Mechanism of the Conversion of Xanthine Dehydrogenase to Xanthine Oxidase

IDENTIFICATION OF THE TWO CYSTEINE DISULFIDE BONDS AND CRYSTAL STRUCTURE OF A NON-CONVERTIBLE RAT LIVER XANTHINE DEHYDROGENASE MUTANT*†‡§

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Mammalian xanthine dehydrogenase can be converted to xanthine oxidase by modification of cysteine residues or by proteolysis of the enzyme polypeptide chain. Here we present evidence that the Cys992 and Cys1324 residues of rat liver enzyme are indeed involved in the rapid conversion from the dehydrogenase to the oxidase. The purified mutants C535A and/or C992R were significantly resistant to conversion by incubation with 4,4'-dithiodipyridine, whereas the recombinant wild-type enzyme converted readily to the oxidase type, indicating that these residues are responsible for the rapid conversion. The C535A/C992R mutant, however, converted very slowly during prolonged incubation with 4,4'-dithiodipyridine, and this slow conversion was blocked by the addition of NADH, suggesting that another cysteine couple located near the NAD⁺ binding site is responsible for the slower conversion. On the other hand, the C535A/C992R/C1316S and C535A/C992R/C1324S mutants were completely resistant to conversion, even on prolonged incubation with 4,4'-dithiodipyridine, indicating that Cys1316 and Cys1324 are responsible for the slow conversion. The crystal structure of the C535A/C992R/C1324S mutant was determined in its demolybdo form, confirming its dehydrogenase conformation.

Xanthine oxidoreductase (XOR),¹ xanthine dehydrogenase (XDH, EC 1.1.1.204), or xanthine oxidase (XO, EC 1.2.3.2) is a complex metalloflavoenzyme that catalyzes oxidation of hypoxanthine to xanthine and xanthine to uric acid with concomitant reduction of NAD⁺ or molecular oxygen. The enzyme is a homodimERIC protein of Mₙ, 300,000 and is composed of independent subunits; each subunit contains one molybdopterin, two non-identical iron sulfur centers ([2Fe-2S] clusters), and one FAD (1, 2, 3). The oxidative hydroxylation of xanthine to uric acid takes place at the molybdenum center, and reducing equivalents thus introduced are transferred rapidly via two iron sulfur centers to FAD, where physiological oxidation occurs (4).

The mammalian enzymes exist in the NAD⁺-dependent form (xanthine dehydrogenase, XDH) in freshly prepared samples from organs under normal conditions, i.e. they exhibit low xanthine/O₂ reductase activity but high xanthine/NAD⁺ reductase activity, even in the presence of O₂ (5, 6). XDH can be converted reversibly to xanthine oxidase (XO) by oxidation of cysteine residues or irreversibly by limited proteolysis (5–13). XO has high reactivity toward O₂ but negligible reactivity toward NAD⁺. As XO can reduce molecular oxygen to superoxide and hydrogen peroxide (1), XO is thought to be one of the key enzymes producing reactive oxygen species (14).

The crystal structures of bovine milk XO and proteolytically produced XO have been solved and showed large conformational differences around the FAD (15). Although the transition seems to occur in a similar way, whether caused by cysteine modification or proteolysis, the identification of the responsible cysteine residues is still a matter of controversy. It is not easy to identify the responsible residues, because the enzyme contains as many as 36 cysteine residues monomer of rat XOR (12), and many residues are modified by common cysteine-modifying reagents, such as 5,5'-dithiobis(nitroenzoic acid) or iodoacetamide (16). It was, however, reported that only four cysteine residues were modified during the conversion from rat liver XDH to XO by titration with 4,4'-dithiodipyridine (4,4'-DTPY) (16). During the titration, two disulfide bonds were suggested to be formed by modification with 4,4'-DTPY, because the addition of 2 mol of 4,4'-DTPY stoichiometrically provides 4 mol of 4-thiopyridone. A similar observation was made by Hunt and Massey (11) with the bovine milk enzyme. Although only four cysteine residues were modified during conversion from XDH to XO by this reagent, it was still difficult to identify the residues, because the modifier was released from the residues involved as part...
of the reaction. We then reported that relatively few cysteine residues were modified upon chemical reaction with FDNB (13). During the initial 10 min of reaction with FDNB, two specific cysteine residues, Cys(535) and Cys(992) were labeled, and these residues were suggested to be involved in the rapid conversion from XDH to XO. Recently, Rasmussen et al. (17) reported the sulfhydryl group modification of bovine milk XOR using radioactive iodoacetic acid, and their results are consistent with our assignment. On the other hand, this interpretation has subsequently been challenged in a report describing gel analyses of the proteolytically cleaved disulfide form of XO (18).

The crystal structure of bovine XOR shows that Cys(992) is situated on the surface of the molecule, but Cys(535) seems to be located in the long linker peptide between the FAD and the molybdopterin domains, although the residue is not visible in the crystal structure most probably due to its flexibility. The proteolytic cleavage site is also on the linker peptide. Based on detailed analyses of crystal structures of reversible XDH and proteolytic XO, as well as site-directed mutagenesis, Kuwabar et al. (19) concluded that the unique amino acid cluster of Phe(545), Arg(535) (corresponding to rat 334), Trp(335), and Arg(427) (rat 426) in the bovine enzyme sits at the center of a relay system that transmits modifications of the linker peptide caused by cysteine oxidation or proteolytic cleavage to the active site loop (Gln(425)–Lys(435)). The movement of the active site loop is considered to be the direct cause of the change in chemical behavior between XDH and XO (15).

In the present work, we describe two cysteine couples, one of which is responsible for rapid and the other for slow conversion from XDH to XO. We also present, at 2.6 Å resolution, the crystal structure of a non-convertible rat mutant XOR in the demolybdo-inactive form and show that its polypeptide chain adopts the XDH conformation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Spodoptera frugiperda (Sf) cells and wild-type circular Autographa californica nuclear polyhedrosis virus (AcNPV) DNA for wild-type XOR were obtained from Invitrogen (20). Bacterial DNA for mutant co-transfection was obtained from Novagen (BacVectorTM 2000). The transfer vector pJPV10Z was kindly provided by Dr. Palmer Taylor (University of California, San Diego). The restriction and modification enzymes were from Takara Shuzo Co., Ltd. (Tokyo, Japan) or New England Biolabs, Inc. The culture media IPL41 or Sf-900II, fetal calf serum, and insect cell lines and expression procedures were performed as described previously (20); the recombinant active XORs and demolybdo-dimeric XORs were separated by affinity column chromatography (23, 24). The enzymes were concentrated and incubated with 5 or 10 mM dithiothreitol (DTT) for 1 h at 25 °C to generate xanthine dehydrogenase forms of XOR, followed by gel filtration to remove excess DTT, as necessary.

**CDC-Galactosidase Assays—** CDC-Galactosidase assays were performed as described previously (20). Enzyme units were calculated on a basis of the amount of β-galactosidase substrate hydrolyzed per minute per milligram of protein.

**Purification Procedures of Recombinant Enzymes—** Purification procedures were performed as described previously (20). The recombinant active XORs and demolybdo-dimeric XORs were separated by affinity column chromatography (23, 24). The enzymes were concentrated and incubated with 5 or 10 mM dithiothreitol (DTT) for 1 h at 25 °C to generate xanthine dehydrogenase forms of XOR, followed by gel filtration to remove excess DTT, as necessary.

**SDS-Polyacrylamide Gel Electrophoresis—** SDS-PAGE was performed as described by Laemmli (25) using 10% polyacrylamide gels. Protein markers purchased from Bio-Rad consisted of a mixture of recombinant proteins (10–250 kDa).

**Enzyme Assays—**Enzyme assays were carried out at 25 °C in 50 mM potassium phosphate buffer, pH 7.8 containing 0.4 mM EDTA in a final volume of 3.0 ml. Xanthine oxidoreductase activities with various electron acceptors were determined by monitoring the absorbance changes as follows: O2 (air-saturated buffer; 295 nm) and NAD+ (500 μM; 340 or 295 nm). NADH-methylene blue (MB) activity was determined by monitoring the absorbance change at 340 nm with 50 μM NADH in the presence of 1 μM methylene blue. The Michaelis–Menten constant for NAD+ was determined from the absorbance at 450 nm using an extinction coefficient of 35.8 μM⁻¹ cm⁻¹ (26) for the native enzyme. XO was converted to XDH by incubation with 5 or 10 mM DTT at 25 °C for 1 h. The dehydrogenase to oxidase ratio (the DO ratio) as defined by Waud and Rajagopalan (8) was determined as the ratio of the absorbance change at 295 nm under aerobic conditions in the presence of NAD+ to that in the absence of NAD+ Activity to flavin ratio (1) was obtained by dividing the change in absorbance/min at 295 nm in the presence of NAD+ under aerobic conditions by the absorbance at 450 nm of the enzyme used in the assay at 25 °C. Photometric experiments were performed with a Hitachi U-3210 spectrophotometer.

**Crystallization and Data Collection of the C535A/C992R/C1324S Mutant of XOR—** The enzyme used for crystallization was further purified on a gel filtration column of TSKS3000XL (Takatori, Co.) just before crystallization to remove aggregated enzyme after folate affinity chromatography. As described previously, the recombinant XOR was expressed in multiple forms (20), and the enzyme used for crystallization was the demolybdo-dimeric form. The enzyme was concentrated to 8 mg/ml in buffer A (20) and incubated with 5 mM DTT for 60 min at 25 °C. Crystals of the mutant XDH were grown by vapor diffusion, equilibrating a mixture of 1 μl of protein and 1 μl of reservoir solution containing 9–11% polyethylene glycol 8000, 0.6 M LiSO4, 5 mM DTT, 1 mM sodium salicylate, 0.4 mM EDTA, 15% glycerol, and 40 mM HEPES (pH 6.20), against 1.5 ml of reservoir solution. Crystals of the enzyme were flash-frozen with their mother liquor as a cryoprotectant and mounted in cryoloops. Diffraction data were collected at beamline BL38B1, SPring8, Harima Garden City,
Expression and Purification of Three Mutants of Rat Liver Recombinant XOR—We reported previously that two cysteine residues, Cys<sup>535</sup> and Cys<sup>992</sup>, were involved in the conversion from XDH to XO, based on the results of chemical modification with FDNB. To confirm the role of these residues, we constructed three mutants of rat XOR, C535A, C992R, and C535A/C992R. In the chicken enzyme, which cannot be converted to XO by any treatment, the residue Cys<sup>992</sup> is replaced by an arginine, whereas the residue Cys<sup>535</sup> is conserved (27). The soluble form of recombinant rat liver XOR was expressed in the baculovirus/Sf9 cell system (20). As described previously, the expressed enzyme consisted of multiple forms; it contained inactive demolybdo-monomeric, inactive demolybdo-dimeric, and active molybdo forms (20). In this study, we used the active enzyme fraction eluted from the first falote affinity chromatography, which removes the demolybdo form, for functional analyses of both wild and mutant enzymes, except in NADH reduction experiments, where the demolybdo form does not influence the results (20). After purification, the activity to flavin ratio value of active XDH at 25 °C was ~130, which is comparable with that of native XOR purified from rat liver without the second affinity chromatography step (23). All of the enzymes were expressed in their XDH form and could be identified as such in crude extracts. Although the wild-type XOR retained negligible XDH activity only for 1 day after the start of purification, the mutant enzymes retained significant XDH activity even seven days after purification; they exhibited 25–50% XDH activity. All of the enzymes, including wild type, could be converted to XDH. Each of these purified recombinant XDHs showed mostly a single band of molecular mass at 150 kDa on SDS-PAGE, although there was a minor amount of proteolytic nicking in the C535A mutant (Fig. 1b). The values of the activity to flavin ratio of these purified enzymes were between 117 and 160, corresponding to a 59–80% active form (the activity to flavin ratio of these purified enzymes were between 117 and 160), which is comparable to the activity to flavin ratio for fully active enzyme is 200 (24), the value of active XDH at 25 °C was ~130, which is comparable to the activity to flavin ratio of these purified enzymes were between 117 and 160.

RESULTS

Conversion of Xanthine Dehydrogenase to Oxidase

We reported previously that two cysteine residues, Cys<sup>535</sup> and Cys<sup>992</sup>, were involved in the conversion from XDH to XO, based on the results of chemical modification with FDNB. To confirm the role of these residues, we constructed three mutants of rat XOR, C535A, C992R, and C535A/C992R. In the chicken enzyme, which cannot be converted to XO by any treatment, the residue Cys<sup>992</sup> is replaced by an arginine, whereas the residue Cys<sup>535</sup> is conserved (27). The soluble form of recombinant rat liver XOR was expressed in the baculovirus/Sf9 cell system (20). As described previously, the expressed enzyme consisted of multiple forms; it contained inactive demolybdo-monomeric, inactive demolybdo-dimeric, and active molybdo forms (20). In this study, we used the active enzyme fraction eluted from the first falote affinity chromatography, which removes the demolybdo form, for functional analyses of both wild and mutant enzymes, except in NADH reduction experiments, where the demolybdo form does not influence the results (20). After purification, the activity to flavin ratio value of active XDH at 25 °C was ~130, which is comparable to the activity to flavin ratio of these purified enzymes were between 117 and 160, corresponding to a 59–80% active form (the activity to flavin ratio of these purified enzymes were between 117 and 160), which is comparable to the activity to flavin ratio for fully active enzyme is 200 (24), the value of active XDH at 25 °C was ~130, which is comparable to the activity to flavin ratio of these purified enzymes were between 117 and 160.

Treatment of Wild-type and C535A/C992R, C535A, and C992R Mutant XORs with 4,4'-DTPY—The purified recombinant wild-type and mutant XOR enzymes were treated with 5 mM DTT to convert them to the XDH type, followed by gel filtration to remove DTT, and the obtained XDHs were treated with 4,4'-DTPY. A typical example is shown in Fig. 1a comparing the recombinant wild-type XOR and C535A/C992R double mutant XOR. The single mutant C535A and C992R XORs also showed similar profiles. Fig. 1a, A, shows the auto-oxidation process of cysteine residues during purification. Both enzymes were converted readily to XDH by 5 mM DTT during 60 min of incubation (Fig. 1a, B). In the case of recombinant wild-type XDH, the XO activity was increased from 20 to 100% within 15 min by treatment with 0.1 mM 4,4'-DTPY, as shown in Fig. 1a, C. The urate formation activity determined in the presence of NAD<sup>+</sup> remained at almost the initial level during the reaction. On the other hand, the conversion process from XDH to XO of the mutant was significantly slower than that of wild-type with 4,4'-DTPY, although even the double mutant was still converted slowly to the oxidase form (Fig. 1a, C) during prolonged incubation; the oxidase activity of C535A/C992R increased from 14 to 52% after 60 min at 25 °C. Resistance to conversion from XDH to XO was also observed in the single mutants C535A and C992R, although the double mutant C535A/C992R was the most resistant (Fig. 1c). These results indicate that, although Cys<sup>535</sup> and Cys<sup>992</sup> are involved in rapid conversion by treatment with 4,4'-DTPY, the conversion from XDH to XO involves not only Cys<sup>535</sup> and Cys<sup>992</sup>, but also other residues. It
should be noted that the oxidase activities after reaction with 4,4'-DTPY were reconverted to the XDH type or DTT-treated native rat liver XDH. As described above, the double mutant C535A/C992R XOR was expressed in its XDH form, but it gradually converted to the level of 50–60% XO activity during the 7–10-day purification period. On the other hand, triple mutants C535A/C992R/C1316S and C535A/C992R/C1324S were also expressed as XDH enzymes, but their XDH/XO activity ratio was not changed over a period of 10 days. When exposed to air, they were not converted to XO enzymes even after a month. Fig. 2b shows the spectra of purified C535A/C992R/C1316S and C535A/C992R/C1324S XORs without DTT treatment before and immediately after reduction with NADH under anaerobic conditions. The reduction level after 20 min (not shown) was the same as in the case of DTT-treated native rat liver XDH. Although we performed the same experiments with DTT-treated C535A/C992R/C1316S or C535A/C992R/C1324S XOR, the spectra were indistinguishable from each other, e.g. they showed the same levels of absorbance bleaching and semiquinone formation at ~600 nm that are typical of the XDH form. Fig. 1e displays the reaction time courses of the mutants C535A/C992R/C1316S and C535A/C992R/C1324S with 4,4'-DTPY. Both mutants showed no activity change at all from XDH to XO; they retained the initial dehydrogenase activity during the whole 1 h reaction period. These results are consistent with the fact that these triple mutants are not converted to XO by sulphydryl oxidation and support the view that Cys1316 and Cys1324 are involved in the slower conversion from XDH to XO by sulphydryl oxidation.

### Crystal Structure of the C535A/C992R/C1324S Mutant of Rat Liver XOR—The C535A/C992R/C1324S mutant of rat liver XOR in the demolybdo form was successfully crystallized, and diffraction data were collected to 2.6 Å resolution. The structure was determined by molecular replacement techniques and refined using molecular dynamics refinement. The asymmetric unit contains one subunit, in contrast to the previously structurally characterized bovine milk XDH, which contains one homodimer. Overall, however, the two structures are very similar (Fig. 3a). In both cases, the monomer consists of three major domains, the N-terminal, iron-sulfur center-binding domain, the FAD-containing intermediate domain, and the largest, the C-terminal domain (12, 15). The latter corresponds to the molybdopterin-binding domain in bovine milk XDH, although the molybdopterin cofactor was absent in this mutant, as already predicted from the results of chemical analysis (20). It is intriguing that the overall structure of the molybdopterin domain of this demolybdo-form mutant is not greatly different from that of the molybdo form of bovine milk XDH, and the amino acid residues surrounding the molybdopterin cofactor are situated at very similar positions and in similar orientations, leaving the space for the molybdopterin cofactor vacant.

Although the electron density of the interdomain linkers was not fully visible in the bovine milk XDH structure (Protein Data Bank code 1FO4), probably because of flexibility, the electron density linking the FAD and the molybdopterin domains was well defined in this mutant even though the B factors of residues Arg528–Gly536 were relatively high (70–84 Å2) compared with the mean B value of 37.3 Å2 for whole
enzyme peptide. This indicates a much reduced, although still recognizable, mobility in this part of the polypeptide chain of the rat enzyme when compared with the bovine one (19). The C992R residue is located at the end of a helix of the molybdopterin domain, and C535A is part of the linker connecting the FAD to the molybdopterin domain (Fig. 3b). The distance between the α carbon atom of residue 992 and that of residue 535 is ~18 Å. As we described previously, there is a unique cluster of amino acids that plays a dual role by forming the core of the relay system for the XDH/XO transition and by gating a solvent channel leading toward the FAD ring (19). The crystal structure of the present rat liver XDH mutant also contains this amino acid cluster, and the loop (Gln422–Lys435) takes the same conformation as in bovine milk XDH. These findings are consistent with the fact that the mutant is in the XDH form. Although the C-terminal stretch of amino acids was inserted into the FAD active site of the neighboring molecule in the bovine crystal structure (15), that C-terminal peptide interacts with the NAD⁺ binding pocket of the same molecule in the crystal structure of the rat enzyme (Fig. 3c). The peptide makes contact with a loop (Glu493–Met503), which seems to support the binding of NAD⁺ to the XDH form. Although electron density for the five amino acid residues between 1319–1323 was missing, the density corresponding to Cys1316 and Ser1324, mutated from Cys1324, was clearly observed in the present structure. The separation of 20.5 Å between Cys1316 and Cys1324 is rather long, and disulfide formation requires a large rearrangement of the flexible 9-amino-acid loop. Thus, it is very likely that the formation of a disulfide bridge between Cys1316 and Cys1324 will result in conformational changes of the C-terminal peptide, interfering with the insertion of this peptide, which seems to be important for pyridine nucleotide binding at the flavin active site.

FIG. 3. a, ribbon diagram of overall dimeric structure of rat XDH C535A/C992R/C1324S mutant. The interdomain loop (Ala529–Ala581) is shown in magenta. The C-terminal loop (Leu1315–Ile1331) is shown in orange. FAD and salicylate are shown as stick models. Fe-S centers are shown as space-filling models. The figures were generated with MOLSCRIPT (28) and RASTER3D (29) software. b, secondary structure elements around the residues that are involved in rapid XDH/XO conversion. The mutated residues Ala535 and Arg992 are shown as stick models. The interdomain loop region (Ala525–Ala581) is shown in magenta, as in a. FAD is shown as a stick model. Fe-S centers are shown as space-filling models. c, the C-terminal region of rat XDH in the NAD⁺ binding cavity. The C-terminal loop (Leu1315–Ile1331) is shown in orange. Cys1316 and Ser1324 are shown as stick models. The loop (Leu493–Met503) next to the C-terminal region is shown in yellow. The active site loop (Gln423–Lys435) is shown in blue. FAD is shown as a stick model. An Fe-S center is shown as a space-filling model. Pyridine moiety of NAD⁺ cofactor binds between the space of the loop (Leu493–Met503) and FAD cofactor.
DISCUSSION

The results of the mutation analysis clearly point to an involvement of both Cys⁵³⁵ and Cys⁹⁹² in the rapid conversion from xanthine dehydrogenase to oxidase. Both mutants C⁹⁹²R and C⁵³⁵A significantly resisted spontaneous conversion of XDH to XO by auto-oxidative disulfide formation or by the sulfhydryl modifier 4,4'-DTDY. As expected, the double mutant C⁵³⁵A/C⁹⁹²R was most resistant to the rapid conversion. Modification with FDNB also results in resistance to conversion, but on prolonged incubation of the mutants, the total activity decreases (data not shown) due to lysine modification near the xanthine binding site (13, 30). The fact that both single mutants resisted conversion suggests that these residues undergo disulfide formation. McManaman and Bain (18) questioned the involvement of Cys⁹⁹² and Cys⁵³⁵ in the XDH to XO conversion after they failed to detect peptides representative of the S-S-linked FAD and molybdopterin domains after proteolysis of oxidatively generated bovine XO. The gel chromatographic analysis that they applied, however, is prone to allow S-S exchange during the experiment when cysteine residues are incompletely alkylated. Involvement of the Cys⁹⁹² residue is also indicated by the x-ray analysis of a bovine XO crystal, which was successfully obtained under special conditions, showing the electron density of a disulfide bond associated with Cys⁹⁹².²

In the crystal structure of the rat liver XDH mutant elucidated in this study, the α carbon atoms of residues 992 and 535 are ~18 Å apart, so a large movement of the linker peptide would be required for disulfide bond formation between Cys⁵³⁵ and the mobile Cys⁹⁹². It should be noted that the distance between the two residues would become feasible for disulfide bond formation if the flexible peptide loop 527–535 were extended to some extent. As summarized in Fig. 4, recent studies of bovine milk XOR by site-directed mutagenesis and more detailed analyses of the crystal structures of bovine milk XDH and XO forms have revealed the presence of a unique amino acid cluster acting as a switch and a solvent gate during the transition between the dehydrogenase and oxidase forms of XOR (19). Tight interactions between the amino acid residues of the cluster are crucial for stabilization of the XDH form of the enzyme. The cluster residues sit at the center of a relay system that transmits modifications of the linker peptide, caused by cysteine oxidation or proteolytic cleavage, to the active site loop (rat enzyme Gln⁴²²–Lys⁴³²), resulting in a dramatic change in the conformation of the latter. Both modifications lead to the removal of Phe⁵⁴⁹ from the cluster, probably the trigger for the subsequent rearrangements. This can be caused either by a change in conformation induced by disulfide formation between Cys⁵³⁵ and Cys⁹⁹² or by proteolysis within the linker peptide. In the Rhodobacter capsulatus XDH, which cannot be converted to an oxidase form, the linker peptide is absent, because the flavin and molybdenum domains reside in separate subunits. The conformation of the R. capsulatus enzyme seems to be stabilized in the XDH form by additional steric factors (31, 32).

Although the C⁵³⁵A/C⁹⁹²R mutant did not display the rapid XDH to XO conversion seen with wild-type enzyme, it was

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² B. T. Eger, E. F. Pai, K. Okamoto, T. Nishino, unpublished observations.
converted slowly by an Src homology modifier, suggesting the existence of another cysteine couple responsible for the slower conversion. Reduction experiments with excess NAND of the C535A/C992R mutants, which had various dehydrogenase activities as a result of prolonged auto-oxidation, suggested that the other cysteine couple might be close to the NAND binding site (Fig. 2). The fact that the slow conversion reaction of (C535A/C992R) XDH to XO with 4,4'-DTPY was completely blocked in the presence of NAND also supports such an interpretation. The crystal structure of the bovine milk enzyme suggested that Cys1316 and Cys1324 (rat numbering) were the most likely candidates for such a couple. The previous results of chemical modification with radioactive FDNB showed that 0.39 mol/FAD of labeled dinitrophenol was incorporated into the C-terminal peptide, which lets it move in and out of the oxidase site. Subsequently, any disturbance, e.g. formation of a bridge, will result in the loss of NAND. Many previous studies have suggested that the slow XDH to XO conversion reaction of C535A/C992R with 4,4'-DTPY was completely blocked in the presence of NAND (Fig. 2a) and that the XO form, which was generated slowly, probably by disulfide formation between the two C-terminal cysteine residues, cannot be reduced by NAND (Fig. 2b). The fact that the electron density of five amino acid residues between 1319 and 1323 was not observed in the crystal structure suggests that this region of the loop is highly disordered and may be able to move to allow disulfide bond formation between Cys1316 and Cys1324. Efforts are under way to grow well diffracting crystals of the fully oxidized form of XO, a form that also contains the Cys1316-Cys1324 disulfide, to obtain a clearer picture of which conformational changes near the flavin cofactor are caused by this oxidative modification.

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