The Mechanism of Assembly and Cofactor Insertion into Rhodobacter capsulatus Xanthine Dehydrogenase*

Silvia Schumann†, Miguel Saggu‡, Nadine Möller§, Stefan D. Anker¶, Friedhelm Lendzian#, Peter Hildebrandt#, and Silke Leimkühler†

From the †Universität Potsdam, Institut für Biochemie und Biologie, Karl-Liebknecht Strasse 24-25, Potsdam 14476, the ‡Technische Universität Berlin, Institut für Chemie, Sekr. PC14, Straße des 17, Juni 135, Berlin 10623, the ¶Center for Cardiovascular Research, Charité, Berlin 10115, and the ##Division of Applied Cachexia Research, Department of Cardiology, Charité CVK, 13353 Berlin, Germany

Rhodobacter capsulatus xanthine dehydrogenase (XDH) is a molybdo-flavoprotein that is highly homologous to the homodimeric mammalian xanthine oxidoreductase. However, the bacterial enzyme has an (αβ)2 heterotetrameric structure, and the cofactors were identified to be located on two different polypeptides. We have analyzed the mechanism of cofactor insertion and subunit assembly of R. capsulatus XDH, using engineered subunits with appropriate substitutions in the interfaces. In an (αβ) heterodimeric XDH containing the XdhA and XdhB subunits, the molybdenum cofactor (Moco) was shown to be absent, indicating that dimerization of the (αβ) subunits has to precede Moco insertion. In an (αβ)2 XDH heterotetramer variant, including only one active Moco-center, the active (αβ) site of the chimeric enzyme was shown to be fully active, revealing that the two subunits act independent without cooperativity. Amino acid substitutions at two cysteine residues coordinating FeSI of the two [2Fe-2S] clusters of the enzyme demonstrate that an incomplete assembly of FeSI impairs the formation of the XDH (αβ)2 heterotetramer and, thus, insertion of Moco into the enzyme. The results reveal that the insertion of the different redox centers into R. capsulatus XDH takes place sequentially. Dimerization of two (αβ) dimers is necessary for insertion of sulfurated Moco into apo-XDH, the last step of XDH maturation.

Mammalian XORs catalyze the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, and exist originally as the dehydrogenase form (XDH, EC 1.17.1.4) but can be converted to the oxidase form (XO, EC 1.1.3.22) either reversibly by oxidation of sulfhydryl residues of the protein molecule or irreversibly by proteolysis (4). XDH shows a preference for NAD+ reduction at the FAD reaction site, whereas XO exclusively uses dioxygen as a terminal electron acceptor, leading to the formation of superoxide and hydrogen peroxide (5). The enzyme has been implicated in diseases characterized by oxygen radical-induced tissue damage, such as postischemic reperfusion injury (6). The oxidation of xanthine takes place at the molybdenum center, and the electrons thus introduced are rapidly distributed to the other centers according to their relative redox potentials (1). The re-oxidation of the reduced enzyme by the oxidant substrate, either NAD+ or molecular oxygen, occurs through FAD (7). The two [2Fe2S] clusters (FeSI and FeSII) are indistinguishable in terms of their absorption spectra, but the midpoint redox potential of FeSII is generally more positive than that of the FeSI center (8, 9). The FeS centers from enzymes of the XO family have been characterized earlier by EPR (10–12). The FeSI center of eukaryotic XOR exhibits a rhombic EPR signal, well observable at temperatures up to 60 K, slightly different from those found in the regular plant-type [2Fe2S] ferrodoxins (9), whereas FeSII exhibits an unusual broad EPR signal that is characteristic of some molybdenum-containing hydroxylases and can be observed only at 20 K or lower temperature.

The crystal structures of the mammalian XDH/XO from bovine milk (3) and the structure of the highly homologous bacterial XDH from Rhodobacter capsulatus (13) have been solved. The bacterial XDH can be expressed in high quantities in a heterologous Escherichia coli system in a highly active form (14). The amino acid sequence of R. capsulatus XDH has a high degree of similarity to eukaryotic XORs (up to 39% identity to bovine milk XOR), however, in contrast to the homodimeric (α), structure of eukaryotic XORs, R. capsulatus XDH has an (αβ)2 heterotetrameric structure, and the cofactors were identified to be located on two different polypeptides: the iron-sulfur clusters and the FAD are bound by the XdhA subunit, and the Moco is bound by the XdhB subunit (15). Despite differences in subunit composition, the folds of bovine XDH and R. capsulatus XDH are highly similar but differ in important details. The NAD+ binding pocket of the bacterial XDH resem-
bles that of the dehydrogenase (XDH) form of the bovine enzyme rather than that of the oxidase (XO) form, and it was shown that R. capsulatus XDH is a true dehydrogenase that is not converted to an oxidase (14). The Moco was found to be deeply buried in the XdhB subunit at the end of a funnel-shaped passage giving access only to substrate molecules like pterins, purins, and aldehydes (13). The FeS clusters of R. capsulatus XDH showed notable differences in comparison to bovine XO (16), with FeSI having the highest redox potential and showing an EPR spectrum with axial symmetry.

Mammalian XORs exist as dimers, and it was reported that the two monomers act as independent catalytic subunits (1). A non-cooperative mechanism was also reported for R. capsulatus XDH (14). Because, with the exception of the monomeric DMSO reductase (17), most molyboenzymes have two independent acting catalytic active sites (18), this raised the old-aged question of why some proteins are dimers. However, a more recent report by Tai and Hwang (19) showed that the two bovine XOR subunits are strongly cooperative in both binding and catalysis.

To investigate whether the two (αβ) subunits of R. capsulatus XDH act independently or whether an intramolecular electron transfer mechanism occurs between the two β-subunits, we have generated an (α)2(β1wt/β2E730A) chimeric XDH variant containing one active XdhB half and one inactive XdhB half. To further characterize the mechanism of assembly and intramolecular electron transfer of R. capsulatus XDH, site-directed mutagenesis was performed modifying the cysteines ligating FeSI and FeSII, and further, the role of GlnA102 for electron transfer from Moco to FeSI was investigated. In addition, amino acid exchanges at the dimer interface of the XdhB subunit were introduced to create an (αβ) dimeric variant of XDH. The influences of the amino acid exchanges on the assembly of the two XDH subunits, cofactor insertion, the kinetic constants, and the EPR properties were investigated. Our results show that the assembly of XDH is a complex process, which occurs in an ordered manner. A model for the assembly and cofactor insertion into R. capsulatus XDH is presented.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions**—*E. coli* TP1000(ΔmobAB) cells (20) were used for the expression of XDH wild type and variants from plasmid pSL207 (16). *E. coli* RK4353(DE3)mobAB− cells (16, 21) were used for heterologous expression of the chimeric R. capsulatus XDH after cotransformation of plasmids pSL239/pAK22 and pSL239/pSS2 (see below). *E. coli* cultures were generally grown in LB medium under aerobic conditions at 30 °C. When required, 1 mM sodium molybdate, 150 μg/ml ampicillin, or 50 μg/ml chloramphenicol were added to the medium.

**Construction of Expression Vectors**—By using PCR mutagenesis the corresponding amino acid exchanges Q102A, Q102G, E7220R/D517R, C44A/C47A, and C134A/C136A were introduced by base pair exchanges into plasmid pSL207 for the expression of the xdhABC genes of R. capsulatus XDH (16).

For the expression of a chimeric (α)2(β1wt/β2E730A) XDH, the amino acid exchange E730A was introduced into plasmid pAK22 (22), containing the xdhB gene as a NdeI/KpnI fragment cloned into vector pTYB2 (New England Biolabs), allowing the expression of XdhB as a C-terminal fusion to an intein tag containing a chitin binding domain. The resulting plasmid was designated pSS2. For coexpression of wild-type xdhB in conjunction with xdhA and xdhC, the plasmid pACYC-duett-1 (Novagen) was used. Primers were designed that allowed cloning of the xdhAB genes into the Ncol and HindIII sites of MCS1 of pACYC-duett-1. In addition, the 3′-HindIII primer contained the coding sequence for six histidines, resulting in an XdhB fusion protein containing a C-terminal His6 tag. Subsequently, an Ndel-KpnI fragment containing the coding sequence of XdhC was cloned into the MCS2 of this vector. The resulting plasmid was designated pSL239.

**Expression and Purification of Different XDH Variants**—Recombinant *R. capsulatus* wild-type XDH was purified using the procedure described by Leimkuhler et al. (14), with affinity chromatography on Sepharose 4B/folate gel as the final step. The generated XDH variants were expressed under the same conditions as the wild-type enzyme and purified by nickel-nitrilotriacetic (NTA), Q-Sepharose, and size-exclusion chromatography. The purified enzymes were concentrated by ultrafiltration, gel filtered using a PD10 gel filtration column (GE Healthcare), equilibrated with 50 mM Tris, 1 mM EDTA, 2.5 mM dithiothreitol, pH 7.5, and stored at −70 °C until used. For expression of chimeric (α)2(β1wt/β2E730A) XDH, *E. coli* RK4353(DE3)mobAB− cells cotransformed with pSL239 and pSS2 were grown in LB medium supplemented with 150 μg/ml ampicillin, 50 μg/ml chloramphenicol, 1 mM molybdate, and 0.02 mM isopropyl-β-D-thiogalactopyranoside until the A600 = 1. These precultures were used 1:500 to start the main culture and subsequently grown at 30 °C until A600 = 0.3 was reached. The cultures were induced with 100 μM isopropyl-β-D-thiogalactopyranoside, and the growth was continued for 24 h at a temperature of 16 °C. Cells were harvested by centrifugation at 8000 × g, the pellet was resuspended in 3 volumes of 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, and cell lysis was performed by several passages through a French Pressure cell. The supernatant was incubated with 1.7 ml of Ni2+ -NTA resin (Qiagen) per 2 liters of cell growth. The slurry was transferred to a column and washed with 10 column volumes of 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, containing 10 mM imidazole, followed by a wash with 10 column volumes of the same buffer containing 20 mM imidazole. His6-tagged XDH was eluted with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, containing 250 mM imidazole. The eluted fractions were analyzed by SDS-PAGE, and the ones containing XDH were combined and dialyzed against 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 8.5. The dialyzed samples were mixed with 7 ml of Chitin matrix (New England Biolabs) and the mixture was incubated for 2 h at 4 °C. The slurry was transferred to a column and washed with 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 8.5. To induce the intein-catalyzed self-cleavage reaction, a buffer with 250 mM Tris, 500 mM NaCl, 1 mM EDTA, 50 mM dithiothreitol, pH 8.5, was added and left on the column for 24 h at 4 °C. Proteins were eluted with the same buffer and analyzed by 10% SDS-PAGE. XDH-containing fractions were combined and...
Assembly of R. capsulatus XDH
dialyzed against 50 mM Tris, 1 mM EDTA, pH 7.5, and stored at −70 °C until used.

Absorption Spectra during Anaerobic Reduction with Xanthine and NADH—XDH in 0.5 ml of 50 mM Tris, 1 mM EDTA, pH 7.5, was incubated in an anaerobic chamber (Coy Lab Systems) for 2 h at 4 °C before either xanthine or NADH was added to a final concentration of 100 μM. Complete reduction was achieved by the addition of 20 mM sodium dithionite. Spectra were recorded in 0.15-ml cuvettes using a Shimadzu UV-2401 PC spectrophotometer.

Enzyme Assays—Routine enzyme assays were carried out at 25 °C in either 50 mM Tris, 1 mM EDTA, pH 7.5, or 50 mM Tris, 0.2 mM EDTA, pH 7.8, monitoring the absorbance change at 340 nm due to reduction of NAD+ to NADH. The enzyme concentration was determined from the absorbance at 465 nm using an extinction coefficient of 31.6 mM−1 cm−1 (14). XDH assays were performed using a Shimadzu UV-2401PC spectrophotometer. 5 mM XDH was incubated with 20–100 μM xanthine as substrate, and 20–100 μM NAD+ or 20–100 μM dichlorphenolindophenol (DCPIP) as electron acceptor.

Metal and Moco/MPT Analysis—The molybdenum and iron contents of the purified proteins were quantified by inductively coupled plasma-optical emission spectroscopy analysis with a PerkinElmer Optima 2100 DV. The samples were wet-ashed at a concentration of 10 μl in a volume of 500 μl by the addition of 500 μM 65% nitric acid and incubated overnight at 100 °C. The samples were further diluted by the addition of 4 ml of water. As reference, the multi-element standard solution XVI (Merck) was used.

Moco was quantified by conversion to Form A as described earlier (23). Moco was extracted from 0.1 μM XDH by the addition of 50 μl of acidic iodine. Following incubation at room temperature for 14 h, excess iodine was removed by the addition of 55 mM 1% ascorbic acid, and the sample was adjusted with 1 ml Tris to pH 8.3. The phosphate monoester of Form A was cleaved by the addition of 16 mM cysteamine hydrochloride, 1 unit of calf intestine alkaline phosphatase. After the addition of 10 μl of acetic acid, Form A was identified and quantified by high-performance liquid chromatography analysis with a C18 reversed phase high-performance liquid chromatography column (4.6×250-mm BDS Hypersil, particle size 5 μm) with 5 mM ammonium acetate, 15% methanol at an isocratic flow rate of 1 ml/min. In-line fluorescence was monitored by an Agilent 1100 series detector with an excitation at 382 nm and emission at 450 nm.

CD Spectroscopy—UV-visible CD spectra of 1.7 mM mg1/2 enzyme samples were recorded in 50 mM Tris, 1 mM EDTA, pH 7.5, using a Jasco J-715 CD spectrophotometer.

EPR Spectroscopy—9.5-GHz X-Band EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a rectangular microwave cavity in the TE102 mode. For temperature control between 6 K and 100 K the sample was kept in an Oxford ESR 900 helium flow cryostat with an Oxford ITC4 temperature controller. The microwave frequency was detected with an EIP frequency counter (Microwave Inc.). The magnetic field was calibrated using an LiLiF standard with a known g-value of 2.002293 ± 0.000002 (24). Samples were prepared as frozen solutions (typically 0.1 mM enzyme) in quartz tubes with 4-mm outer diameter. Chemical reduction, to generate the reduced Fe(II)Fe(III) clusters, has been performed with a 20-fold excess of sodium dithionite. Baseline corrections, if required, were performed by subtracting a background spectrum, obtained under the same experimental conditions from a sample containing only a buffer solution. Simulations of the experimental EPR spectra have been carried out with the program EasySpin (25). Second integrals from the simulated FeSI and FeSII spectra were used to estimate the relative amount of both clusters in the respective samples.

RESULTS

Characterization of an R. capsulatus XDH Mutant Affecting the Binding Site of FeSI—The two non-identical [2Fe2S] clusters located in the XdhA domain of R. capsulatus XDH are coordinated by the amino acid motifs Cys47–Xn–Cys106–Xn–Cys134–Arg–Cys136 for FeSI and Cys44–Xn–Cys730–Xn–Cys87–Xn–Cys63 for FeSII (Fig. 1). FeSI is the cluster in proximity to the pterin ring of Moco, whereas FeSII is in proximity to the FAD (13, 16). It has been reported before that a mutation of Arg135 in R. capsulatus XDH, being located in-between the two conserved cysteine residues Cys134 and Cys136 of FeSI resulted in the production of two different forms of XDH: an (αβ)2 heterotetrameric form indistinguishable from the wild-type protein and an (αβ) heterodimeric form lacking FeSII (16). Thus alterations in FeSI seem to influence the correct assembly of the protein. To characterize alterations in the FeSI binding site on XDH by GluB

![FIGURE 1. Residues involved in coordination of Moco and FeSI of R. capsulatus XDH. Shown is the coordination of Moco and FeSI at the active site of R. capsulatus XDH by GluB, GlnA, CysA, CysB, and CysA, using the coordinates from the Protein Data Bank (accession number 1JRO).](Image 325x627 to 553x734)
Assembly of *R. capsulatus* XDH

region, with a shift in the absorbance peak from 465 to 450 nm (Fig. 2A). Also, the absorbance of the FeS clusters and Moco at 320 nm was strongly influenced. However, because the iron content was shown to be 62%, this finding suggests that the protein variant lacks a significant amount of FeSI and contains a modified version of the FeSI cluster in a small amount of the protein. Steady-state kinetics of the XDH-CA134A/CA136A variant showed no xanthine-oxidizing activity with NAD\(^+\) or DCPIP as electron acceptor. In contrast to amino acid exchanges in the ligating cysteines for FeSI, it was not possible to purify a double variant of the ligands Cys\(_A\)\(^{44}\) and Cys\(_A\)\(^{47}\) for FeSI substituted by alanine, due to the instability of the variant (data not shown).

Generation of an (αβ) Dimeric XDH Variant by Amino Acid Exchanges in the Dimer Interface—Because the absence or a modified FeSI prevented the formation of the XDH (αβ)\(_2\) heterotetramer and a modified FeSI influenced the stability of the protein, cofactor insertion into XDH seems to be a coordinated process. To investigate whether Moco insertion into XDH precedes dimerization of the XdhB subunit to form the (αβ)\(_2\) heterotetramer, amino acid exchanges in the dimer interface region of XdhB were introduced. The crystal structure of *R. capsulatus* XDH suggested that introducing positively charged amino acids in the dimer interface region might interrupt dimerization (13). Thus a variant of XDH was generated exchanging Asp\(_B\)\(^{220}\) and Glu\(_B\)\(^{517}\) to arginine (Fig. 2C). Size exclusion chromatography identified that the XDH-DB220R/E\(_B\)\(^{517}\)R variant was mainly purified as an (αβ) heterotetramer after heterologous expression in *E. coli* (Fig. 2D). The small peak at 11 min showed that a very small portion of the protein existed as a heterotetramer (Fig. 2D). Compared with native XDH, the UV-visible absorption spectra of the protein variant showed that the heterodimer also displayed a diminished absorbance in the visible region, with a shift in the absorption peak from 465 to 450 nm (Fig. 2C). Also, the absorbance of the FeS clusters and Moco at 320 nm was strongly influenced; however, the absorbance changes differed from the XDH-C\(_A\)-134A/C\(_A\)-136A variant (Fig. 2A). The iron content was shown to be 81%, suggesting an almost full complement of FeS clusters (Table 1). Analysis of the molybdenum and MPT content of the variant indicated that the protein was purified in a form containing only residual amounts of Moco (Table 1), showing similarities to the XDH-C\(_A\)-134A/C\(_A\)-136A variant.

Investigations of the FeS Clusters of (αβ) Heterodimeric and (αβ)\(_2\) Heterotetrameric XDH Variants Using EPR Spectroscopy—Because of the differences in UV-visible spectra of *R. capsulatus* XDH and the XDH-C\(_A\)-134A/C\(_A\)-136A and XDH-DB220R/E\(_B\)\(^{517}\)R variants, the EPR properties of the FeS clusters were investigated. Fig. 3A shows the EPR spectra of the FeS clusters of dithionite-reduced *R. capsulatus* wild-type XDH (trace A), together with the corresponding simulations (traces B–E). The spectra show signals from the reduced FAD cofactor.

---

**TABLE 1**

| Metal content and kinetic parameters for *R. capsulatus* XDH and variants C\(_A\)-134A/C\(_A\)-136A, E\(_B\)-220R/D\(_B\)-517R, Q\(_A\)-102A, and Q\(_A\)-102G |
|---|
| **Xanthine-NAD activity**\(^a\) | **Xanthine:DCPIP activity**\(^b\) | **Iron, molybdenum, and MPT content**\(^c\) |
| \(k_{cat}\) | \(K_m^{\text{Xanthine}}\) | \(K_m^{\text{NAD}}\) | \(k_{cat}\) | \(K_m^{\text{DCPIP}}\) | Fe\(^d\) | Mo\(^e\) | MPT\(^f\) |
| \(s^{-1}\) | \(\mu M\) | \(\mu M\) | \(s^{-1}\) | \(\mu M\) | % |
| Wild-type XDH | 77.5 ± 5.4 | 44.2 ± 9.0 | 32.8 ± 2.9 | 66.1 ± 4.3 | 82.3 ± 9.7 | 95 | 95 | 95 |
| C\(_A\)-134A/C\(_A\)-136A | ND\(^g\) | ND | ND | ND | ND | ND | ND | ND |
| E\(_B\)-220R/D\(_B\)-517R | ND | ND | ND | 34.3 ± 3.9 | 80.9 ± 15.8 | 99 | 66 | 68.5 |
| Q\(_A\)-102A | 39.5 ± 3.5 | 29.3 ± 5.7 | 40.2 ± 9.9 | 31.6 ± 4.6 | 80.9 ± 18.0 | 94 | 58 | 60 |
| Q\(_A\)-102G | 40.6 ± 1.1 | 22.7 ± 2.2 | 37.3 ± 3.3 | ND | ND | 81 | 2 | 4.1 |

---

\(^a\) Kinetic data were recorded in 50 mM Tris, 0.2 mM EDTA, pH 7.8, by varying the concentrations of xanthine and NAD\(^+\).

\(^b\) Kinetic data were recorded in 50 mM Tris, 0.2 mM EDTA, pH 7.8, by varying the concentrations of DCPIP.

\(^c\) Iron and molybdenum were determined by inductively coupled plasma-optical emission spectroscopy using a multi-element standard.

\(^d\) MPT was quantified after conversion to the stable oxidized fluorescent degradation product Form A. 100% was set to a control XDH with a full complement of Moco.

\(^e\) ND, none were detectable.
Assembly of R. capsulatus XDH

**FIGURE 3.** EPR spectra of XDH wild-type and variants. In A: EPR spectra of XDH wild type. Trace A, experimental spectrum of reduced XDH wild-type at 20 K. Trace B, corresponding simulation of the complete experimental spectrum made by addition of the different spectral contributions shown in Traces C–E. The Moco (Mo\(^{\text{V}}\)) was neglected in the simulation. Trace C, simulation of FeSI using an isotropic Gaussian line width of 2.8 mT and \(g\)-values of \(g_x, y, z = 2.017, 1.921, 1.921\). Trace D, simulation of FeSII with \(g\)-values of 2.070, 1.97, and 1.90 and line width of 4 mT. Trace E, simulation of the flavin semiquinone with an isotropic \(g\)-value of \(g = 2.001\) and a line width of 3.9 mT. Trace F, experimental spectrum of XDH wild type at 60 K where FeSI is no longer observed. Experimental conditions: 40 microwatts of microwave power (20 K), 160 microwatts of microwave power (60 K), 1 mT of modulation amplitude, and 12.5-kHz modulation frequency. In B: EPR spectra of FeSI and FeSII centers in XDH-CA134A/CA136A and XDH-EB220R/DB517R variants. Trace A, experimental spectrum of FeSI and FeSII in wild-type XDH at 20 K. Trace B, experimental spectrum of CA134A/CA136A and EB220R/DB517R variants. Trace A, experimental spectrum of FeSI and FeSII in wild-type XDH at 20 K. Trace B, experimental spectrum of CA134A/CA136A mutant, relative ratio of FeSI/FeSII is ~0.1. Trace C, simulation of CA134A/CA136A variant. Trace D, experimental spectrum of EB220R/DB517R mutant. Trace E, simulation of EB220R/DB517R variant with a relative ratio of FeSI/FeSII is ~1:1. The spectrum in B shows only little contribution from an FAD radical, because FAD is mostly double reduced under the experimental conditions (dithionite reduction at 5 °C and pH 8.0). Experimental conditions: 1 milliwatt of microwave power; 1 mT of modulation amplitude, 12.5-kHz modulation frequency, and temperature of 20 K.

**TABLE 2** Simulation parameters for R. capsulatus XDH wild-type and variants C\(_{\text{A}}\)/C\(_{\text{A3}}\) and E\(_{\text{b}}\)/D\(_{\text{b}}\)

| Sample          | Cluster                   | \(g\)-values | Line width | mT   |
|-----------------|---------------------------|---------------|------------|------|
| CA134A/CA136A   | FeSI                      | 2.016         | 1.926      | 1.924| 2.5  |
| CA134A/CA136A   | FeSII                     | 2.085         | 1.99       | 1.907| 5.5  |
| E\(_{\text{b}}\)/D\(_{\text{b}}\)517R | FeSI                      | 2.017         | 1.921      | 1.921| 2.8  |
| CA134A/CA136A   | FeSII                     | 2.070         | 1.97       | 1.900| 4.0  |
| CA134A/CA136A   | FeSII                     | 2.088         | 1.921      | 1.921| 3.4  |
| CA134A/CA136A   | FeSI                      | 2.048         | 1.96       | 1.900| 4.0  |
| CA134A/CA136A   | FeSII                     | 2.031         | 1.94       | 1.890| 2.2  |

- For values obtained in this work, errors for wild-type XDH are ±0.004 for FeSI and ±0.008 for FeSII. \(g\)-value for FeSII has a larger error due to overlap with the Mo(V) signal.
- The \(g\)-strain was included in the simulation with 0.025, 0.025, and 0.005 for \(g_x, g_y, g_z\), and \(g_{e}\).
- The \(g\)-strain was included in the simulation with 0.0, 0.0, 0.12, and 0.05 for \(g_x, g_y, g_z\), and \(g_{e}\).

The \(g\)-values of the wild-type FeSI signals are similar to the ones of the de-Moco form, only the line widths are somewhat larger. The double integrated simulations for the single iron-sulfur clusters (Traces C and D) display a ratio of 1:1 indicating the presence of both clusters in the same amount in the protein. The flavin semiquinone (FAD) has been simulated by using an isotropic \(g\)-value of 2.0 and a line width of 3.9 milliteslas (mT). This line width is larger as compared with that from usual flavins (~1.9 mT) (11) and points to a magnetic interaction observed at low temperatures with the metal centers. At 80 K the line width of FAD is only 2.1 mT, in accordance to that from other FAD cofactors (11). The Moco (Mo\(^{\text{V}}\)) has been neglected in the simulations.

The EPR spectra from the FeSI and FeSII clusters of variants CA134A/CA136A and XDH-DB517R differ from those of the wild type, although broad similarities remain (Fig. 3B). The spectrum of variant CA134A/CA136A (trace B, complete simulation in trace C) shows predominantly FeSI, which exhibits \(g\)-values, shifted closer together, and lines, showing broadening due to \(g\)-strain. The FeSI signal of the CA134A/CA136A variant is very weak. The relative EPR signal intensity (second integral) of FeSI versus FeSII (~0.1) is much smaller as...
for the wild type (1.0) (Fig. 3A, trace A) indicating a loss of a significant fraction of FeSI in this variant. This is also reflected in the reduced iron content of 62%, determined for this variant (Table 1).

For the D_α_220R/E_β_517R variant (trace D) a spectrum more similar to that from the wild type was observed, with strong signals from FeSI. Both signals, from FeSI and from FeSII, are similar to that of the wild type, except for a smaller value of \( g_x \) and slightly shifted values of \( g_y \) and \( g_z \) for FeSII. Furthermore, the line width is increased due to \( g \)-strain. The integrated signal intensities from FeSII versus FeSI, estimated from second integrals (trace E shows the complete simulation) show a relative ratio close to 1:1 indicating the presence of almost equal amounts of both clusters in this variant, consistent with the iron content of 81% (Table 1).

Iron, which may be bound in an unspecific way to the protein, can be sometimes observed in enzymes with active iron centers, in particular in variants, when the protein structure may be modified or in the worst case damaged. In its oxidized form this unspecific iron, Fe(III), usually gives a strong EPR signal with \( g = 4 \). We recorded therefore EPR spectra over the full field range from 50 to 380 mT from all samples in the oxidized form, prior to reduction with dithionite. In all cases we found only marginal amounts of \( g = 4 \) signal, corresponding at maximum (D_α_220R/E_β_517R variant) to <2% as compared with the EPR signals from the reduced FeSI and FeSII centers. This shows that practically all of the iron was present in the form of FeSI and/or FeSII clusters.

**CD Spectroscopy**—To obtain more information on the FeS centers in wild-type XDH and the D_α_220R/D_β_517R and C_β_134A/C_β_136A variants, CD spectra were measured in the visible region in both the reduced and oxidized forms (Fig. 4). The spectrum of the oxidized wild-type enzyme exhibited strong negative dichroic bands at \( \sim 350 - 400 \) nm and \( 520 - 580 \) nm, and intensive positive bands between 400 and 500 nm (Fig. 4A). From the various maxima and infections, transitions can be identified at \( 374 (-), 434 (+), 470 (+), \) and \( 552 (-) \) nm. On the reduction with dithionite, the spectrum changes markedly with less intense transitions at \( 371 (-), 409 (+), 461 (-), \) and \( 573 (-) \) nm. The visible CD spectra of reduced and oxidized *R. capsulatus* wild-type XDH are very similar in shape and intensity to those of *Comamonas acidovorans* XDH (26). The spectra of the two variants were measured and compared with wild-type XDH. As seen in Fig. 4, the CD spectra derive largely from the FeS centers, thus, they provide further information about the content of correctly assembled FeSI and FeSII in the enzymes. CD \( \varepsilon_434 \) and \( \varepsilon_470 \) values for wild-type XDH, the D_α_220R/D_β_517R variant, and the C_β_134A/C_β_136A variant were determined from the spectra and used to calculate the amount of FeS centers in the variants, assuming the FeS center are the sole contributor to the CD spectrum at 434 nm and 470 nm. As obvious in Fig. 4 (A and B), the spectra of variant D_α_220R/D_β_517R in comparison to wild-type XDH have essentially the same form, but show different intensities. The intensity for the D_α_220R/D_β_517R variant is \( \sim 80\% \) of the wild-type XDH spectrum, consistent with the reduced iron content of the enzyme. However, apparently both FeSI and FeSII are present at lower concentrations. The spectrum of the D_α_220R/D_β_517R

![CD spectroscopy. CD spectra were recorded in the oxidized state (solid lines) and after reduction with sodium dithionite (dotted lines) from 1.7 mg/ml wild-type XDH (A), 1.7 mg/ml XDH-D_α_220R/D_β_517R (B), and 1.7 mg/ml XDH-C_β_134A/C_β_136A (C). Spectra were recorded in 50 mM Tris, 1 mM EDTA, pH 7.5.](image-url)
Amino Acid Substitutions in the GlnA<sub>102</sub> Ligand of the Molybdenum Cofactor Bound to the XdhB Subunit—Especially mutations in the ligands for FeSI seemed to strongly influence the assembly of XDH and the subsequent insertion of Moco into the (αβ)<sub>2</sub> heterotetrameric protein. The amino acid GlnA<sub>102</sub>, which lies in proximity to FeSI (Fig. 1), was identified to be the only residue of the XdhA subunit with direct contact to the pterin ring of Moco (13). To analyze whether GlnA<sub>102</sub> is involved in the electron transfer reaction or influences the assembly of the XdhA and XdhB subunits, we changed this residue to alanine and glycine by site-directed mutagenesis. Size exclusion chromatography identified that the XDH-QA<sub>102</sub>G and XDH-QA<sub>102</sub>A variants were expressed as an (αβ)<sub>2</sub> heterotetramer. The UV-visible absorption spectra of the protein variant in comparison to native XDH showed little difference in the region around 300–350 nm (Fig. 5). Analysis of the molybdenum and iron content revealed that the protein contained a full complement of iron, whereas the molybdenum content corresponded well with the MPT content determined to be 60% for both variants. The kinetic parameters at pH 7.8 of the XDH-QA<sub>102</sub>G and XDH-QA<sub>102</sub>A variant with either NAD<sup>+</sup> or DCPIP as electron acceptors are given in Table 1. The <i>k<sub>cat</sub></i><sub>xanthine</sub> and <i>K<sub>m</sub></i><sub>xanthine</sub> for NAD<sup>+</sup> as electron acceptor for the protein variants were found to be very similar, whereas the values with DCPIP as electron acceptor varied between the two variants and the wild-type XDH. Because no major differences in comparison to the wild-type enzyme were determined, it is rather unlikely that the electron transfer pathway from Moco to FeSI occurs via this amino acid residue.

Generation of an XDH Variant Containing Only One Active Site per (αβ)<sub>2</sub> Tetramer—So far it was believed that both of the two identical (αβ) heterodimers of <i>R. capsulatus</i> XDH act independently during catalysis (14). To analyze whether cooperativity exists in <i>R. capsulatus</i> XDH of an intramolecular electron transfer occurs between two XdhB subunits, we created an XDH variant containing two different active sites in the (αβ)<sub>2</sub> heterotetramer: while one site contained the wild-type structure, the second active site contained the amino acid exchange E730A, which was previously shown to result in a fully inactive enzyme (14). The chimeric protein was created by fusion of two different affinity tags to the C terminus of the XdhB subunit (see “Experimental Procedures”). The two different versions of XdhB, containing a His<sub>6</sub> tag and an intein tag, were co-expressed together with the XdhA and XdhC subunits, and purified first by Ni-NTA chromatography and then by chitin affinity chromatography. The two affinity purification steps should separate all assembled proteins containing either two active wild-type XdhB subunits or two inactive XdhB-E730A subunits from the chimeric XdhB-wt/E730A variant. Fig. 6 shows a Coomassie-stained SDS-PAGE gel of the different purifications steps of the (αβ)<sub>2</sub>(wt/E730A) chimeric XDH. For better comparison, the wild-type XDH was purified according to the same procedure (Fig. 6). Analysis of the Moco content showed that both proteins were purified in a form containing similar amounts of Moco with 76% for wild-type XDH and 70% for the (αβ)(wt/E730A) chimeric XDH (Table 3). For further char-
characterization of the activity of both proteins, reduction spectra with xanthine and NADH were recorded. Immediately after mixing the reduction level with xanthine as substrate resulted in ~46 and 26% of the initial absorbance at 465 nm for wild-type XDH and the (α)2(β, wt/β,E730A) chimeric XDH, respectively. Reduction with NADH showed a level of 66% for wild-type XDH and 74% for the (α)2(β, wt/β,E730A) chimeric XDH (Table 3). Because the reduction with NADH results in a reduced XDH to the four-electron state, both proteins are fully functional at the FAD site and the [2Fe2S] clusters. However, the reduction with xanthine indicated that only 46% of wild-type XDH contained the terminal Mo=S ligand required for activity. In comparison, the (α)2(β, wt/β,E730A) chimeric XDH was only reduced to a level of 26%, which is about half of the activity determined for wild-type XDH expressed simultaneously under the same conditions.

Steady-state Kinetics of the (α)2(β, wt/β,E730A) Chimeric XDH Variant—Steady-state kinetics of wild-type R. capsulatus XDH, in comparison to the (α)2(β, wt/β,E730A) chimeric XDH, were performed by varying the concentrations of xanthine and NAD (see “Experimental Procedures”). From the data for the wild-type XDH, the $k_{\text{cat}}$ and $K_m$ values were obtained. The kinetic parameters for the wild-type XDH and the chimera are presented in Table 4.

### Table 3: Determination of the MPT content and the level of reduction with xanthine and NADH of R. capsulatus wild-type XDH and the (α)2(β, wt/β,E730A) chimeric XDH variant

| Reduction with | Reduction with | MPTa |
|---------------|---------------|------|
| xanthineb     | NADHb         |      |
| %             | %             |      |
| Wild-type XDH | 46            | 66   |
| (α)2(β, wt/β,E730A) | 26            | 74   |

a XDH in 0.5 ml of 50 mM Tris, 1 mM EDTA, pH 7.5, was reduced with either 100 μM xanthine or 100 μM NADH. Complete reduction was achieved by the addition of 20 mM sodium dithionite.

b MPT was quantified after conversion to the stable oxidized fluorescent degradation product Form A. 100% was set to a control XDH with a full complement of Moco.

### Table 4: Kinetic parameters for R. capsulatus wild-type XDH and the (α)2(β, wt/β,E730A) chimeric XDH variant

|         | $k_{\text{cat}}$ | $K_m$ | $K_m$ | $K_m$ | $K_m$ | $K_m$ |
|---------|-----------------|-------|-------|-------|-------|-------|
|         | s⁻¹ M⁻¹ s⁻¹ μM | s⁻¹ μM | s⁻¹ μM | s⁻¹ μM | s⁻¹ μM |
| Wild-type XDH | 38.8 ± 3.4    | 36.2 ± 7.3 | 1.07 | 62.2 ± 4.5 | 1.07 |
| (α)2(β, wt/β,E730A) | 19.7 ± 0.4 | 27.0 ± 1.4 | 0.73 | 31.3 ± 1.0 | 0.7 |

### DISCUSSION

Bioconversion of the functional form of XOR is clearly a multistep process. In the case of the homodimeric mammalian XOR, this requires the incorporation of FAD, 2x[2Fe2S] centers, and Moco into each subunit (1). To date it has not been investigated whether this occurs during the synthesis of the protein or post-translationally before or after dimer formation. Bioconversion of R. capsulatus XDH is even more complicated, requiring the assembly of an (αβ)2 heterotetramer, which is a dimer of dimers. Here, the FAD and [2Fe2S] centers are inserted into the XdhA subunit, whereas the Moco is bound to the XdhB subunit (15). Our studies suggest that insertion of the three different redox centers into R. capsulatus XDH is a complex process that occurs in an ordered manner (Fig. 6). The data both from EPR and CD show that in the XDH-CA134A/C136A variant a significant amount of FeSI is missing. Only ~10% of FeSI is missing in this variant. Because the XDH-CA134A/C136A variant was found to have mainly an (αβ) dimeric structure with a little portion remaining as (αβ)2 heterotetramer (Fig. 2A), we conclude that the absence of an active FeSI cluster results in a structure unable to form the XDH(αβ)2 heterotetramer. Also Moco was missing in this protein variant. Previous studies showed that a stable Moco-free (αβ)2 heterotetrameric form of XDH can be purified from an E. coli moaA-deficient strain (22). Thus, we conclude that Moco insertion is the last step of XDH assembly and occurs after the formation of the (αβ)2 heterotetramer.

To analyze whether intramolecular electron transfer occurs from Moco via the residue Gin102, which is in close proximity to the ligand Cin103 of FeSI and the only residue of the XdhA subunit involved in coordination of Moco, Gin102 was substituted by alanine or glycine. Because no changes of the steady-state kinetics were found, any impact of Gin102 on the intramolecular electron transfer rates can be ruled out. Modification of the cysteine residues Cys104 and Cys47 coordinating FeSI result in an unstable protein. Surprisingly, modification of two amino acid residues at the dimer interface of the XdhB subunit to positively charged residues results in a stable (αβ) dimeric XDH form. Because the dimeric protein contained both FeSI and FeSII and FAD but no Moco, this is additional evidence that Moco insertion might occur after the formation of the (αβ)2 heterotetramer. However, we cannot rule out the possibility that the structure of the (αβ) heterodimer was altered by the two amino acid exchanges in such a manner that both dimerization via the XdhB subunit and Moco insertion were influenced, although FAD and FeS insertion are not affected.

An altered structure of the (αβ) dimer can be inferred from the slightly modified EPR spectrum of FeSI from the XDH-E522R/D517R variant and the larger EPR line width (g-strain). However, the CD and EPR data show that both FeSI and FeSII are present, and possibly the structure of the (αβ)2 dimer is stabilized and thus slightly changed after formation of the (αβ)2 heterotetramer.

The failure to express the XdhA subunit separately as reported before (16) implies that XdhA has to dimerize with the...
XdhB subunit to form a stable complex prior to insertion of FAD and the two [2FeS] clusters. Also, the separately purified XdhB subunit, which was stabilized by specific purification conditions, has been shown to be monomeric and free of Moco (22).

We conclude that the presented data support the view that the assembly of XDH is a highly ordered process, which involves the synthesis of the XdhA and XdhB subunits, the dimerization of both subunits, the insertion of FeSI, FeSII, and FAD into the XdhA subunit, dimerization of two (αβ) dimers via the XdhB subunit, and finally, insertion of sulfurred Moco into XdhB by aid of XdhC. When one of the FeS centers is incorrectly assembled, the protein remains an (αβ) heterodimer form, which is incompetent for Moco insertion.

FIGURE 7. Model for the assembly of R. capsulatus XDH. The assembly of R. capsulatus XDH involves the synthesis of the XdhA and XdhB subunits, the dimerization of both subunits, the insertion of FeSI, FeSII, and FAD into the XdhA subunit, dimerization of two (αβ) dimers via the XdhB subunit, and finally, insertion of sulfurred Moco into XdhB, resulting in an active enzyme (Fig. 7). The biosynthesis of Moco is additionally a complex process involving more than a dozen different proteins (27), with the insertion of sulfurred Moco by the XdhC protein being the last step of XDH maturation (28). This step is strictly regulated in R. capsulatus, because in vivo di-oxo Moco is not inserted into R. capsulatus XDH (29). Thus XdhC performs two reactions: (i) to ensure that Moco is sulfurred by the interaction with the l-cysteine desulfurase NiFs4 (30) before insertion into XDH and (ii) to insert the sulfurred Moco in the formed (αβ)2 heterotramer of XDH. Because Moco is deeply buried in the protein, it is also believed that XdhC acts as a chaperone being involved in proper folding of XDH after Moco insertion (29).

In contrast to a previous report on bovine XO (19), we have shown that both (αβ) dimers of R. capsulatus XDH act independently without cooperativity or intramolecular electron transfer between the XdhB subunits. We were able to purify a chimeric (α)2(βw1/βw2E730A) XDH with one active XdhB subunit and one inactive XdhB subunit (containing the amino acid exchange E730A at the active site). Although the $K_m$ values for the substrates xanthine and NAD remained unaltered, $k_{cat}$ for xanthine was reduced to 50% compared with the wild-type protein, ruling out any cooperativity or intramolecular electron transfer between the active subunits. This result is in agreement with early reports for bovine XO, suggesting that both subunits of bovine XO carry out catalysis independently (1). However, these findings are in contrast to a recent report by Tai and Hwang (19) who demonstrated that binding of slow substrates like 6-formylpterin at one active site affects the binding affinity and catalysis rate at the other active site. This finding may be taken as an indication for a difference in subunit interaction between R. capsulatus XDH and bovine XO. It would be, therefore, instructive, to apply the same approach used in this work also to bovine XO to unambiguously confirm or rule out a cooperative mechanism for the mammalian enzyme.

The present results for R. capsulatus XDH demonstrate that dimerization of the (αβ) subunits is required to stabilize a structure of the protein that makes the protein suitable for Moco insertion. In contrast to our results, a sulfite oxidase variant was identified in a human patient containing the amino acid exchange G473D in impaired dimerization of the protein but still contains a full complement of Moco (31). For this protein variant it was shown that misfolding prevented dimerization of sulfite oxidase, thereby preventing an efficient electron transfer between the Moco domain and the heme domain of sulfite oxidase (31). So far, no specific chaperone/scaffold protein for Moco insertion like the XdhC protein for XDH has been reported for sulfite oxidase, thus a “quality control” is missing for sulfite oxidase ensuring that the enzyme adopts the correct conformation suitable for Moco insertion. This observation together with our data supports the idea that dimerization of molyboenzymes is important for stabilizing a structure for the intramolecular electron transfer between various cofactors in most molyboenzymes.

Acknowledgment—We thank Annika Krause for help with cloning.

REFERENCES

1. Hille, R. (1996) Chem. Rev. 96, 2757–2816
2. Choi, E.-Y., Stockert, A. L., Leimkühler, S., and Hille, R. (2004) J. Inorg. Biochem. 98, 841–848
3. Enroth, C., Eger, B. T., Okamoto, K., Nishino, T., and Pai, E. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10723–10728
4. Nishino, T., Okamoto, K., Kawaguchi, Y., Horii, H., Matsumura, T., Eger, B. T., Pai, E. F., and Nishino, T. (2005) J. Biol. Chem. 280, 24888–24894
5. Nishino, T. (1994) J. Biochem. (Tokyo) 116, 1–6
6. McCord, J. M. (1985) New Engl. J. Med. 312, 159–163
7. Komai, H., Massey, V., and Palmer, G. (1969) J. Biol. Chem. 244, 1692–1700
8. Hunt, J., Massey, V., Dunham, W. R., and Sands, R. H. (1993) J Biol. Chem. 268, 18685–18691
9. Palmer, G., and Massey, V. (1969) J. Biol. Chem. 244, 2614–2620
10. Canne, C., Stephan, I., Finsterbusch, J., Lingens, F., Kappl, R., Fetzner, S., and Huttermann, I. (1997) Biochemistry 36, 9780–9790
11. Parschat, K., Canne, C., Huttermann, I., Kappl, R., and Fetzner, S. (2001) Biochim. Biophys. Acta 1544, 151–165
12. Kappl, R., Sielker, S., Rangelova, K., Wegner, J., Parschat, K., Huttermann, I., and Fetzner, S. (2006) Biochemistry 45, 14853–14868
13. Truguilo, J. I., Theis, K., Leimkühler, S., Rappa, R., Rajagopalan, K. V., and...
14. Leimkuhler, S., Stockert, A. L., Igarashi, K., Nishino, T., and Hille, R. (2004) *J. Biol. Chem.* **279**, 40437–40444
15. Leimkuhler, S., Kern, M., Solomon, P. S., McEwan, A. G., Schwarz, G., Mendel, R. R., and Klipp, W. (1998) *Mol. Microbiol.* **27**, 853–869
16. Leimkuhler, S., Hodson, R., George, G. N., and Rajagopalan, K. V. (2003) *J. Biol. Chem.* **278**, 20802–20811
17. Schindelin, H., Kisker, C., Hilton, J., Rajagopalan, K. V., and Rees, D. C. (1996) *Science* **272**, 1615–1621
18. Kisker, C., Schindelin, H., and Rees, D. C. (1997) *Annu. Rev. Biochem.* **66**, 233–267
19. Tai, L. A., and Hwang, K. C. (2004) *Biochemistry* **43**, 4869–4876
20. Palmer, T., Santini, C.-L., Iobbi-Nivol, C., Eaves, D. J., Boxer, D. H., and Giordano, G. (1996) *Mol. Microbiol.* **20**, 875–884
21. Johnson, J. L., Indermaur, L. W., and Rajagopalan, K. V. (1991) *J. Biol. Chem.* **266**, 12140–12145
22. Neumann, M., Schulze, M., Junemann, N., Stocklein, W., and Leimkuhler, S. (2006) *J. Biol. Chem.* **281**, 15701–15708
23. Johnson, J. L., Hainline, B. E., Rajagopalan, K. V., and Arison, B. H. (1984) *J. Biol. Chem.* **259**, 5414–5422
24. Stemsans, A., and Vangorp, G. (1989) *Phys. Rev. B Condens. Matter* **39**, 2864–2867
25. Stoll, S., and Schweiger, A. (2006) *J. Magn. Reson.* **178**, 42–55
26. Xiang, Q., and Edmondson, D. (1996) *Biochemistry* **35**, 5441–5450
27. Rajagopalan, K. V. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 674–679, ASM Press, Washington, DC
28. Neumann, M., Stocklein, W., and Leimkuhler, S. (2007) *J. Biol. Chem.* **282**, 28493–28500
29. Leimkuhler, S., and Klipp, W. (1999) *J. Bacteriol.* **181**, 2745–2751
30. Neumann, M., Stocklein, W., Walburger, A., Magalon, A., and Leimkuhler, S. (2007) *Biochemistry* **46**, 9586–9595
31. Wilson, H. L., Wilkinson, S. R., and Rajagopalan, K. V. (2006) *Biochemistry* **45**, 2149–2160