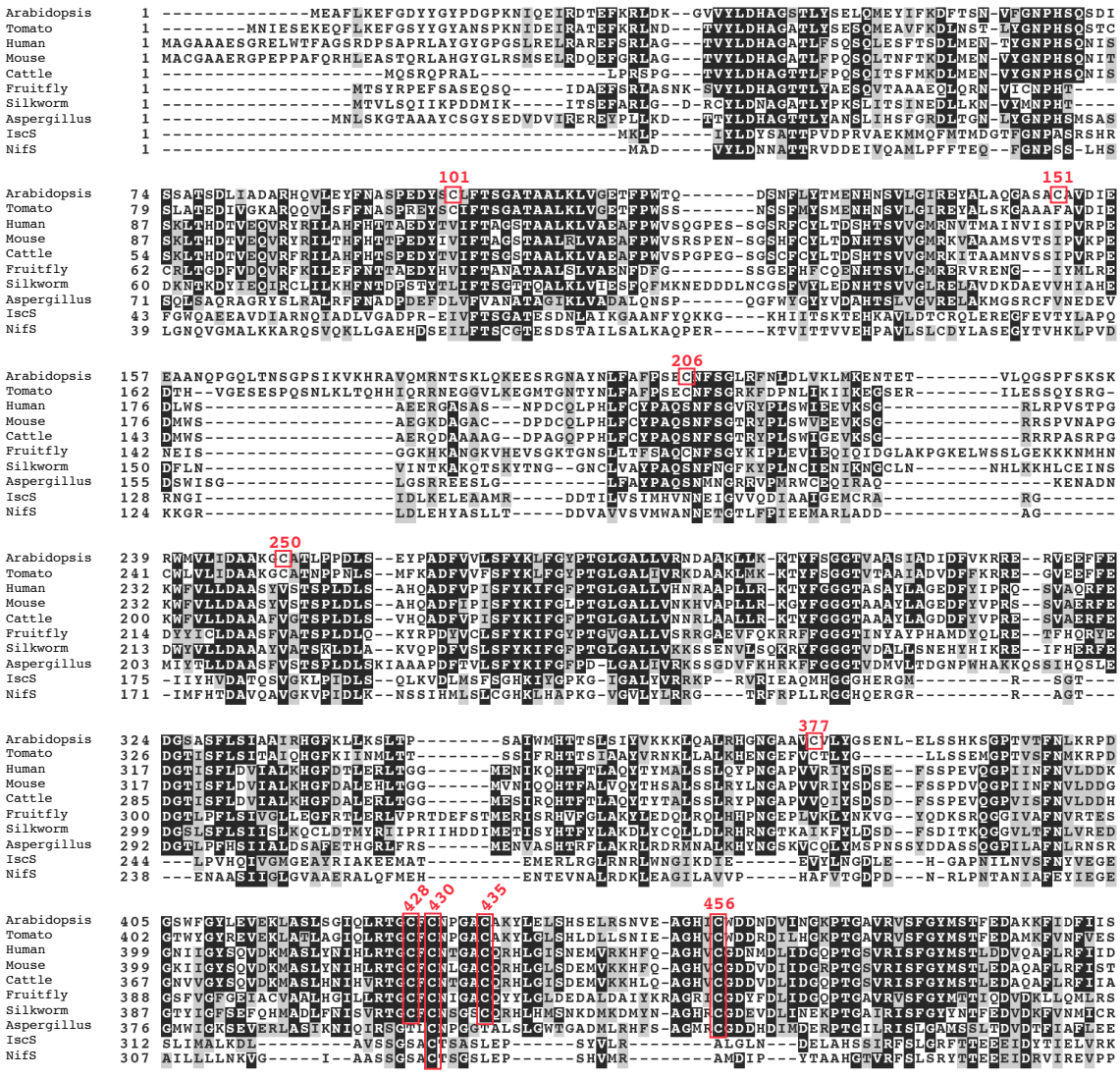
Fig. 7: Theoretical structure of ABA3-NifS. (**A**) Overall structure of one monomer of the N-terminal domain of the Moco sulfurase ABA3. (**B**) Enlarged section of the active site of ABA3 with the ring structure of the PLP cofactor indicated on the upper right side. The sulfur atoms of cysteines are depicted as yellow balls with the position of the respective cysteine indicated. Cysteine430 forms the active site together with the PLP cofactor and is positioned on top of a loop, which is hold together by a putative disulfide bridge between cysteine428 and cysteine435. Cysteine206 is placed in the background close to cysteine430.

Supplemental Figure 1



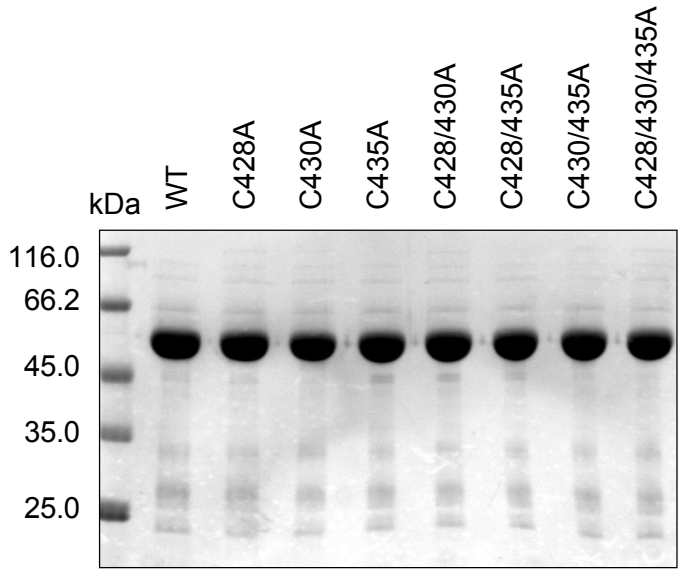
Suppl. Fig. 1: Quantification of surface-exposed cysteines and bound persulfides at ABA3-NifS. (**A**) 1,5-I-AEDANS-based quantification of surface-exposed cysteines. ABA3-NifS was pretreated with the indicated concentrations of *N*-ethylmaleimide prior to co-incubation with 1,5-I-AEDANS and electrophoresis on 12% denaturing polyacrylamide gels. Exposure to UV light identifies that 1,5-I-AEDANS has no access to ABA3-NifS when 1 mol protein was pretreated with 2 moles *N*-ethylmaleimide (upper gel), thereby confirming the existance of two surface-exposed cysteine residues at ABA3-NifS. After exposure to UV light, the gel was stained with Coomassie Brilliant Blue staining to show equal loading of lanes (lower gel). (**B**) Quantification of ABA3-NifS-bound persulfides by cyanolysis. Persulfides were released from ABA3-NifS by cyanide treatment and quantified as thiocyanate (n=4). The PLP saturation of ABA3-NifS was determined based on the PLP-specific absorbance at 420 nm and served as internal reference for the ABA3-NifS amount.

Supplemental Figure 2



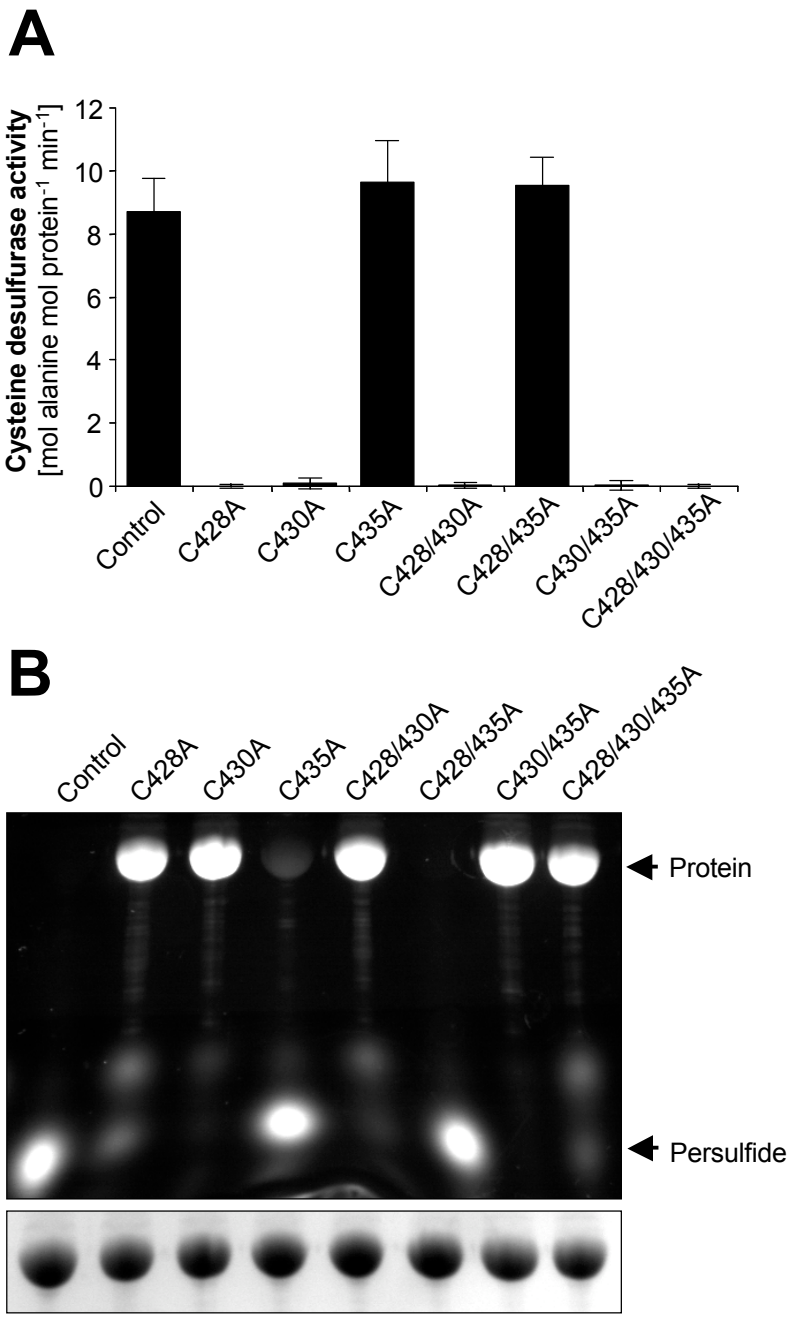
Suppl. Fig. 2: Sequence comparison of eukaryotic molybdenum cofactor sulfurase NifS-like domains. Sequences of the following proteins and organisms were used: Arabidopsis= *Arabidopsis thaliana* ABA3; tomato = *Lycopersicon esculentum* FLACCA; human = *Homo sapiens* HMCS; Mouse = *Mus musculus* MOCOS; 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.

Supplemental Figure 3



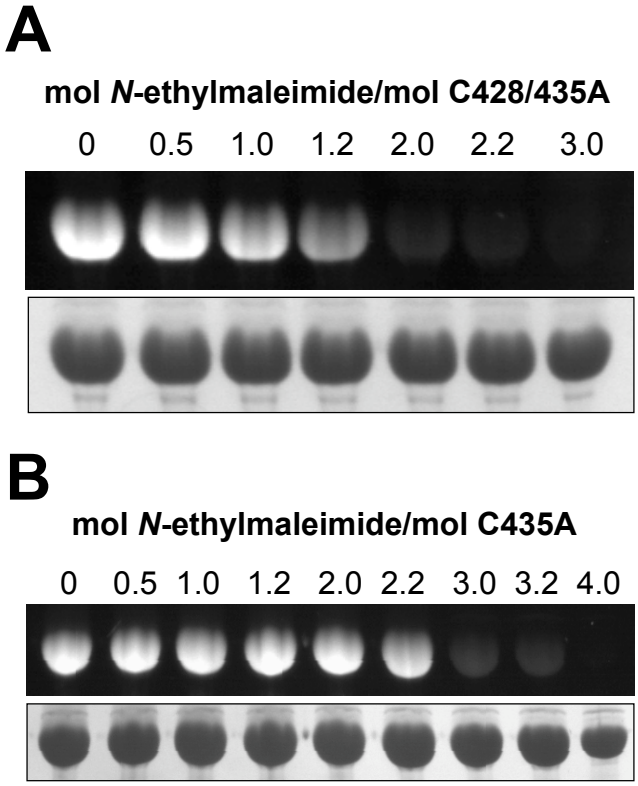
Suppl. Fig. 3: Polyacrylamide control gel of purified ABA3-NifS variants of the triple-cysteine motif. Each lane was loaded with 12 µg of the respective ABA3-NifS variant purified by affinity chromatography. After electrophoresis on a 12% denaturing polyacrylamide gel, the gel was stained with Coomassie Brilliant Blue.

Supplemental Figure 4



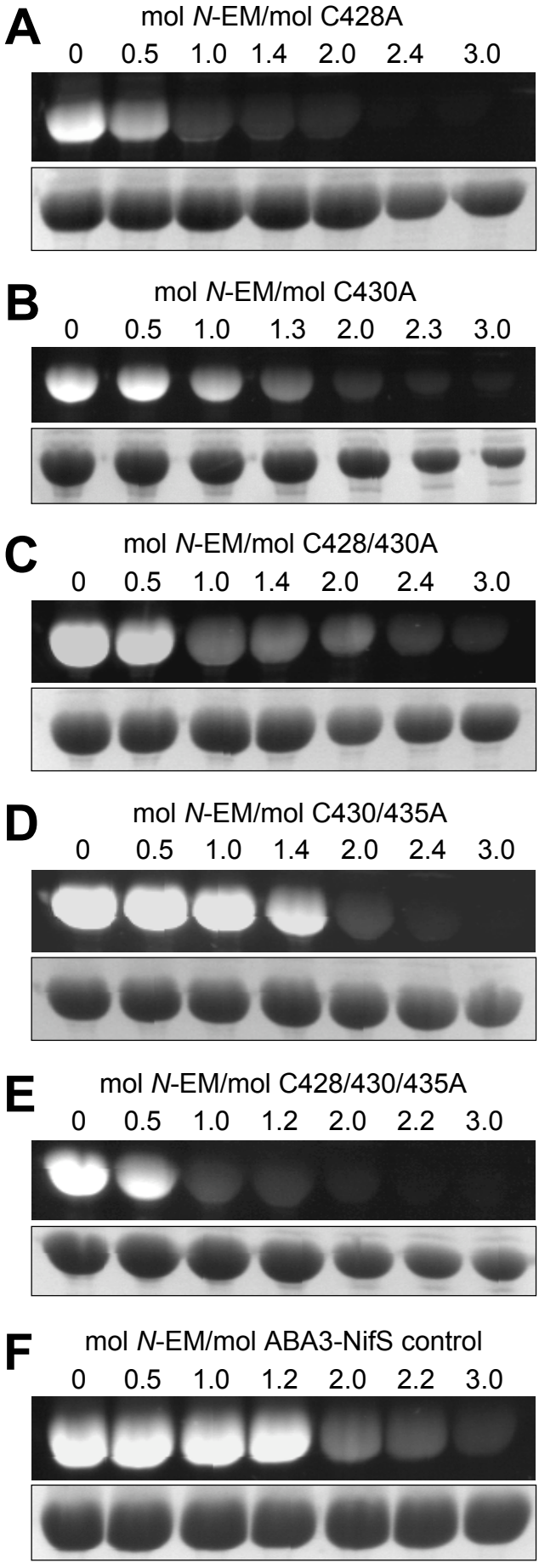
Suppl. Fig. 4: Persulfide-binding capacities of cysteine428, cysteine430, and cysteine435 variants. (**A**) Cysteine desulfurase activity and (**B**) persulfide formation capacity of ABA3-NifS variants with deficiencies in the triple cysteine motif (control: wild-type ABA3-NifS). After treatment with L-cysteine as substrate for persulfide formation and subsequent gel electrophoresis, exposure to UV light identified persulfide formation capacity only for the ABA3-NifS control and the variants C435A and C428/435A (upper gel). 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 bands can clearly be distinguished when exposed to UV light as 1,5-I-AEDANS/persulfide bands originally have a cyan colour whereas free 1,5-I-AEDANS appears lime-green (not shown). After exposure to UV light, the gel was stained with Coomassie Brilliant Blue staining to show equal loading of lanes (lower gel).

Supplemental Figure 5



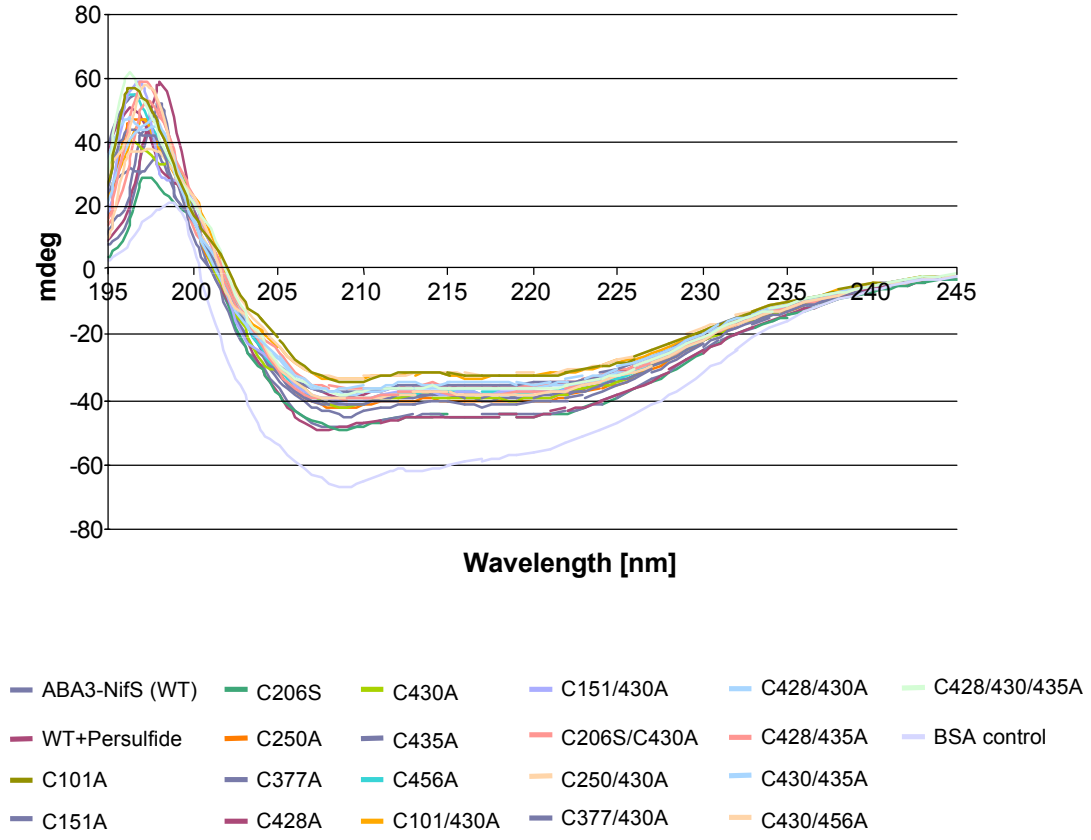
Suppl. Fig. 5: 1,5-I-AEDANS-based quantification of surface-exposed cysteines of the active ABA3-NifS variants C435A and C428/435A. The ABA3-NifS variants C428/435A (**A**) and C435A (**B**) were treated with different concentrations of *N*-ethylmaleimide to provoke the inhibition of 1,5-I-AEDANS binding. In case of the C428/435A variant (A), the existance 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 2 or more moles *N*-ethylmaleimide (upper gel). In case of the C435A variant (B), 3 or more moles *N*-ethylmaleimide 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).

Supplemental Figure 6



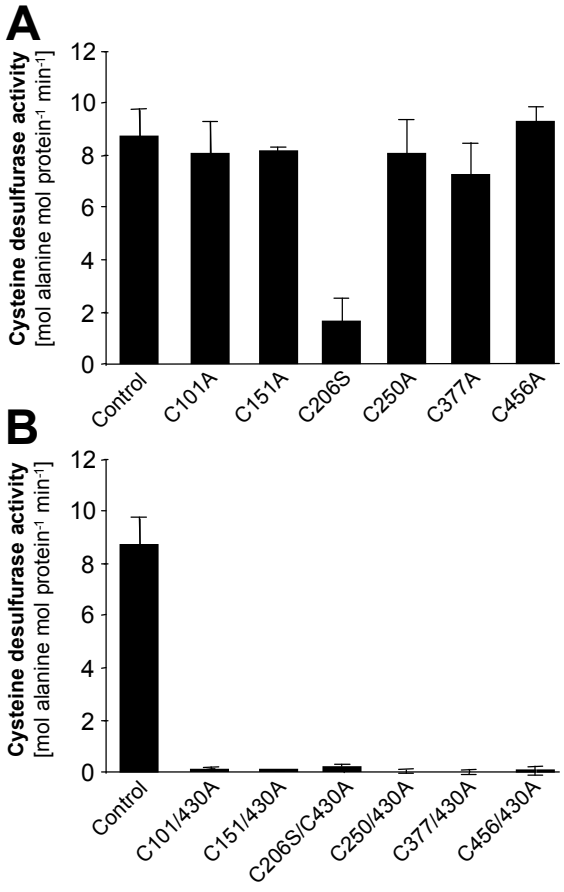
Suppl. Fig. 6: Quantification of surface-exposed cysteines of inactive ABA3-NifS variants. The ABA3-NifS variants C428A (**A**), C430A (**B**), C428/430A (**C**), C430/435A (**D**), C428/430/435A (**E**), and an ABA3-NifS control protein (**F**) were pretreated with the indicated concentrations of *N*-ethylmaleimide (*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 harbors 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 (respective upper gel). After exposure to UV light, each gel was stained with Coomassie Brilliant Blue to show equal loading of lanes (respective lower gel).

Supplemental Figure 7



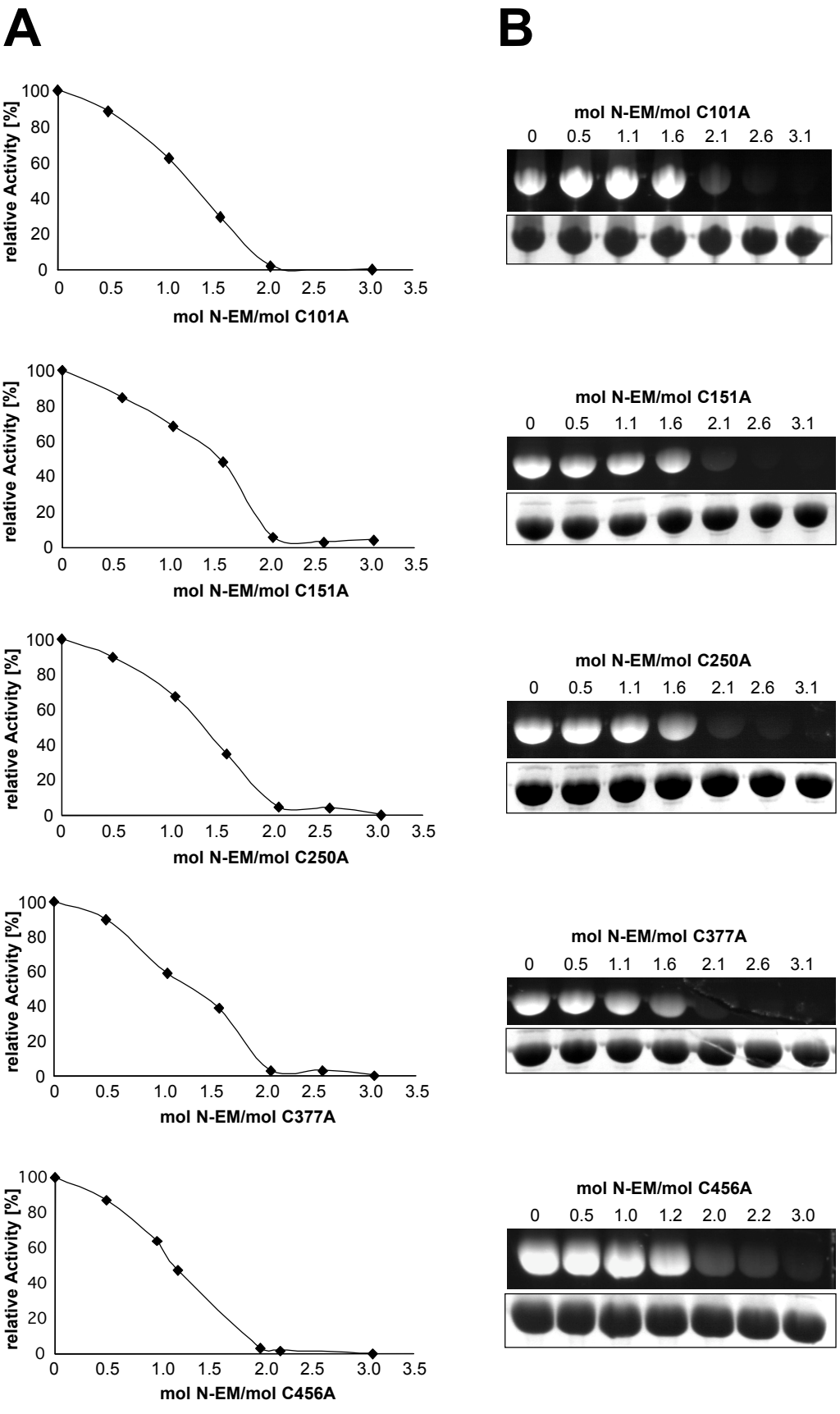
Suppl. Fig. 7: Circular dichroism spectra of ABA3-NifS and cysteine variants.

Supplemental Figure 8



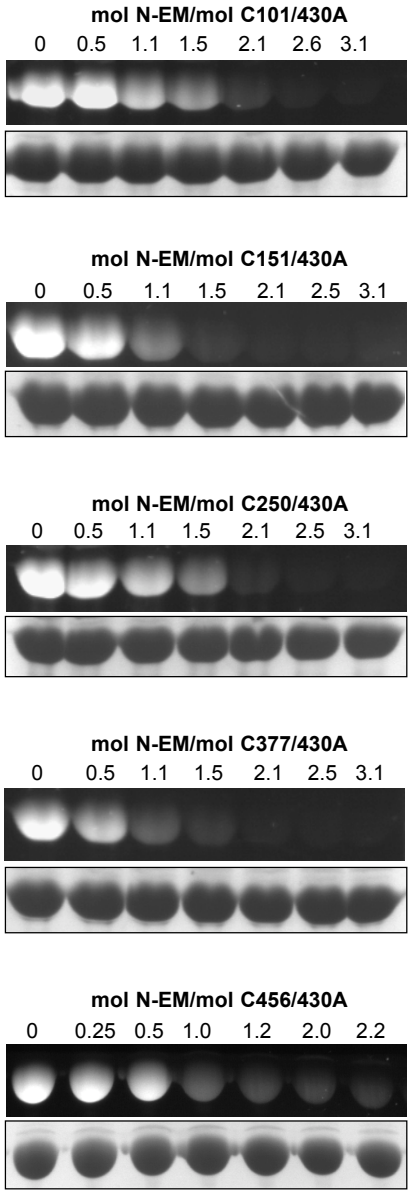
Suppl. Fig. 8: Cysteine desulfurase activity of remaining ABA3-NifS cysteine variants. (**A**) Activities of the variants with single mutations in cysteine101, cysteine151, cysteine206, cysteine250, cysteine377, and cysteine456. (**B**) Activities of the variants with simultaneous mutations in either cysteine101, cysteine151, cysteine206, cysteine250, cysteine377, or cysteine456, and cysteine430 (control: wild-type ABA3-NifS).

Supplemental Figure 9



Suppl. Fig. 9: Quantification of surface-exposed cysteines of the remaining ABA3-NifS cysteine variants. (**A**) N-ethylmaleimide-dependent inhibition of cysteine desulfurase activity. (**B**) N-ethylmaleimide-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).

Supplemental Figure 10



Suppl. Fig. 10: 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-ethylmaleimide-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 perfomed to show equal loading of lanes.

Suppl. Tab. 1: 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.

ABA3-NifS variant	Retention time (mL)	Determined molecular mass (kDa)	Multiple of theoretical monomer mass (57.3 kDa)
ABA3-NifS control	13.892	127.392	2.22
ABA3-NifS-C101A	13.941	124.452	2.17
ABA3-NifS-C151A	13.931	125.046	2.18
ABA3-NifS-C206S	13.854	129.719	2.26
ABA3-NifS-C250A	13.959	123.389	2.15
ABA3-NifS-C377A	13.950	123.919	2.16
ABA3-NifS-C428A	13.603	146.199	2.55
ABA3-NifS-C430A	13.752	136.180	2.38
ABA3-NifS-C435A	13.989	121.638	2.12
ABA3-NifS-C456A	13.943	124.333	2.17