Biochemical Characterization of Molybdenum Cofactor-free Nitrate Reductase from *Neurospora crassa*

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Background: Eukaryotic nitrate reductase maturation is poorly understood.

Results: Binding of molybdenum cofactor to apo-nitrate reductase is independent from other prosthetic groups.

Conclusion: Active site formation of eukaryotic nitrate reductase is an autonomous process intrinsically tied to nitrate reductase dimerization.

Significance: The understanding of molybdenum cofactor-dependent enzyme maturation is of significance as molybdenum enzymes are involved in essential cellular processes.

Nitrate reductase (NR) is a complex molybdenum cofactor (Moco)-dependent homodimeric metalloenzyme that is vitally important for autotrophic organism as it catalyzes the first and rate-limiting step of nitrate assimilation. Beside Moco, eukaryotic NR also binds FAD and heme as additional redox active cofactors, and these are involved in electron transfer from NAD(P)H to the enzyme molybdenum center where reduction of nitrate to nitrite takes place. We report the first biochemical characterization of a Moco-free eukaryotic NR from the fungus *Neurospora crassa*, documenting that Moco is necessary and sufficient to induce dimer formation. The molybdenum center of NR reconstituted in vitro from apo-NR and Moco showed an EPR spectrum identical to holo-NR. Analysis of mutants unable to bind heme or FAD revealed that insertion of Moco into NR occurs independent from the insertion of any other NR redox cofactor. Furthermore, we showed that at least in vitro the active site formation of NR is an autonomous process.

In autotrophic organisms the major pathway for assimilating inorganic nitrogen is the nitrate assimilation pathway, with nitrate reductase (NR) catalyzing the first and rate-limiting step. NR is a molybdenum cofactor (Moco)-dependent enzyme that shares its cofactor with a family of four other plant enzymes that are involved in sulfate detoxification, purine catabolism, and abscisic acid biosynthesis (1). As a unifying characteristic, these enzymes catalyze two-electron transfer (redox) reactions involving a molybdenum atom coordinated in Moco (2). This cofactor is a unique pterin derivative conserved among all kingdoms of life, and with the exception of the bacterial nitrogenase, it is found in all enzymes that hold a molybdenum atom in their active site. In addition to Moco, eukaryotic NRs harbor with heme and FAD two other prosthetic groups. For nitrate reduction, NR uses NAD(P)H as an electron donor that provides two electrons to the FAD cofactor that are subsequently transferred to the heme group and finally reduce the molybdenum center of the enzyme (3). In eukaryotes, NRs form a family of enzymes that share a high degree of sequence homology. Differences exist concerning the use of NADH or NADPH as electron donors for nitrate reduction. NADH-specific NR forms are found in higher plants and algae, in contrast to the less frequently occurring NAD(P)H-bispecific NR forms found in higher plants, algae, and fungi. NR forms specific for NADPH are only found in fungi (4). Bacterial NRs are completely different from their eukaryotic counterparts both in sequence and in structural composition. Eukaryotic NR is only functional as a homodimer. The NR monomer has a size of ~100 kDa and harbors its three redox cofactors, Moco, heme, and FAD, in three structurally distinct domains (4, 5) (Fig. 1A). Furthermore, there is a dimerization domain that is located C-terminal to the Moco binding domain. The NAD(P)H and FAD binding domains form the so-called cytochrome b reducing fragment (CbR). Combination of the heme binding domain with the CbR fragment builds the so called cytochrome c reducing fragment (CcR). Both CbR and CcR are functional units of the NR protein capable to reduce artificial electron acceptors like ferricyanide or cytochrome c, whereby the former accepts electrons directly from the CbR fragment and the latter is reduced by the CcR fragment (Fig. 1A). Other than the cofactor housing domains, the connecting hinge regions as well as an N-terminal extension preceding the Moco domain are not conserved among eukaryotic NRs (Fig. 1B). In plants the hinge 1 region, which connects the Moco and heme domain, was shown to be important for NR activity regulation (6), and likewise the N-terminal extension is thought to possibly have a
function in the post-transcriptional regulation of NR activity (7).

There are indications for Moco to function in the dimerization process of NR (4, 8–11); however, contrary results indicate that the dimerization domain functions autonomously (12). The insertion of Moco into molybdenum-containing enzymes is an ill-defined process. Also, the insertion of other prosthetic groups during the maturation of this class of metalloenzymes is...
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poorly understood. The reason for this gap in knowledge is that the study of cofactor insertion was hampered by the availability of well defined, stable apo-enzyme proteins in sufficiently large amounts. So far the recombinant expression and purification of a Moco-free eukaryotic NR was not reported, and all attempts to characterize the oligomerization state of Moco-free NR were constrained to whole cell extracts of Moco-deficient mutants from plants and fungi.

Virtually nothing is known about the influence of the two remaining redox active cofactors heme and FAD on the formation of the physiologically active, homodimeric eukaryotic NR. Also the sequence of redox cofactor incorporation into eukaryotic NR and likewise the underlying principles are still an open question.

In this work we characterized the recombinant Moco-free eukaryotic NR from the filamentous fungus Neurospora crassa. Biochemical characterization of the apo-enzyme revealed that Moco is solely sufficient to induce NR dimer formation. Furthermore, the sequence of prosthetic group insertion was found to be independent from Moco bound to NR.

EXPERIMENTAL PROCEDURES

Cloning of the Neurospora NR—The gene encoding Neurospora NR has been identified by Okamoto et al. (13), and the Neurospora gene-locus NCU05298 has been assigned to the NR encoding sequence (14, 15). Therefore, we cloned the gene of Neurospora NR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) with primers derived against the locus NCU05298. The oligonucleotides designed to amplify the coding DNA sequence of Neurospora NR and its variants Neurospora NR variant H654A/H677A, codons 654 and 677 (CAT) and 677 (CAC) were changed to GCC, resulting in the conversion of both histidines to alanines. Three single amino acid variants with altered FAD binding characteristics were generated based upon the pQE-80L vector (Qiagen GmbH). Different E. coli strains were used for recombinant NR production, thus allowing the expression of Moco-free NR (E. coli strain RK5204 (17)), molybdopterin (MPT)-containing NR (E. coli strain RK5206 (17)), or Moco containing NR (E. coli strain TP1000 (18)). Expression of Neurospora NR was carried out in LB medium containing 50 μg/ml ampicillin at 22 °C. For expression in TP1000 cells, 10 mM sodium molybdate was additionally added. After cell density reached an A600 nm = 0.1, NR expression was induced with 20 μM isopropyl-1-thio-β-D-galactopyranoside, and cells were allowed to grow aerobically for 20 h. For production of NR CcR fragments, E. coli BL21 cells were used. Expression was carried out in LB medium containing 50 μg/ml ampicillin at 30 °C. After cell density reached an A600 nm = 0.2, cells were induced with 50 μM isopropyl-1-thio-β-D-galactopyranoside and allowed to grow aerobically for 20 h. Cell lysates were achieved by two passages through a French pressure cell. Upon this, cells were sonicated for 1 min on ice. Cell lysis was carried out at 4 °C. After centrifugation, double-tagged proteins were purified at 4 °C under native conditions. In the first purification step Ni-NTA Superflow resin (Qiagen) was used according to the manufacturer’s instructions. Cell lysis buffer contained 100 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole, 2% (v/v) glycerol (pH 8.0). Washing steps were carried out using washing buffer containing 100 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 2% (v/v) glycerol (pH 8.0). Proteins were eluted in elution buffer (100 mM Tris-HCl, 150 mM NaCl, 250 mM imidazole, 2% (v/v) glycerol (pH 8.0)). All buffers were degassed before use. Eluted fractions were pooled and subsequently loaded on Strep-Tactin Superflow® high capacity resin (IBA). Washing steps were carried out using washing buffer containing 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 2% (v/v) glycerol. Proteins were eluted in elution buffer (100 mM Tris-HCl, 150 mM NaCl, 20 mM D-desthiobiotin). Purity of the eluted protein was routinely confirmed by Coomassie Blue staining after SDS-PAGE. Pure protein fractions were concentrated (Vivaspin 6, Sartorius AG) and stored in 20-μL aliquots in liquid nitrogen.

Size Exclusion Chromatography—Purified recombinant proteins were analyzed by gel filtration chromatography using an analytical Superdex 200 column (GE Healthcare) connected to an Äkta purifier system (Amersham Biosciences). As running buffer, 100 mM Hepes-KOH, 150 mM NaCl, 5 mM EDTA, and 5% (v/v) glycerol (pH 7.5) was chosen. Molecular weight standards (GE Healthcare) were used for calibration according to the manufacturer’s instruction.
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**CD Spectroscopy**—CD spectra were recorded on a Jasco Model J-810 spectropolarimeter (Jasco) at 20 °C. CD spectra of purified protein preparations were recorded at protein concentrations of 1 μM in 50 mM sodium phosphate, 100 mM NaF, and 2% (v/v) glycerol (pH 7.2) using quartz glass cuvettes of 1-mm cell path length between 350 and 180 nm at 0.1-nm intervals. A minimum of 10 scans was recorded, and base-line spectra were subtracted from each spectrum. Data analysis was performed using CDPro software (19).

**Analytical Ultracentrifugation**—Sedimentation velocity experiments were carried out in a Beckman Coulter ProteomeLab XL I analytical ultracentrifuge at 35,000 rpm and 20 °C in a buffer containing 0.15 M NaCl, 1 mM EDTA, and 50 mM Tris-HCl (pH 8.0) using an An-50 Ti rotor. Concentration profiles were measured using the manufacturer’s data acquisition software ProteomeLab XL-I Graphical User Interface Version 6.0 (firmware 5.06) and the UV absorbance scanning optics at 280 nm. Experiments were performed in 3- or 12-mm double sector centerpieces filled with 100 or 400 μl of sample, respectively. Due to thermal equilibration of the rotor before the start of the run, diluted samples were incubated at least 2 h at 20 °C before centrifugation. Because it was recently found that version 6.0 of the data acquisition software records incorrect elapsed time in the data files, the program SEDFIT was used to determine correction factors and to correct scan times (20). Correction factors were in the range of 1.076–1.078. Data analysis was performed using a model for diffusion-deconvoluted differential sedimentation coefficient distributions (c(s) distributions) implemented in SEDFIT (21). Partial specific volume, buffer density, and viscosity were calculated by the program SEDNTERP (22) and were used to correct the experimental sedimentation coefficients to $s_{20,w}$. Contributions of bound cofactors to partial specific volume of the enzyme were not taken into account. Protein concentrations were determined spectrophotometrically using the absorption coefficients at 280 nm as calculated from amino acid composition (23) and are given in monomers throughout the text.

**EPR Spectroscopy**—For EPR spectroscopy proteins were incubated in 100 mM HEPES-KOH, 150 mM NaCl, 5 mM EDTA, 5% (v/v) glycerol (pH 7.5) containing 50 μM FAD and 20 mM KNO$_3$. Reduction of NR molybdenum was initiated by the addition of 20 mM NADPH. Samples were incubated for 25 s at room temperature and frozen in an ethanol/liquid nitrogen mixture. EPR spectra were recorded at X band frequency (9.4314 GHz) with a Bruker model EMX-6/1 spectrometer equipped with a standard TE102 rectangular cavity. The temperature was maintained at 77 K by immersion of the samples in partially silvered liquid nitrogen finger Dewar. A correction for the deviation of the magnetic field measured by the EMX-032T Hall probe at the position of the sample was calculated from the $g$ value of the strong pitch ($g = 2.0028$) and the microwave frequency of the ER-041–1161 counter. Spin concentrations were calculated by double integration and comparison with a 1 mM Cu$^{2+}$EDTA standard under non-saturating conditions (i.e. a microwave power of <0.2 milliwatt for Mo$^{5+}$ and <20 micro-watts for Cu$^{2+}$). A correction for the signals of $^{95}$Mo and $^{97}$Mo nuclear hyperfine split signals as well as a correction for the undetectable signals of the $^{95}$Mo and $^{97}$Mo hyperfine split signals was made. To obtain a suitable signal-to-noise ratio for comparison of spectral shape and intensity, multiple spectra (up to 32) were recorded at a 2-milliwatt microwave power and 0.65-milliTesla modulation amplitude (modulation frequency, 100 kHz).

**Quantification of Moco/MPT**—The amount of total Moco/MPT was quantified by HPLC FormA analysis as described earlier (24).

**Inductively coupled Plasma Mass Spectrometry (ICP-MS)**—Molybdenum content of NR was quantified with Agilent 7700 Series ICP-MS (Agilent Technologies) according to a standard calibration curve of inorganic molybdenum (Fluka). The detection limit of ICP-MS for molybdenum ranged between 1 and 20 μg/liter. Protein solutions and standards were mixed automatically with rhodium as an internal standard. All values were corrected for the molybdenum content of control samples consisting of buffer alone. All data were collected and processed using MassHunter work station software.

**Quantification of Heme**—The amount of heme bound to NRs was quantified using a QuantChrom heme assay kit purchased from GENTAUTM, and changes in absorbance at 400 nm were measured in a TECAN sunriseTM microplate reader. For calculation of NR, heme content UV-visible absorption measurements were performed with a PerkinElmer Life Sciences Lambda 25 spectrophotometer using $ε = 120,000$ M$^{-1}$ cm$^{-1}$ at 412 nm for heme in oxidized redox state (25) and $ε = 156,760$ M$^{-1}$ cm$^{-1}$ at 280 nm for NR.

**Nitrate Reducing Activity**—Nitrate reducing activity was determined according to Evans and Nason (26) with slight modifications. For typical enzyme assays, 50–600 ng of purified NR was incubated in 50 mM sodium phosphate, 200 mM NaCl, 5 mM EDTA (pH 7.2) buffer containing 1 mM NADPH, 10 mM potassium nitrate, 50 μM FAD. The reaction was started with potassium nitrate and incubated at room temperature for 2–30 min. Enzyme assays were terminated by the addition of 0.6 M zinc acetate. For quantification of nitrite converted from nitrate, 2 volumes of 1% (w/v) sulfanilamide in 3 M HCl and 2 volumes 0.02% (w/v) $N$-(1-naphthyl)-ethylenediamine dihydrochloride were added. After incubation for 10 min at room temperature, the solutions were centrifuged (13,000 × g) for 5 min, and the absorbance was measured at 540 nm using TECAN sunriseTM microplate reader. According to nitrite standard curve NR activity ($μmol$ of nitrite/min × mg of protein) was calculated.

**NADPH-dependent Cytochrome c Reducing Activity**—NR NADPH-dependent cytochrome c reducing activity was determined according to Garrett and Nason (27). For a typical cytochrome c assay, 100–500 ng of purified NR were incubated in 50 mM sodium phosphate, 200 mM NaCl, 5 mM EDTA (pH 7.2) buffer containing 5 μM FAD, 82 μM cytochrome c, and 0.1 mM NADPH. Cytochrome c reductase activity was measured by observing the increase in absorbance at 550 nm with a PerkinElmer Life Sciences Lambda 25 spectrophotometer. The molar extinction coefficient for cytochrome c of $ε = 19,600$ M$^{-1}$ cm$^{-1}$ (reduced-oxidized) was used to calculate the enzyme activity ($μmol$ of reduced cytochrome c/min × mg NR). Conversion of NADPH was followed at 340 nm and quantified by its extinction coefficient, $ε = 6,220$ M$^{-1}$ cm$^{-1}$ (Sigma).
NR in Vitro Reconstitution—For reconstitution of Moco-free NR various amounts of purified Moco carrier protein (MCP) and apo-NR were co-incubated in degassed 100 mM Heps-KOH, 150 mM NaCl, 5 mM EDTA, 5 mM glutathione, 5% (v/v) glycerol (pH 7.5) buffer for 3 h at room temperature or at 4 °C overnight. MCP was expressed and purified as described earlier (28). NR in vitro reconstitution was analyzed using HPLC-based FormA quantification, NR activity measurements, and dimer formation (SEC) as described above.

Isothermal Titration Calorimetry—Isothermal titration Calorimetry (ITC) titrations were carried out at 25 °C using a MicroCal VP-ITC instrument (GE Healthcare). Before the titration, proteins and FAD were dialyzed against 100 mM Tris HCl, 150 mM NaCl, 1 mM EDTA buffer (pH 8.0) at 4 °C for 2 days. After dialysis, the protein concentration was determined using the Bradford protein assay (29). The FAD concentration was determined spectrophotometrically using a molar extinction coefficient of 11,300 m^−1 cm^−1 at 450 nm. The reference cell of the ITC instrument was filled with dialysis buffer. In a typical experiment the respective CcR fragment (20–35 μM) in the ITC cell was titrated with FAD (0.5 mM) loaded into the ITC syringe. The first injection (2 μl, obtained from analysis) was followed by 19 injections of 10 μl, with 210-s intervals between injections. The cell contents were stirred at 307 rpm to provide immediate mixing. The data were collected automatically and base-line correction, peak integration, and binding parameters were performed using the ORIGIN analysis software. Normalized area data were plotted in kcal/mol of injectant versus the molar ratio of FAD/CcR. Non-linear fitting of data points was performed with the one set of sites binding model, and binding constants were calculated.

RESULTS

Prosthetic Group Content of Recombinant Holo- and Moco-free Neurospora NR—Neurospora NR was purified by two-step affinity chromatography from the Moco accumulating bacterial strain TP1000 (18), the MPT accumulating bacterial strain RK5206 (17), and the Moco/MPT free bacterial strain RK5204 (17). Protein preparations obtained this way were analyzed by SDS-PAGE and CD spectroscopy (Fig. 2, A and B). Comparison of the spectra revealed no significant differences so that one can conclude that all NR proteins purified possess a highly similar secondary structure. Likewise yield and purity of the proteins obtained were comparable, thus allowing carrying out a comparative biochemical characterization of the different NR forms. In a first set of experiments, we quantified the contents of prosthetic groups (i.e. Moco/MPT, heme, and FAD) of two different, holo-, apo-, and MPT-NR preparations, respectively. Moco was determined in NR preparations derived from TP1000. Therefore, the molybdenum content of these preparations was determined using ICP-MS, revealing on average 0.78 ± 0.11 MPT per monomer (Fig. 2C) as quantified by HPLC-based FormA analysis (Fig. 2D (24)). RK5204-derived NR preparations contained neither Moco nor MPT. In the following we will use the term holo-NR for Moco-containing NR. The term apo-NR will be used for Moco/MPT-free NR, whereas the term MPT-NR will be used for NR co-purified with MPT. Next we asked whether or not the NR heme content is related to the presence of Moco/MPT. To answer this, NR-bound heme was quantified using two different methods. At first protein-bound heme was quantified by using the molar extinction coefficient of cytochrome b$_3$, a method that is commonly used to quantify NR heme content (25). As a second method, a chromogenic assay was taken to quantify NR-bound heme. In this way, heme saturation of holo NR was found to be in the range of 0.89 ± 0.04 molecules heme per monomer (determination was based on the extinction coefficient) or 0.97 ± 0.01 molecules heme per monomer (determination by chromogenic assay) (Fig. 2E). MPT-NR showed essentially the same heme stoichiometries as identified for holo-NR. However, apo-NR displayed a slightly lower heme binding stoichiometry, i.e. 0.72 ± 0.01 molecules heme per monomer (via extinction coefficient) or 0.8 ± 0.03 molecules heme per monomer (via chromogenic assay) (Fig. 2F). Therefore, neither Moco nor MPT has a significant influence on the stoichiometries of NR to bind heme. Subsequently we also quantified the amount of FAD co-purified with holo-, MPT- and apo-NR using UV-visible spectroscopy (30). Results from these experiments showed that none of the tested NRs was co-purified with FAD (data not shown). It is noteworthy that FAD deficiency was already reported for purified NRs from various other species (31, 32).

NADPH-dependent Cytochrome c Reducing Activity of Recombinant Neurospora Holo-NR—Hitherto expression of eukaryotic holo-NR was preferentially carried out in the methylothrophic yeast Pichia pastoris, yielding active recombinant holo-NR (3, 33). Recombinant N. crassa holo-NR purified from E. coli is likewise functional. For its nitrate reducing activity, the corresponding $K_m$ value was found to be 0.25 mM with a $V_{max}$ of 4.32 μmol of nitrite/mn/mg of protein (Fig. 3E). The $K_m$ determined was similar to the $K_m$ values documented for the endogenous NR purified from Neurospora and the NR purified from the fungus Aspergillus nidulans. However, differences exist regarding the $V_{max}$ values of these NR species (Fig. 3E).

NADPH-dependent Cytochrome c Reducing Activity of Recombinant Neurospora NR—Other than holo-NR, MPT- and apo-NR have no nitrate reducing activity. However, all three NR species were purified with approximately equimolar amounts of heme (Fig. 2E). In the following we tested apo-NR and MPT-NR for their NADPH-dependent cytochrome c reducing activity, thus monitoring the electron flow from FAD to heme (Fig. 3D). As a control, the NADPH-dependent cytochrome c reducing activity of holo-NR was recorded. Correlating with its nitrate reducing activity, holo-NR also showed, as expected, NADPH-dependent cytochrome c reducing activity (Fig. 3D, B and C). Likewise, apo- and MPT-NR showed cytochrome c reducing activity (Fig. 3B). However, when compared with holo-NR, apo-NR had a marginally lower (~10% reduced) cytochrome c reducing activity, which correlates to its ~15% reduced heme content (Fig. 2E). Consequently we reason that...
heme bound to apo- and MPT-NR is fully capable of transferring NADPH-derived electrons.

Oligomerization State of Recombinant Neurospora NR—Next we wanted to characterize the oligomerization state of holo-, apo-, and MPT-NR. After a two-step affinity purification, SEC revealed that the NR protein pools purified from strains TP1000 and RK5206 contained dimeric NR exclusively (Fig. 4A). In contrast, protein preparations from RK5204 contained dimeric as well as monomeric NR, with both forms appearing in an ~1:2 molar ratio (Fig. 4A). However, the ratio of dimeric to monomeric NR varied significantly from preparation to preparation. Next we asked whether or not heme is important for NR dimerization. Therefore, cytochrome c reductase activities of the main peak fractions of monomeric and dimeric apo-NR
were determined and found to be invariant, thus showing that each fraction contains NR proteins with identical heme binding stoichiometries (Fig. 4, B and C). Therefore, the lack of Moco/MPT is solely responsible for monomer formation of apo-NR.

Analysis of NR Protein Variants by Analytical Ultracentrifugation—To further characterize the oligomerization state of the different NR variants, we examined the proteins in a concentration range from 0.6 to 7.7 μM by sedimentation velocity experiments in the analytical ultracentrifuge. Holo- and MPT-NR sedimented predominantly as a single species with an $s_{20,w}$ of 8.3 and 8.1 S, respectively, independently of the protein concentration used (Fig. 5, A and B). The continuous c(s) distribution model in the program SEDFIT (21) was used to determine the molar mass of the proteins from the diffusion broadening of the sedimenting boundary. At a concentration of 2.6 μM, molar masses of 220 and 213 kg/mol were obtained for holo- and MPT-NR, respectively. Because a molar mass of 114.5 kg/mol can be calculated from amino acid composition, both proteins exist predominantly as dimers in solution. This is consistent with the results for the native enzyme purified from N. crassa (34). For these dimers frictional ratios of 1.65 and 1.68 can be calculated from the sedimentation coefficients. Because for a globular hydrated protein a frictional ratio of 1.1–1.2 is expected (35), both proteins deviate substantially from the shape of a sphere with the holoenzyme being slightly more compact than the MPT form. All three NR variants show, in addition to the dimeric form, a species sedimenting with about 11–12 S, which might represent a small amount of tetrameric enzyme (Fig. 5). A tendency of NR to form tetramers without significant impact on functionality has been described previ-
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FIGURE 4. Oligomerization state of recombinant NR. A, 200 μg of recombinant NR containing Moco (holo-NR) or molybdopterin (MPT-NR) or no Moco or MPT (apo-NR) were subjected to SEC. mAU, milliabsorbance units. B, shown is an elution profile of holo-NR as shown in A. In addition to absorption at 280 nm, the optical density at 412 nm is shown. Cytochrome c reducing activity was determined for fractions F2 and F6 as shown in C. Determination of cytochrome c reducing activity was carried out as described under “Experimental Procedures.”

FIGURE 5. Characterization of different NR variants by analytical ultracentrifugation. Different concentrations of molybdenum cofactor (Moco) containing, molybdopterin (MPT) containing and Moco/MPT free (apo) nitrate reductase (NR) were analyzed in sedimentation velocity runs at 35,000 rpm and 20 °C. To achieve better comparability, all sedimentation coefficient distributions have been converted to 12-mm path lengths. Whereas holo- and MPT-NR exist predominantly as dimers in solution, in apoNR both monomers and dimers are clearly populated. Unexpectedly, the monomer/dimer ratio did not change significantly when protein concentration was varied by a factor of 20, implying that both oligomerization states are not in equilibrium.

Because the NR preparations were not completely saturated with Moco/MPT (Fig. 2C), these fractions might correspond to monomeric enzyme devoid of these co-factors. Consistent with the SEC results, c(s) distributions of apo-NR, in contrast to those of holo- and MPT-NR, show two major species (Fig. 5C). A faster one sedimenting with essentially the same sedimentation coefficient as MPT-NR dimers and a slower one with an

ally (3). Furthermore, a small fraction of holo- and MPT-NR sedimented with an $s_{20,w}$ value about 5–6 S (Fig. 5, A and B).
MCP was identified to be 2 molecules MCP per NR monomer.

Because the NR dimer interface has a buried area of about 2000 Å² per monomer and is spotted with conserved charged (Arg-294, Lys-314, Glu-497, Arg-523, Glu-535) and polar amino acids (e.g. Val-381, Thr-384, Arg-406, Arg-454, Glu-456, Tyr-472), which form intermolecular salt bridges and hydrogen bonds. The hydrophobicity of the interface leads to a significant free energy gain upon dimerization and creates a strong bias toward the dimer.

Moco is no direct part of the interface but strengthens it by stabilizing a small all-helical domain, which spans amino acids Asn-372 to Ile-409 and contributes about 500 Å² to the buried area. This domain was identified by a thorough B-factor analysis of 2BIH and the fact that it is the only part of the interface with an independent fold. According to our PISA analysis, it plays an essential role for complex formation, and its elimination renders the dimer unstable.

Quantification of Moco Transfer—Results from the in vitro reconstitution experiments demonstrate that monomeric apo-NR dimerizes upon Moco addition, yielding physiologically active NR. As a next step, the amount of Moco transferred to apo-NR had to be quantified. To address this question, we enriched reconstitution-competent, monomeric apo-NR by SEC (data not shown) and co-incubated it with various amounts of MCP. NR activity was measurable upon co-incubation with MCP and increased with the amount of MCP used for co-incubation (Fig. 8A).

Maximum NR activity was observed upon co-incubation of one monomer apo-NR with ~0.35 molecules of Moco. In the following, monomeric apo-NR was co-incubated with the 4-fold stoichiometric excess of Moco bound to MCP. After co-incubation, strep-tagged NR was quantitatively separated from MCP by Strep-Tactin affinity chromatography as demonstrated by SDS-PAGE analysis (Fig. 8B). Subsequently the amount of Moco/MPT co-purified with NR was determined HPLC-based, thus revealing an average Moco/MPT binding stoichiometry of 0.34 ± 0.04 molecules per NR monomer (Fig. 8C). Therefore, in vitro reconstituted NR has a significantly lower Moco binding stoichiometry as recombinant holo-NR.

EPR Studies of Reconstituted NR—Because EPR spectroscopy is an extremely sensitive tool to probe the microenvironment of molybdenum, we employed this technique to detect Moco transfer from MCP to apo-NR. First, suitable conditions to reproducibly elicit Mo⁵⁺ EPR signals in N. crassa holo-NR had to be established. Early work on spinach NR used incubation with an excess of NADPH and nitrate to generate an axial Mo⁵⁺ EPR signal (38).
As judged from g values and proton superhyperfine parameters, this signal is characteristic for NR as EPR signals with almost identical parameters were detected in partially reduced Candida nitratophila (39) and Chlorella vulgaris NR (40). 

N. crassa holo-NR also showed the characteristic nearly axial Mo\textsuperscript{5+} EPR signals (Fig. 9, g = 1.998, 1.971, 1.969). Upon exchange of the solvent for D\textsubscript{2}O, the coupling of the single, solvent exchangeable proton vanished (A\textsubscript{4\textsubscript{H}} = 1.2, 1.0, and 1.8 millitesla, respectively).

Our data confirm the ample information on N. crassa NR (41). Double integration showed that under these conditions 5–10% of the molybdenum could be trapped in the EPR active 5+ state. In the EPR spectral region around g = 1.97, at which the sharp proton-superhyperfine split NR Mo\textsuperscript{5+} signal is best detected, neither holo-MCP nor apo-NR exhibits EPR spectral features upon the addition of nitrate and NADPH. But after transfer of the Moco from holo-MCP to apo-NR, the presence of Moco with a microenvironment identical to holo-NR could unmistakably be inferred from the characteristic EPR signal. Based on the amplitude in comparison to holo-NR, at least 34% of apo-NR could be activated by holo-MCP. This value compares favorably with the activity data as shown in Fig. 8.

FIGURE 6. Moco-dependent dimerization of NR. A, 100 μg of recombinant Moco-free NR (apo-NR) were co-incubated with the 2-fold stoichiometric excess of the MCP with bound Moco (holo-MCP) and Moco-free MCP (apo-MCP), respectively. After co-incubation, the reaction mixture was separated using SEC. B, 2 mg of apo-NR were subjected to SEC, and fractions F12-F15 were collected. Moco-dependent NR reconstitution activity and cytochrome c reducing activity were determined for these fractions as shown in C. For Moco-dependent NR reconstitution, 200 ng of holo-MCP were co-incubated with 500 ng of apo-NR, resulting in a 2.5 stoichiometric excess of MCP over NR. NADPH-dependent nitrate reduction and cytochrome c reduction was carried out as described under “Experimental Procedures.” D, shown is SDS-PAGE analysis of SEC fractions F12–F15. E, 400 ng of recombinant apo-NR were co-incubated for 3 h at room temperature with increasing amounts of recombinant holo-MCP in degassed reconstitution buffer, and Moco-dependent NR activity was recorded as described.
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The Sequence of Prosthetic Group Insertion into Neurospora NR—The timing of prosthetic group insertion into some prokaryotic molybdenum enzymes is known to be crucial for their maturation (42). To test for a mandatory sequence of prosthetic group insertion into NR, we first analyzed a NR protein defective in heme binding. Therefore, the NR variant H654A/H677A (13) was created by site-directed mutagenesis. The mutant protein was purified from the Moco-accumulating bacterial strain TP1000. CD spectroscopy showed no negative effects of the introduced mutations on protein secondary structure in comparison to the wild type protein (data not shown).

Upon chromogenic-based detection, no heme was detectable in H654A/H677A protein preparations, documenting the successful construction of a heme-free NR. The Moco/MPT content of NR H654A/H677A purified from TP1000 (determined HPLC-based) was found to be 0.81 molecules of Moco ± 0.04 per monomer (Fig. 10C), thus resembling the value quantified for the wild type NR. The molybdenum content as determined by ICP-MS revealed on average >80% saturation. Therefore, we conclude that exclusively Moco is bound to NR H654A/H677A. These findings closely resemble the Moco binding properties of the NR wild type protein.

Consequently we reason that Moco insertion into NR occurs independently from the presence of heme. In reverse, wild-type-like heme binding was observed for Moco/MPT-free NR (Fig. 2E), thus documenting that NR heme binding is independent from Moco/MPT binding.

As a next step the influence of FAD on the insertion of Moco and heme, respectively, was determined. NR has a ferredoxin reductase-type FAD binding domain (43). Therefore, FAD binding mutants were constructed considering known FAD binding mutants of other enzymes with ferredoxin reductase-type FAD binding sites as well as FAD binding mutants of N. crassa and Arabidopsis thaliana NR (4, 44). Accordingly, N. crassa NR was mutated, yielding FAD binding mutants R778E, Y780A, and G811V, respectively. To show that these NR variants do not bind FAD, we quantified the FAD binding properties of an N-terminal-truncated NR variant consisting of the FAD and heme binding domain (thus forming the NR CcR fragment). Expression and purification of each CcR variant was successful, yielding the highest amounts of pure protein as documented by SDS-PAGE analysis (Fig. 10D). In the following we determined the effects of the introduced mutations in the FAD domain on the protein secondary structure using CD spectroscopy. No differences as compared with the CcR wild type CD-spectra were detectable (data not shown). Subsequently, the FAD binding properties of NR CcR fragments were quantified using ITC. By this, a FAD $K_d$ value of 0.61 ± 0.05 μM was revealed for wild type CcR (Fig. 10A). However, ITC-based no FAD binding was detectable for CcR R778E and Y780A variants (data not shown). Next we quantified FAD binding properties of the heme-free CcR variant H654A/H677A, revealing an FAD $K_d$ value of 0.52 ± 0.03 μM (Fig. 10B). Therefore, FAD binding to the NR CcR fragment is independent from heme binding.

In the inverse experiment we asked whether or not heme binding to NR is independent from FAD binding. Therefore, the amount of heme co-purified with NR variant R778E being unable to bind FAD was quantified. In comparison to wild type protein, NR R778E had a very similar heme content with a binding stoichiometry of 0.78 ± 0.07 molecules heme per monomer (data not shown). Next we quantified FAD binding properties of the heme-free CcR variant H654A/H677A, revealing an FAD $K_d$ value of 0.52 ± 0.03 μM (Fig. 10B). Therefore, FAD binding to the NR CcR fragment is independent from heme binding.

In the next section the influence of FAD on the insertion of Moco and heme, respectively, was determined. NR has a ferredoxin reductase-type FAD binding domain (43). Therefore, FAD binding mutants were constructed considering known FAD binding mutants of other enzymes with ferredoxin reductase-type FAD binding sites as well as FAD binding mutants of N. crassa and Arabidopsis thaliana NR (4, 44). Accordingly, N. crassa NR was mutated, yielding FAD binding mutants R778E, Y780A, and G811V, respectively. To show that these NR...

FIGURE 7. Stereo view of the dimerization interface of a N. crassa NR model based on the structure of Moco-bound NR from P. angusta. The model was computed using the one-to-one threading option of the Phyre2 server. The left monomer is colored by the potential on its solvent-accessible surface as calculated by pdb2pqr (53) (scale ranging from −5 to 5 kT e$^{-1}$). The right monomer is shown in schematic representation with the small all-helical domain colored in green. Moco is shown in ball-and-stick representation. The figure was prepared with PyMOL (54).
of Moco as does the wild type protein, thus demonstrating that Moco binding to NR is independent from FAD binding.

**DISCUSSION**

In the past eukaryotic NR has been characterized intensively, revealing the multidomain character of this complex metalloenzyme. Although detailed knowledge is available for the holoenzyme (3–5, 28), little is known about its redox cofactor assembly. This gap of knowledge is mainly due to insufficient amounts of well defined stable apo-enzyme proteins available for analysis.

However, 40 years ago (45) a stable apo-NR was identified in the whole cell extract of the N. crassa nit-1 mutant. nit-1 extracts were found to be a valuable tool for Moco research (11) as the addition of physiologically active Moco results in forma-
tion of active NR, thus representing the only enzymatic assay system available for detecting physiologically active Moco.

Structural elucidation of the eukaryotic NR (46) revealed that Moco is deeply buried within the protein, at the end of the substrate funnel. This finding led to the questions of (i) how Moco is inserted, and (ii) considering that besides Moco, heme and FAD are also NR redox cofactors, is there a mandatory sequence for NR redox cofactor assembly? In this study we addressed both questions using the *N. crassa* NR as model enzyme. In a first set of experiments we characterized the Moco insertion process using recombinant Moco-free apo-NR and found that Moco is sufficient to induce NR dimer formation in this fully defined *in vitro* system. Consistently, cofactor-induced dimerization was already previously observed in the undefined whole cell extracts of the *nit-1* system (45, 47, 48), but its sole dependence on Moco was not provable in this system.

How can Moco-induced NR dimer formation be explained? To address this question we built a structural model of *N. crassa* NR on the basis of our data set for the molybdenum domain of *Pichia* NR and analyzed its dimer interface. By this, we identified a small domain that provides ~25% of the buried interface area.

We speculate that this domain is flexible and that it triggers dimerization upon Moco binding. In the absence of Moco it adopts a conformation unfit for dimerization, and as a result, the monomer becomes the abundant species. In the presence of Moco, the all-helical domain becomes part of the interface, and therefore, the equilibrium shifts toward the dimer. Once formed, the dimer is kinetically locked, thus precluding accessibility to the Moco binding site.

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We postulate that the monomer is also kinetically locked until dimerization is triggered. Due to the abundance of Moco under natural conditions, NR is usually found to be Moco-loaded and, hence, dimeric. The emergence of both forms could result from NR overexpression in a Moco-free *E. coli* strain. It cannot further be ruled out that certain *E. coli* metabolites with weak affinity occupy the Moco binding site and hence trigger dimerization.

Analytical ultracentrifugation revealed that monomeric apo-NR is not in equilibrium with dimeric apo-NR, thus substantiating our model of NR dimerization. We were not able to verify the proposed model because as yet apo-NR crystallization failed. Furthermore, solely monomeric apo-NR is reconstitution competent, whereas dimeric apo-NR is not,
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which is best explained by missing accessibility of the Moco binding site.

Monomeric apo-NR binds substoichiometric Moco amounts (~35% Moco saturation) and is, therefore, different from recombinant holo-NR that is co-purified with an approximately equimolar Moco amount (~81% Moco saturation). EPR studies with reconstituted NR revealed no differences in the molybdenum micro environment as compared with the recombinant holoenzyme, documenting that NR active site formation is an autonomous process. How can we explain the substoichiometric Moco binding upon NR reconstitution? To ensure stoichiometric Moco binding to NR, a yet unidentified factor(s) (e.g., an enzyme-specific chaperone) may be required. However, hitherto molybdenum enzyme-specific chaperons are only known for the prokaryotic molybdenum enzymes xanthine dehydrogenase, trimethylamine oxidoreductase, and NR A (NarGHI) (42). Efficient maturation of these enzymes essentially depends on the cognate chaperones XDHc, TorD, and NarJ, respectively. However, Ilbert et al. (49) report that activation of TorA does not completely depend on the presence of the chaperone TorA in vitro but is significantly enhanced by it.

We speculate that likewise Moco insertion into NR is enhanced by a yet unknown factor(s). Because recombinant NR contains equimolar amounts of Moco, we expect these factors either to be evolutionarily conserved, as is Moco biosynthesis (1), or to be functionally redundant in the expression host E. coli. Our defined in vitro reconstitution system will be a valuable tool for the identification and characterization of this factor(s).

Likewise, as compared with the undefined N. crassa nit-1 system, the in vitro system is a more suitable tool for the detection of physiologically active Moco. Hitherto, detection and quantification of physiologically active Moco was constrained, as the whole-cell nit-1 system also contains other proteins with a Moco binding site, either involved in Moco biosynthesis, Moco transfer, or Moco usage. These error sources are not present in the defined in vitro system containing solely Moco and apo-NR. Functional NR is a homo-dimeric enzyme consisting of two monomers that are built up from three distinct cofactor binding domains. This finding suggests that Moco insertion needs to be synchronized with the insertion of the remaining redox cofactors. For the bacterial molybdenum enzyme NR A (NarGHI) an obligatory sequence of metal cofactor insertion was shown (50), documenting its complex and intricate assembly process. However, we did not find any indication for a mandatory sequence of eukaryotic NR cofactor assembly, which allows us to conclude that the three NR redox cofactors are bound autonomously by their respective domains. Furthermore, we cannot exclude that NR cofactor assembly is intimately connected to protein translation, thus defining the sequence of cofactor insertion and preventing the formation of cofactor-free NR protein.

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REFERENCES

1. Mendel, R. R., and Kruse, T. (2012) Cell biology of molybdenum in plants and humans. Biochim. Biophys. Acta 1823, 1568–1579
2. Schwarz, G., Mendel, R. R., and Ribbe, M. W. (2009) Molybdenum cofactors, enzymes, and pathways. Nature 460, 839–847
3. Campbell, W. H. (2001) Structure and function of eukaryotic NAD(P)H: nitrate reductase. Cell. Mol. Life Sci. 58, 194–204
4. Campbell, W. H. (1999) Nitrate reductase structure, function, and regulation. Bridging the gap between biochemistry and physiology. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 277–303
5. Campbell, W. H., and Kinghorn, K. R. (1990) Functional domains of assimilatory nitrate reductases and nitrate reductases. Trends Biochem. Sci. 15, 315–319
6. Lillo, C., Meyer, C., Lea, U. S., Provan, F., and Oltedal, S. (2004) Mechanism and importance of post-translational nitrate regulation of nitrate reductase. J. Exp. Bot. 55, 1275–1282
7. Nussaume, L., Vincentz, M., Meyer, C., Boutin, J. P., and Caboche, M. (1995) Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. Plant Cell 7, 611–621
8. Poll, A. M., Cherel, I., Gonneau, M., and Leydecker, M. T. (1991) Biochemical characterization of cnx nitrate reductase-deficient mutants from Nicotiana plumbaginifolia. Plant Sci. 76, 201–209
9. McDonald, D. W., and Coddington, A. (1974) Properties of the assimilatory nitrate reductase from Aspergillus nidulans. Eur. J. Biochem. 46, 169–178
10. De Vries, S. E., Dirks, R., Mendel, R. R., Schaart, G. J., Feenstra, W. J. (1986) Biochemical characterization of some nitrate reductase deficient mutants of Nicotiana plumbaginifolia. Plant Science 44, 105–110
11. Nason, A., Lee, K. Y., Pan, S. S., Ketchum, P. A., Lambert, A., and De Vries, J. (1991) In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate:nitrate reductase from a Neurospora mutant and a component of molybdenum enzymes. Proc. Natl. Acad. Sci. U.S.A. 68, 3242–3246
12. Mertens, I. A., Shiraishi, N., and Campbell, W. H. (2000) Recombinant expression of molybdenum reductase fragments of plant nitrate reductase at high levels in Pichia pastoris. Plant Physiol. 123, 743–756
13. Okamoto, P. M., Fu, Y. H., and Marzluf, G. A. (1991) Nit-3, the structural gene of nitrate reductase in Neurospora crassa. Nucleotide sequence and regulation of mRNA synthesis and turnover. Mol. Gen. Genet. 227, 213–223
14. Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. D., Jaffe, D., FitzHugh, W., Ma, L. J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S., Nielsen, C. B., Butler, J., Endrizzi, M., Qui, D., Ianakiev, P., Bell-Pedersen, D., Nelson, M. A., Werner-Washburne, M., Selitrennikoff, C. P., Kinsey, J. A., Braun, E. L., Zelter, A., Schulte, U., Kothe, G. O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gniere, S., Kamal, M., Kamvysselis, M., Maucler, E., Bieke, C., Rudd, S., Frishman, D., Krystofova, S., Rasmussen, C., Mettenberg, R. L., Perkins, D. D., Kroken, S., and Cogni, C. (2003) The genome sequence of the filamentous fungus Neurospora crassa. Nature 422, 859–868
15. Borkovich, K. A., Alex, L. A., Yarden, O., Freitag, M., Turner, G. E., Read, N. D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plefosky, N., Plamann, M., Goodrich-Tanigaki, M., Schulte, U., Manshaupt, G., Nargang, F. E., Radford, A., Selitrennikoff, C., Galagan, J. E., Dunlap, J. C., Loros, J. J., Kothe, G. O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gniere, S., Kamal, M., Kamvysselis, M., Maucler, E., Bieke, C., Rudd, S., Frishman, D., Krystofova, S., Rasmussen, C., Mettenberg, R. L., Perkins, D. D., Kroken, S., and Cogni, C. (2003) The genome sequence of the filamentous fungus Neurospora crassa. Nature 422, 859–868
16. McCluskey, K. (2003) The Fungal Genetics Stock Center. From molds to molecules. Adv. Appl. Microbiol. 52, 245–262
17. Stewart, V., and MacGregor, C. H. (1982) Nitrate reductase in Escherichia coli K-12. Involvement of chlC, chlE, and chlG loci. J. Bacteriol. 151, 788–799
18. Palmer, T., Santini, C.-L., Iobbi-Nivol, C., Eaves, D. J., Boxer, D. H., and
Giordano, G. (1996) Involvement of the narf and mob gene products in distinct steps in the biosynthesis of the molybdooenzyme nitrate reductase in *Escherichia coli*. *J. Mol. Microbiol.* 20, 875–884.

19. Seeraram, N., and Woody, R. W. (2004) Computation and analysis of protein circular dichroism spectra. *Methods Enzymol.* 383, 318–351.

20. Zhao, H., Ghirlando, R., Piszczek, G., Curth, U., Brautigam, C. A., and Schuck, P. (2013) Recorded scan times can limit the accuracy of sedimentation coefficients in analytical ultracentrifugation. *Anal. Biochem.*, in press.

21. Schuck, P. (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamll equation modeling. *Biophys. J.* 78, 1606–1619.

22. Laue, M. T., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1991) *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, pp. 90–125, Cambridge, UK.

23. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 4, 2411–2423.

24. Schwarz, G., Boxer, D. H., and Mendel, R. R. (1997) Molybdenum cofactor biosynthesis. The plant protein Cnx1 binds molybdopterin with high affinity. *J. Biol. Chem.* 272, 26811–26814.

25. Redinbaugh, M. G., and Campbell, W. H. (1985) Quaternary structure and composition of squash NADH:nitrate reductase. *J. Biol. Chem.* 260, 3380–3385.

26. Evans, H. J and Nason, A. (1952) The effect of reduced triphosphopyridine nucleotide on nitrate reduction by purified nitrate reductase. *Arch. Biochem. Biophys.* 39, 234–235.

27. Garrett, R. H., and Nason, A. (1967) Involvement of a B-type cytochrome in the assimilatory nitrate reductase of *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.A.* 58, 1603–1610.

28. Fischer, K., Llamas, A., Tejada-Jimenez, M., Schrader, N., Kuper, J., Ataya, F. S., Galvan, A., Mendel, R. R., Fernandez, E., and Schwarz, G. (2006) Function and structure of the molybdenum cofactor carrier protein from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 281, 30186–30194.

29. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

30. Maceroux, P. (1999) UV-visible spectroscopy as a tool to study flavoproteins. *Methods Mol. Biol.* 131, 1–7.

31. Pan, S. S., and Nason, A. (1978) Purification and characterization of homogeneous assimilatory reduced nicotineamide adenine dinucleotide phosphate-nitrate reductase from *Neurospora crassa*. *Biochim. Biophys. Acta* 523, 297–313.

32. Minagawa, N., and Yoshimoto, A. (1982) Purification and characterization of the assimilatory NADPH-nitrate reductase of *Aspergillus nidulans*. *J. Biochem.* 91, 761–774.

33. Barbier, G. G., Joshi, R. C., Campbell, E. R., and Campbell, W. H. (2004) Purification and biochemical characterization of simplified eukaryotic nitrate reductase expressed in *Pichia pastoris*. *Protein Expr. Purif.* 37, 61–71.

34. Garrett, R. H., and Nason, A. (1969) Further purification and properties of *Neurospora* nitrate reductase. *J. Biol. Chem.* 244, 2870–2882.

35. Lebowitz, J., Lewis, M. S., and Schuck, P. (2002) Modern analytical ultracentrifugation in protein science. A tutorial review. *Protein Sci.* 11, 2067–2079.

36. Kelley, L. A., and Sternberg, M. J. (2009) Protein structure prediction on the Web. A case study using the Phyre server. *Nat. Protoc.* 4, 363–371.

37. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797.

38. Gutteridge, S., Bray, R. C., Notton, B. A., Fido, R. J., and Hewitt, E. J. (1983) Studies by electron paramagnetic resonance spectroscopy of the molybdenum centre of spinach (*Spinacia oleracea*) nitrate reductase. *Biochem. J.* 213, 137–142.

39. Kay, C. J., Barber, M. J., Solomonson, L. P., Kau, D., Cannons, A. C., and Hipkin, C. R. (1990) Spectroscopic, thermodynamic, and kinetic properties of *Candida nitratophila* nitrate reductase. *Biochem. J.* 272, 545–548.

40. Kay, C. J., and Barber, M. J. (1989) EPR and kinetic analysis of the interaction of halides and phosphate with nitrate reductase. *Biochemistry* 28, 5750–5758.

41. Orme-Johnson, W. H. (1980) *Molybdenum and molybdenum-containing enzymes* (Coughlan, M. P., ed.), pp. 327–344, Pergamon Press, New York.

42. Magalon, A., Fedor, J. G., Walburger, A., and Weiner, J. H. (2011) Molybdenum enzymes in bacteria and their maturation. *Coord. Chem. Rev.* 255, 1159–1178.

43. Dym, O., and Eisenberg, D. (2001) Sequence-structure analysis of FAD-containing proteins. *Protein Sci.* 10, 1712–1728.

44. Gonzalez, C., Brito, N., and Marzulf, G. A. (1995) Functional analysis by site-directed mutagenesis of individual amino acid residues in the flavin domain of *Neurospora crassa* nitrate reductase. *Mol. Gen. Genet.* 249, 456–464.

45. Nason, A., Antoine, A. D., Ketchum, P. A., Frazier, W. A., 3rd, and Lee, D. K. (1970) Formation of assimilatory nitrate reductase by *in vitro* intercistronic complementation in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.A.* 65, 137–144.

46. Fischer, K., Barbier, G. G., Hecht, H. J., Mendel, R. R., Campbell, W. H., and Schwarz, G. (2005) Structural basis of eukaryotic nitrate reduction. Crystal structures of the nitrate reductase active site. *Plant Cell* 17, 1167–1179.

47. Ketchum, P. A., Cambier, H. Y., Frazier, W. A., 3rd, and Madamsky, C. H., and Nason, A. (1970) *In vitro* assembly of *Neurospora* assimilatory nitrate reductase from protein subunits of a *Neurospora* mutant and the xanthine oxidizing or aldehyde oxidase systems of higher animals. *Proc. Natl. Acad. Sci. U.S.A.* 66, 1016–1023.

48. Ketchum, P. A., and Sevilla, C. L. (1973) *In vitro* formation of nitrate reductase using extracts of the nitrate reductase mutant of *Neurospora crassa*, nit-1, and *Rhodospirillum rubrum*. *J. Bacteriol.* 116, 600–609.

49. Ilbert, M., Mèjean, V., Giudici-Orticoni, M. T., Samama, J. P., and Iobb-Nivol, C. (2003) Involvement of a mate chaperone (TorD) in the maturation pathway of molybdoenzyme TorA. *J. Biol. Chem.* 278, 28787–28792.

50. Lanciano, P., Vergnes, A., Grimaldi, S., Guigliarelli, B., and Magalon, A. (2007) Biogenesis of a respiratory complex is orchestrated by a single accessory protein. *J. Biol. Chem.* 282, 17468–17474.

51. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) *Clustal W* and *Clustal X Version 2.0*. *Bioinformatics* 23, 2947–2948.

52. Horner, R. D. (1983) Purification and comparison of nit-1 and wild-type NADPH:nitrate reductases of *Neurospora crassa*. *Biochim. Biophys. Acta* 744, 7–15.

53. Dolinski, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDBePQR. An automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* 32, W665–W667.

54. The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.