Binding of Sulfurated Molybdenum Cofactor to the C-terminal Domain of ABA3 from Arabidopsis thaliana Provides Insight into the Mechanism of Molybdenum Cofactor Sulfuration

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The molybdenum cofactor sulfurase ABA3 from Arabidopsis thaliana is needed for post-translational activation of aldehyde oxidase and xanthine dehydrogenase by transferring a sulfur atom to the desulfo-molybdenum cofactor of these enzymes. ABA3 is a two-domain protein consisting of an NH2-terminal NifS-like cysteine desulfurase domain and a C-terminal domain of yet undescribed function. The NH2-terminal domain of ABA3 decomposes L-cysteine to yield elemental sulfur, which subsequently is bound as persulfide to a conserved protein cysteinyl residue within this domain. In vivo, activation of aldehyde oxidase and xanthine dehydrogenase also depends on the function of the C-terminal domain, as can be concluded from the A. thaliana aba3/sir3-3 mutant. sir3-3 plants are strongly reduced in aldehyde oxidase and xanthine dehydrogenase activities due to a substitution of arginine 723 by a lysine within the C-terminal domain of the ABA3 protein. Here we present first evidence for the function of the C-terminal domain and show that molybdenum cofactor is bound to this domain with high affinity. Furthermore, cyanide-treated ABA3 C terminus was shown to release thiocyanate, indicating that the molybdenum cofactor bound to the C-terminal domain is present in the sulfurated form. Co-incubation of partially active aldehyde oxidase and xanthine dehydrogenase with ABA3 C terminus carrying sulfurated molybdenum cofactor resulted in stimulation of aldehyde oxidase and xanthine dehydrogenase activity. The data of this work suggest that the C-terminal domain of ABA3 might act as a scaffold protein where prebound desulfo-molybdenum cofactor is converted into sulfurated cofactor prior to activation of aldehyde oxidase and xanthine dehydrogenase.

Molybdenum enzymes catalyze diverse redox reactions in the global carbon, nitrogen, and sulfur cycles (1). In all eukaryotic molybdenum enzymes, the molybdenum atom is coordinated by the dithiolene group of molybdopterin, thus forming the molybdenum cofactor (Moco) (2). According to the coordination chemistry of the molybdenum ligand, eukaryotic molybdenum enzymes can be divided into two groups; Moco with two additional oxo-ligands and a protein-derived cysteinyl sulfur is bound by enzymes of the sulfite oxidase family, whereas enzymes of the xanthine oxidase family have one oxygen, one inorganic sulfur, and one hydroxyl group ligated to the pterin-chelated molybdenum of the active enzyme. Among the four different molybdenum enzymes known in higher plants, sulfite oxidase and nitrate reductase belong to the sulfite oxidase family, whereas aldehyde oxidase (AO) and xanthine dehydrogenase (XDH) are members of the xanthine oxidase family (3). Although it is believed that all of these molybdenum enzymes basically incorporate the same type of Moco, only AO and XDH, but not enzymes of the sulfite oxidase family, require a final enzyme-dependent post-translational modification of the molybdenum center for activity (4). During this modification step, an oxo-ligand of the Moco in inactive AO and XDH enzymes is substituted by a sulfur atom in order to activate AO and XDH.

The first insight into the mechanism of Moco sulfuration was obtained by cloning and biochemical characterization of the Moco sulfurase protein ABA3 from Arabidopsis thaliana (5, 6). The NH2-terminal domain of ABA3 (ABA3-NifS) shares significant similarities to NifS-like cysteine desulfurases, whereas the C-terminal domain did not exhibit striking similarities to any other protein, except other Moco sulfurases and the recently identified mitochondrial amidoxime-reducing component (7). It was shown that, as typical for NifS-like enzymes, ABA3-NifS binds a pyridoxal phosphate cofactor that is essential for activity (6). Furthermore, L-cysteine and L-selenocysteine are decomposed by ABA3-NifS, with L-cysteine representing the preferred substrate with a Km value 4 times lower than that for the selenium substrate. During the decomposition of L-cysteine, L-alanine is released, and elemental sulfur is generated. The sulfur is immediately coupled to a conserved cysteine resi...
idue of ABA3-NifS, thus forming a protein-bound persulfide. Co-incubation of purified ABA3-NifS and cyanide-inactivated AOα from *A. thaliana* as target enzyme in the presence of L-cysteine resulted in activation of the AOα protein, indicating that the persulfide sulfur was transferred from the NifS-like domain of ABA3 to the Moco of AOα. *In vitro*, the presence of the C-terminal domain is not required for sulfuration of xanthine oxidase family enzymes; however, there is strong evidence that it is needed *in vivo*. The tomato *flacca* mutant with a mutation in the Moco sulfurase C-terminal domain (8) is strongly reduced in root AO and XDH activities and does not reveal any activities in the shoots (9). Very recently, the *sir3* mutant *sir3-3* was isolated by a sirtinol resistance screen, which is based on reduced AO activities (10). *sir3-3* was found to have a single point mutation in the C-terminal domain of the *aba3* coding sequence, likewise indicating that the function of the C-terminal domain is required for proper activation of AO (and XDH). It was hypothesized that the function of the C-terminal domain of Moco sulfurases is related to the recognition of the respective target enzymes or one of the protein-bound cofactors (5, 11). Other authors discussed the possibility of the C-terminal domain functioning as sulfur mediator during Moco sulfuration (8, 12).

In this work, we report the biochemical and functional characterization of the C-terminal domain (ABA3-CT) of the Moco sulfurase ABA3 from *A. thaliana*. We assayed the protein for bound molecules that might be transferred to the ABA3 target enzymes AO and XDH. Moreover, the effect of the *sir3-3* mutation was studied in plants as well as on the level of the recombinant protein. Finally, we discuss a possible role for ABA3-CT during Moco sulfuration.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—A 921-bp open reading frame encoding the C-terminal domain of ABA3 was subcloned into pQE80 (Qiagen, Hilden, Germany) as described before (6). The amino acid exchange R723K was introduced into ABA3-CT by PCR-based site-directed mutagenesis, and the respective cDNA was cloned into the BamHI site of pQE80, resulting in an NHEI-terminal His6 tag fusion of ABA3-CT/R723K.

**Expression and Purification of ABA3-CT, ABA3-CT/R723K, AOα, and AtXDH1 from *A. thaliana***—Expression of ABA3-CT and ABA3-CT/R723K was performed in freshly transformed *Escherichia coli* TP1000 cells (13). Cells were grown aerobically in LB medium in the presence of 100 μg/ml ampicillin at 22 °C to an *A*600 = 0.1 before induction with 15 μM isopropyl-β-D-thiogalactopyranoside and the addition of 1 mM sodium molybdate. After induction, cells were grown for a further 20 h at 22 °C. Expression in *E. coli* RK5206 and RK5204 (14) was done likewise but in the absence of sodium molybdate. Overexpression of recombinant His6-tagged AOα (kindly provided by T. Koshiba, Tokyo, Japan) and AtXDH1 in the yeast *Pichia pastoris* was performed as described in Refs. 15 and 16, respectively. Cells were harvested by centrifugation and stored at −70 °C until use. Cell lysis was achieved by several passages through a French pressure cell followed by sonication for 5 min. After centrifugation, His6-tagged protein was purified on a nickel-nitrilotriacetic acid superflow matrix (Qiagen, Hilden, Germany) under native conditions at 4 °C according to the manufacturer’s manual and eluted in elution buffer (50 mM sodium phosphate, pH 6.0, containing 300 mM sodium chloride, 250 mM imidazole, 10% glycerol). Eluted fractions were electrophoretically separated on 12% (for ABA3-CT or 7.5% (for AOα and AtXDH1) SDS-polyacrylamide gels and stained by Coomassie Brilliant Blue.

**NADPH Nitrate Reductase Reconstitution**—Extracts from the *Neurospora crassa nit-1* mutant were prepared as described in Ref. 17 and stored in aliquots at −70 °C. All reconstitutions were performed in *nit-1* buffer (50 mM sodium phosphate, 200 mM NaCl, and 5 mM EDTA, pH 7.2) in the presence of 2 mM reduced glutathione and 5 mM sodium molybdate where appropriate. The reconstitution assay was performed in a 40-μl reaction volume containing 20 μl of gel-filtered *nit-1* extract. Complementation was carried out anaerobically for 2 h at room temperature. After the addition of 20 mM NADPH and incubating for 10 min, reconstituted NADPH-nitrate reductase activity was determined as described in Ref. 17.

**Chemical Detection of Moco and Molybdopterin**—Moco and its metal-free precursor molybdopterin (MPT) were detected and quantified by converting them to the stable oxidation product FormA-dephospho according to Ref. 18. Oxidation, dephosphorylation, QAE chromatography, and HPLC analysis were performed as described in detail in Ref. 19. FormA-dephospho was quantified by comparison with a standard isolated from xanthine oxidase for which the absorptivity was *ε*350 = 13,200 M⁻¹ cm⁻¹ (18).

**Enzyme Assays**—Plant material was squeezed at 4 °C in 2 volumes of extraction buffer (100 mM potassium phosphate, 2.5 mM EDTA, 5 mM dithiothreitol, pH 7.5), sonicated, and centrifuged. Enzyme activities of AO and XDH in plant extracts were detected by activity staining after native PAGE, as previously described in Ref. 20 for AO and in Ref. 16 for XDH. *In vitro* superactivation of recombinant AOα and AtXDH1 by ABA3-CT was performed aerobically in a total volume of 40 μl of 20 mM Tris/HCl, pH 8.0. 0.5 μg of AOα or 2 μg of AtXDH1 were incubated with 10, 20, and 50 μg of Moco-loaded ABA3-CT for 30 min at 22 °C, followed by native PAGE and activity staining with indole-3-carboxaldehyde as substrate for AOα or hypoxanthine as substrate for AtXDH1 according to Refs. 15 and 16. The relative densities of the resulting activity bands were determined by using ImageJ software version 1.38 from the National Institutes of Health (available on the World Wide Web).

**Immunoblot Analysis**—For immunoblot analysis, AOα and ABA3-CT proteins were excised from 7.5% native polyacrylamide gels and subjected to 12% SDS-PAGE. After gel blotting, a primary monoclonal anti-His6 antibody from mouse (1:1000 dilution) and a secondary hors eradish peroxidase-conjugated anti-mouse Ig was used (Sigma; 1:10,000 dilution) to detect chemiluminescence using the ECL system (Amersham Biosciences).

**Determination of Protein Concentrations**—Concentrations of total soluble protein were determined by use of Roti Quant solution (Roth, Karlsruhe, Germany) according to Ref. 21.
**C-terminal Domain of ABA3**

**Wave Scan of ABA3-CT**—Absorption spectroscopy was carried out in elution buffer using an Ultrospec 2100 pro (Amersham Biosciences).

**Inductively Plasma-coupled Mass Spectrometry (ICP-MS)**—For analyses of the molybdenum concentrations, a temperature- and pressure-controlled microwave digestion was performed (CEM Mars 5). The samples were prepared in closed vessels by HNO₃ with a concentration of 6% and heated up in three steps to 200 °C (120, 160, and 200 °C). The temperature of 200 °C was maintained for 10 min. For measurement by ICP-MS, samples were diluted to 1% HNO₃. A Micromass Platform ICP-MS was used. The hexapole ion optic cell was rinsed with hydrogen as a collision gas and with helium as a deceleration gas (both 4.1 ml/min). The hexapole bias was set to −2.0 V. The rate of plasma flow was 15.4 liters/min with an intermediate gas of 1.45 liters/min and a nebulizer gas flow rate of 0.94 liters/min. The reference factor power was 1.3 kilowatts. The sample rate was varied (0–12 μm) during measurement, the following equation was used for determination of the k₂₀ value.

\[
\frac{[AB]}{[A]_{\text{total}}} = \frac{[B]}{[B] + K_0}
\]  

(Eq 1)

**Stabilization of Moco/MPT by ABA3-CT**—A stabilizing effect on Moco/MPT by ABA3-CT was analyzed with 4.8 mg of protein in 600 μl of elution buffer incubated at 4 °C or 22 °C, respectively. For each approach, two samples of equal volume (10 μl each) were taken at the indicated time points, and FormA-dephospho was determined for each sample as described above.

**Plant Material**—Arabidopsis thaliana wild-type and mutant plants aba3-1, aba3-2, 13.5, and sir3-3 were grown in pots containing low nutrient soil in an AR-36L Arabidopsis growth chamber (Percival Scientific) at 10 h light/14 h darkness, 21 °C, and 70% relative humidity for 4 weeks before harvesting leaf material.

**Chemical Sulfuration of Plant Extracts**—Reconstitution of AO and XDH activities in plant extracts was performed according to Ref. 23. 1 g of leaf material was squeezed at 4 °C in 2 ml of extraction buffer (100 mM potassium phosphate, 2.5 mM EDTA, and 5 mM dithiothreitol, pH 7.5), sonicated, and centrifuged. Ammonium sulfate was added to 1.5 ml of the supernatant to a final concentration of 40%. After centrifugation, the ammonium sulfate-precipitated proteins were resolved in 400 μl of extraction buffer and desalted on Sephadex G-50 Nick columns. 0.4 ml of the desalted extract as well as 0.5 M solutions of sodium sulfide and sodium dithionite were made anaerobic by degassing under vacuum and purging with nitrogen. For reconstitution, sulfide and dithionite were added through a septum to the anaerobic extracts to final concentrations of 20 mM. The reaction mixture was incubated for 30 min at 37 °C and desalted on Sephadex G-50 Nick columns before protein concentrations were determined. Finally, 150 μg of total protein of each extract were subjected to native PAGE and stained for AO and XDH activity as described above.

**RESULTS**

**Heterologous Expression and Characterization of ABA3-CT**—ABA3-CT was expressed in E. coli TP1000, yielding a monomeric His₆-tagged protein with a molecular mass of ~35 kDa. Since the E. coli strain TP1000 is characterized by high accumulation of the eukaryotic form of Moco, purified ABA3-CT was analyzed for its ability to bind Moco or its metal-free precursor MPT, respectively. In fact, FormA-dephospho, the stable oxidation product of both Moco and MPT, could be identified on ABA3-CT expressed in TP1000 (Table 1). FormA-
dephospho was also detected when ABA3-CT was expressed under identical conditions in the MPT-accumulating and Moco-free E. coli strain RK5206. However, the amount was reduced to 10–20% of that of the protein derived from TP1000 (data not shown).

Identification of Sulfurated Moco on ABA3-CT—ABA3-CT was analyzed for biologically active Moco by use of the nit-1 reconstitution assay. This assay is based on the transfer of Moco derived from an exogenous source to the nitrate reductase apoprotein of the Moco-deficient N. crassa nit-1 mutant, whereby reconstitution of NADPH-dependent nitrate reductase activity is achieved (15). When omitting supplementary molybdate from the reaction mixture, only active Moco and not molybdenum-free MPT can be detected, since only Moco is capable of reconstituting NADPH-nitrate reductase activity. In the presence of molybdate, however, MPT is nonenzymatically converted to Moco, thereby enabling reconstitution of NADPH-nitrate reductase activity as well. ABA3-CT as purified after heterologous expression in E. coli TP1000 yielded a specific NADPH-nitrate reductase activity of 533 ± 46 nmol nitrite/(mg min) in the absence of molybdate, whereas in the presence of molybdate, a specific activity of 1411 ± 150 nmol nitrite/(mg min) was observed (n = 3). Although the majority of bound pterin obviously is represented by nit-1-inactive cofactor, these results indicate that more than 30% of the total cofactor bound to ABA3-CT is represented by nit-1-active Moco, which was able to reconstitute NADPH-nitrate reductase activity in the nit-1 extract.

Upon quantifying molybdenum contents of ABA3-CT by ICP-MS, 25% of the ABA3-CT monomers were found to contain molybdenum when the cells were expressed in the presence of 1 mM molybdate (Table 1). The same protein fractions showed an average FormA saturation of about 35%, indicating that at least two-thirds of the MPT molecules bound to ABA3-CT contain molybdenum and thus resemble Moco. Since from these experiments it was not clear whether the Moco bound to ABA3-CT is present as Moco, and about 64% of the Moco contains an additional terminal sulfur ligand, as essentially required by enzymes of the xanthine oxidase family. When ABA3-CT was expressed in the absence of supplementary molybdate, FormA as well as molybdenum contents were extremely reduced, and no thiocyanate formation was detected (Table 1), indicating that 1) molybdenum supply during expression in E. coli enhances Moco assembly on ABA3-CT, and 2) the sulfur detected as thiocyanate must derive from Moco. The latter is further supported by the finding that ABA3-CT expressed in the MPT-accumulating (i.e. Moco-free) E. coli strain RK5206 did not release thiocyanate upon cyanide treatment (data not shown).

High Affinity Binding of MPT/Moco to ABA3-CT—A possible stabilizing effect of Moco by ABA3-CT was investigated under aerobic conditions at different temperatures. Under the conditions as described under “Experimental Procedures,” the half-life of Moco/MPT at 22 °C was about 67 h (Fig. 1A). At later time points, precipitation of ABA3-CT protein was observed, whereby further measurements were prohibited. When performing the experiment at 4 °C, the half-life was enhanced to 125 h with more than 33% Moco/MPT still detectable after 334 h. The final fractions of both experiments were found to

| Protein          | FormA | Molybdenum | SCN⁻ |
|------------------|-------|------------|------|
|                   | mol/mol | mol/mol   | mol/mol |
| ABA3-CT + MoO₄⁻ | 0.35 ± 0.058 | 0.25 ± 0.02 | 0.16 ± 0.052 |
| ABA3-CT - MoO₄⁻ | 0.104 ± 0.013 | 0.014 ± 0.001 | ND |

C-terminal Domain of ABA3

FIGURE 1. Characteristics of Moco-binding to ABA3-CT. A, stabilizing effect of ABA3-CT on Moco/MPT at 4 °C (solid line) and 22 °C (dashed line). The halflife of Moco/MPT bound to ABA3-CT is indicated by the dotted horizontal line. B, high affinity binding of Moco to ABA3-CT. Bound Moco/MPT per ABA3-CT monomer in relation to unbound Moco/MPT in solution.
contain intact Moco, since both fractions were able to reconstitute NADPH-nitrate reductase activity in extracts of the N. crassa nit-1 mutant in the absence and presence of additional molybdate (data not shown). These data suggest that ABA3-CT very efficiently protects Moco/MPT from rapid and irreversible degradation by oxidation.

In order to determine a dissociation constant ($k_d$) for the binding of Moco/MPT to ABA3-CT, 6 μM cofactor-free purified ABA3-CT expressed in *E. coli* expression strain RK5204 was co-incubated with different concentrations (0–12 μM) of Moco/MPT extracted from recombinant *C. reinhardtii* MCP, which can be purified in large amounts and which specifically binds Moco rather than MPT. After coinubation, the samples were transferred to a 10,000 molecular weight cut-off concentrator, and unbound cofactor was separated from ABA3-CT-bound cofactor by ultracentrifugation. Moco/MPT within the flow-through was immediately converted to the stable oxidation product FormA and quantified by HPLC. As a control, the same experiment was performed with free Moco/MPT in the absence of ABA3-CT. After quantification of free Moco/MPT in the flow-through of samples incubated in the presence and absence of ABA3-CT, determination of a $k_d$ value for cofactor binding to ABA3-CT was possible (Fig. 1B). The amount of Moco/MPT bound to ABA3-CT and the concentrations of free ABA3-CT were determined according to the calculated concentrations of free and total Moco/MPT and the known amount of total ABA3-CT. The maximum Moco/MPT saturation of ABA3-CT under the given experimental conditions was determined to be 0.89, suggesting a Moco/MPT to ABA3-CT ratio of 1:1. A $k_d$ value of 0.55 ± 0.14 μM was obtained, whereby high affinity binding of Moco/MPT to ABA3-CT is demonstrated.

Spectroscopic Properties of Moco-containing ABA3-CT—UV-visible spectra of Moco-loaded ABA3-CT showed absorption around 315 nm, between 350 and 400 nm, and also between 450 and 550 nm when the protein was analyzed directly after aerobic purification (Fig. 2A). Very similar to this, also the anaerobically purified protein revealed absorption around 315 nm and between 350 and 400 nm directly after purification. However, in contrast to the aerobically purified protein, the third absorption band of anaerobically purified protein was shifted to the range between 500 and 580 nm. Upon oxidation in air for 20 h, the spectra of both aerobically and anaerobically purified protein developed the same distinct absorption around 315 nm and maxima at 395 and 465 nm, indicating that Moco bound to ABA3-CT is sensitive to oxidation, with the most sensitive absorption range between 350 and 580 nm. The addition of reducing agents like sodium dithionite (Fig. 2B) and oxidizing agents like potassium cyanide (Fig. 2C) to ABA3-CT for 2 or 20 h did not cause significant changes in the overall absorption. However, oxidation proceeded faster and was more pronounced in the presence of potassium cyanide in comparison with sodium dithionite. This effect has also been observed when using other reducing agents like dithiothreitol and β-mercaptoethanol or oxidizing agents like ferricyanide, respectively (supplemental Fig. 1). When fully oxidized ABA3-CT was titrated with sodium dithionite under anaerobic conditions, the absorption between 500 and 580 nm that has been observed earlier for anaerobically prepared protein was reconstituted (Fig. 3). These results not only confirm the redox sensitivity of this particular absorption band but also indicate that ABA3-CT is obtained in a (nearly) fully reduced state when purified under anaerobic conditions and in a partially reduced state when purified under aerobic conditions. It is noteworthy that the terminal sulfur ligand of the ABA3-CT-bound Moco obviously has no influence on the spectroscopic properties of the protein since the addition of cyanide, which is known to release the terminal sulfur ligand from the Moco of xanthine oxidase family enzymes (see “Experimental Procedures”), did not cause specific alterations of the spectrum.

Similar to plant (24) and animal sulfite oxidase proteins (25) and to the recently described Moco carrier protein MCP from *C. reinhardtii* (22), the absorption around 395 nm is likely to derive from the ene-dithiolate group of bound Moco.
tion around 500 nm has been ascribed to a cysteine-to-molybdenum charge transfer band (25) for the Moco-binding domain of rat sulfite oxidase, and we therefore assume that the absorption of Moco-loaded ABA3-CT between 450 and 500 nm also is caused by a ligand-to-molybdenum charge transfer band. This assumption is supported by the observation that ABA3-CT obtained from E. coli RK5206, which accumulates metal-free MPT and is unable to generate Moco, lacks absorption in this particular range (supplemental Fig. 2). In contrast, no influence has been observed on the absorption at 315 nm, indicating that the origin of this absorption band is unrelated to the molybdenum-metal. Rather, it should derive from the MPT-moiety, since ABA3-CT, expressed in the MPT/Moco-free E. coli strain RK5204, is lacking any of the absorptions typically observed for Moco-loaded ABA3-CT (data not shown). Like for Moco-bound ABA3-CT, absorption between 500 and 580 nm has been observed also for MCP from C. reinhardtii (22). Since this absorption is also absent in the ABA3-CT protein expressed in MPT-accumulating E. coli RK5206, it is probably related to the molybdenum center like the absorption between 450 and 500 nm.

The sir3-3/R723K Variant of ABA3-CT Is Affected in Moco Binding—Recently, a new aba3 mutant from A. thaliana, sir3-3, was isolated by a specific screening procedure based on the resistance of the mutant plant toward sirtinol treatment (10). The sir3-3 mutant was found to contain a G-to-A transversion at nucleotide 2168 within the coding region that caused an exchange from arginine to lysine at position 723 (R723K) within the C-terminal domain of the ABA3 protein. Since it was unclear whether and to what extent the mutation affects the function of ABA3, the activities of its target enzymes AO and XDH were analyzed in extracts of sir3-3 plants and compared with AO and XDH activities of other aba3 mutant plants, including aba3-1, aba3-2, and 13.5 (Fig. 4A). Although aba3-1 presents a single substitution of glycine 469 by glutamic acid within the NifS-like domain of ABA3, the mutants aba3-2 and 13.5 are characterized by deletions within the aba3 gene.

accompanied by framaoffs and early truncation of the aba3 open reading frame (5). In contrast to extracts of aba3-1, aba3-2, and 13.5, where no AO and XDH activities have been detected, extracts of sir3-3 mutants showed a residual XDH activity, indicating that the sir3-3 mutation is leaky to a certain extent. In order to prove whether the lack of the terminal sulfur ligand is the primary cause of AO and XDH deficiency in the sir3-3 mutant, as was shown for the mutants aba3-1 and aba3-2 (23), leaf extracts of all mutants were treated anaerobically with sodium sulfide and sodium dithionite (B). In gel activity staining for AO was performed by using a combination of indole-3-carboxaldehyde and 1-naphthaldehyde as substrate; XDH activity was developed in the presence of hypoxanthine as substrate.

FIGURE 3. UV-visible spectrum of ABA3-CT after anaerobic titration with sodium dithionite. Fully oxidized ABA3-CT expressed in E. coli TP1000 was made anaerobic prior to nine successive additions of equal amounts of anaerobic sodium dithionite solution. The spectrum of ABA3-CT before the addition of sodium dithionite is indicated by the heavy solid line, and the spectrum of ABA3-CT after the last addition of sodium dithionite is indicated by the heavy dashed line. Spectra of intermediate additions are indicated by light solid lines.

FIGURE 4. Reconstitution of AO and XDH activities in extracts of sir3-3 mutants by in vitro sulfuration. 150 μg of total extracts of the A. thaliana aba3 mutants aba3-1, aba3-2, 13.5, and sir3-3 were separated on native PAGE either without (A) or subsequent to anaerobic treatment with sodium sulfide and sodium dithionite (B). In gel activity staining for AO was performed by using a combination of indole-3-carboxaldehyde and 1-naphthaldehyde as substrate; XDH activity was developed in the presence of hypoxanthine as substrate.
**C-terminal Domain of ABA3**

**TABLE 2**

Moco/MPT and terminal sulfur contents of the ABA3-CT/R723K protein

| FormA/protein | Thiocyanate/FormA |
|---------------|-------------------|
| ABA3-CT control | 0.243 ± 0.057 |
| ABA3-CT/R723K | 0.142 ± 0.035 |

* Due to the low absorption at 460 nm (<0.002), the values obtained during this experiment did not allow absolute quantification.

3/R723K mutation into heterologously expressed ABA-CT, it was found that the altered protein bound less Moco/MPT relative to the control protein (Table 2). Besides significant reduction of Moco/MPT, a strong reduction of thiocyanate formation has also been observed for the R723K variant, suggesting that the amount of terminal sulfur is drastically reduced and that most, if not all, of the Moco bound to this protein is present in the desulfo-form.

**Activation of AOα and AtXDH1 by Moco-containing ABA3-CT**—The separately expressed NifS-like domain of ABA3 was shown previously to be capable of activating recombinant AOα in vitro, whereas ABA3-CT did not show a stimulating effect on the activity of AOα (6). However, the ABA3-CT protein used for those assays was expressed under different conditions and did not contain Moco. In the present work, AOα and AtXDH1 as purified after heterologous expression in *P. pastoris* were co-incubated with Moco-loaded ABA3-CT for 30 min at 22 °C prior to determination of AO and XDH activities. In fact, Moco-loaded ABA3-CT superactivated the activity of both AOα and AtXDH1, indicating a stimulating function of ABA3-CT, which led to the activation of so far inactive forms of AOα and AtXDH1 (Fig. 5, A and B). The appearance of a new activity band indicates the formation of a complex generated in the presence of Moco-loaded ABA3-CT and probably consisting of ABA3-CT and its target enzymes AOα and AtXDH1. In order to prove this assumption, the newly appearing activity band was excised from the native polyacrylamide gel and subjected to an additional SDS-PAGE. Subsequent immunoblotting using anti-His antibodies showed that this band in fact contained both proteins, ABA3-CT and AOα (Fig. 5C). Such complex formation has been observed earlier in co-incubation experiments with full-length ABA3 and AtXDH1 (16). In contrast, co-incubation of ABA3-NifS with AOα or AtXDH1, respectively, did not result in formation of such a complex (6), indicating that the C-terminal domain of ABA3 rather than the NifS-like domain mediates interaction with the target enzymes of ABA3. To our surprise, ABA3-CT was found to accumulate also in the lower activity band after co-incubation with AOα (Fig. 5C), which may indicate the general necessity of a protein-protein interaction for activation of AOα.

**DISCUSSION**

The process of Moco sulfuration in eukaryotes, as required by molybdenum enzymes of the xanthine oxidase family, depends on the specific function of Moco sulfurate enzymes. All eukaryotic Moco sulfurate known to date generally feature two domains (5, 8, 11, 26–28), of which only the NH2-terminal NifS-like domain is basically understood, since it was shown to be responsible for the mobilization of sulfur from L-cysteine (6). Furthermore, in vitro ABA3-NifS alone was shown to be able to sulfurate the Moco of recombinant AOα (6). However, it was known that in vivo besides the NifS-like domain also the C-terminal domain of Moco sulfuratases is required for activation of AO and XDH. For the Moco sulfurate mutant *flacca* of tomato, a 6-bp deletion within the C-terminal domain was reported to be the primary cause of combined AO and XDH deficiency (8). Recently, the *aba3* mutant *sir3-3* from *A. thaliana* has been predicted to be deficient in AO-activity, since it presents an impaired capacity to oxidize the sirtinol derivative 2-hydroxy-1-naphthaldehyde to 2-hydroxy-1-naphthoic acid, which activates auxin signaling (10). This mutant harbors a point mutation within the C terminus coding region of *aba3*, leading to a substitution of the strictly conserved arginine 723 of the ABA3 protein by a lysine (R723K). Interestingly, a human xanthinuria
type II patient was described as carrying the same mutation in the C-terminal domain of the human Moco sulfurase HMCS (29), except that the conserved arginine is exchanged for a cysteine residue (R776C). Both mutations not only prove the importance of the C-terminal domain for Moco sulfuration in general but also underline the specific relevance of the motif including the conserved arginine residue 723 in ABA3 or 776 in HMCS, respectively. However, experimental information about the contribution of the C-terminal domain for activation of AO and XDH was not available as yet. It was suggested that the C-terminal domain might have a role in recognizing the target enzymes of Moco sulfuration, thereby serving as a bridge to allow sulfur transfer from the NiFS-like domain to the Moco of AO and XDH (5, 11). The existence of an invariant cysteine residue within the C-terminal domain (cysteine 758 in ABA3), which is also conserved in YiiM proteins of bacteria, led others to assume that the C-terminal domain might be a sulfur carrier domain that receives sulfur mobilized by the NiFS-like domain in the form of a persulfide on its conserved cysteine and delivers it for the formation of sulfurated Moco (12). However, no such persulfide formation has been shown on the C-terminal domain of a Moco sulfurase protein up to now.

The present work provides evidence that the C-terminal domain of ABA3 actually serves as the scaffold for assembly of sulfurated Moco and that binding of sulfurated Moco to the C-terminal domain is a basis for activation of AO and XDH. In fact, Moco/MPT binding to ABA3-CT is highly specific, as indicated by the protective effect of ABA3-CT on Moco/MPT expressed as a high half-life of ABA3-CT-bound Moco/MPT. A $k_d$ of 0.55 μM, which is similar to those observed for the separately expressed domains of the Moco biosynthetic protein Cnx1 with $k_d$ values of 0.1 μM (Cnx1G) and 1.6 μM (Cnx1E), respectively (19), further confirms the specificity of Moco/MPT binding to ABA3-CT. With a $k_d$ of about 3.5 μM (31), also XDHC from *Rhodobacter capsulatus* has Moco/MPT-binding characteristics similar to ABA3-CT. It is a common feature of Cnx1 and XDHC that the Moco generated by Cnx1 or bound by XDHC, respectively, has to be passed on to the apo-forms of molybdenum enzymes for converting them into the respective holo-forms. Although the direct acceptor proteins of Cnx1-generated Moco are not identified as yet, it is known that bacterial XDHC proteins are required for the co-translational insertion of sulfurated Moco into apo-XDH (30–33), which was shown to depend on an interaction between XDHC and the XDHB subunit of XDH (31). Although ABA3-CT comprises similar properties like XDHC, namely binding of sulfurated Moco and the ability to interact with its target enzymes, its function is markedly different from the function of XDHC; the activity of ABA3-CT for activation of AO and XDH is required post-translationally rather than co-translationally as in case of XDHC. Evidence for this assumption has been obtained by the characterization of the *A. thaliana sir3-3*/R723K mutation, which causes dramatic reduction of AO and XDH activities. Since the activities of both enzymes were restored in extracts of *sir3-3* plants by sulfide/dithionite treatment, it was proven that Moco is present in both enzymes but in its desulfo-form. We have therefore to conclude that the C-terminal domain of ABA3 is not required for the initial insertion of Moco into the apoproteins of AO and XDH but exclusively for the post-translational sulfuration of Moco-containing holoenzymes.

Recombinantly expressed ABA3-CT harboring the *sir3-3*/R723K mutation was shown to accumulate less than 60% Moco/MPT relative to control proteins, and the amounts of terminal sulfur bound to the Moco of the R723K variant were found to be below the detection limit. It appears unlikely that the loss of Moco sulfuration activity in the *sir3-3* mutants is due to the decrease of ABA3-CT-bound total Moco, since 60% of residual Moco should be sufficient for providing significant Moco sulfuration activity for activation of AO and XDH if this Moco is sulfurated at least partially. However, considering that total Moco/MPT bound to ABA3-CT is about 45% sulfurated (Table 1), one can assume that the decrease of Moco/MPT by more than 40% and the concomitant loss of terminal sulfur are caused by the reduced capacity of the ABA3-CT/R723K protein to bind sulfurated Moco. Therefore, our results rather indicate that the primary cause of AO and XDH deficiency in the *sir3-3* mutant is the decreased capacity to bind sulfurated Moco, and they also suggest that arginine 723 is involved in binding of the sulfurated Moco. Furthermore, the results obtained from the ABA3-CT/R723K variant allow us to hypothesize that the C-terminal domain of ABA3 has two different species of Moco bound, one with a terminal sulfur ligand and another without this additional ligand. The amounts by which Moco/MPT and the terminal sulfur are decreased in the R723K variant might imply that only the binding affinity for sulfurated Moco is reduced, whereas the affinity for desulfo-Moco remains unaltered.

The NiFS-like domain of ABA3 abstracts sulfur from L-cysteine and binds it in the form of a persulfide (6). It was suggested that in vivo the NiFS-like domain of ABA3 transfers its persulfide to the conserved cysteine 758 to generate another persulfide at the C-terminal domain, which subsequently is transferred to the Moco of AO and XDH (12). However, different from this model, our results suggest that if a persulfide is bound to the C-terminal domain of ABA3, this persulfide sulfur is subsequently ligated to the molybdenum atom of the Moco bound to the C-terminal domain of ABA3 rather than to the Moco of AO and XDH. This of course raises the question of how the sulfur finally is transferred from the C-terminal domain of ABA3 to AO and XDH. Two possibilities can be discussed: 1) the terminal sulfur of the Moco bound to the C-terminal domain of ABA3 is transferred as such to the Moco of AO and XDH, or 2) the whole sulfurated Moco molecule is transferred from the C-terminal domain of ABA3 to AO and XDH. Since AO and XDH already contain desulfo-Moco before being processed by ABA3, the latter alternative demands replacement of this desulfo-Moco by the sulfurated Moco of ABA3. The fact that ABA3-CT is able to superactivate recombinant AOα and AtXDH1 neither excludes nor supports one of both possibilities. Hence, it confirms that the C-terminal domain is capable of transferring sulfur to AO and XDH proteins, either in a low molecular form or in the higher molecular form as sulfurated Moco.
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