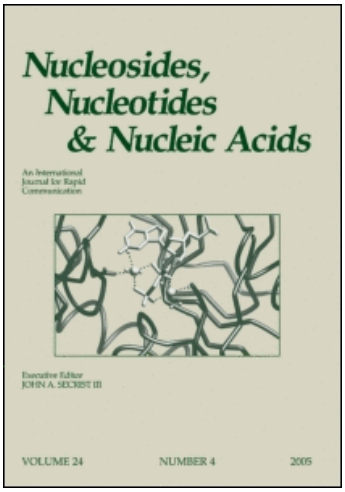

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MECHANISM OF TRANSITION FROM XANTHINE DEHYDROGENASE TO XANTHINE OXIDASE: EFFECT OF GUANIDINE-HCL OR UREA ON THE ACTIVITY

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□ *Mammalian xanthine oxidoreductase can be converted from the dehydrogenase to the oxidase form, either reversibly by formation of disulfide bridges or irreversibly by proteolytic cleavage within the xanthine oxidoreductase protein molecule. A tightly packed amino acid cluster stabilizes the dehydrogenase form, and disruption of this cluster is accompanied with rearrangement of the active site loop. Here, we show that the conversion occurs in the presence of guanidine-HCl or urea. We propose that xanthine dehydrogenase and oxidase are in a thermodynamic equilibrium that can be shifted by disruption of the amino acid cluster with a denaturant.*

Keywords Xanthine dehydrogenase; xanthine oxidase; super oxide; active oxygen

INTRODUCTION

Xanthine oxidoreductase (XOR) catalyzes the oxidation of hypoxanthine to xanthine or xanthine to uric acid in the metabolic pathway of purine degradation.[1,2] The animal enzymes are homodimers of molecular mass around 290 kDa, with each of the monomers containing one molybdopterin cofactor, two non-identical [2Fe-2S] centers, and one flavin adenine dinucleotide (FAD) cofactor.[2,3] which are located in the corresponding C-terminal 85 kDa, N-terminal 20 kDa, and intermediate 40 kDa domains, respectively.[4–6] The oxidation of xanthine takes place at the molybdopterin center and the electrons thus introduced are rapidly transferred to FAD via the Fe/SI and Fe/SII centers.[7] The reoxidation of the reduced enzyme by the oxidant substrate, NAD^+or molecular oxygen, occurs through FAD.[7,8] The mammalian enzymes can be converted from xanthine dehydrogenase (XDH), which uses NAD^+ as its oxidant substrate, to the xanthine oxidase (XO) form, which exclusively uses O_2 as its oxidant substrate.[9–11] This conversion occurs either reversibly by formation of disulfide bridges or irreversibly by proteolytic cleavage within the XOR

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protein molecule.[2,11–16] Based on site-directed mutagenesis studies of rat XOR expressed in a *Spodoptera frugiperda* (Sf9)/insect cell system, the two disulfide bonds responsible for the reversible conversion were identified as Cys535-Cys992 and Cys1316-Cys1324.[17] The site of tryptic cleavage responsible for irreversible conversion was identified as Lys551 in the linker peptide between the FAD and the molybdopterin domains.[4,5]

The crystal structures of both the XDH and XO forms of the enzyme from bovine milk[6] or rat liver enzymes[17,18] have been determined, and the structural differences between the two forms have been described.[17–19] A mechanism for the transition from XDH to XO has been proposed based on a detailed comparison of the XDH and XO structures, in addition to the results of site-directed mutagenesis studies.[18,19] Mechanistic models focus on an amino acid cluster formed by the residues Arg334, Trp335, Arg426, and Phe549 (amino acid numbers are those for the rat enzyme). Held together mostly by π-cation interactions, this cluster sits at the center of a relay system that can transmit modifications of the linker peptide, *e.g.*, disulfide formation or proteolytic cleavage of the linker peptide between the FAD and molybdopterin domains, to the FAD-approaching active site loop (rat enzyme Gln422-Lys432),[6] resulting in a dramatic change of the loop's conformation, as well as of the electrostatic environment around FAD.[6] During these conformational transitions, the cluster acts not only as a transmitter, as described above, but also as a solvent gate.[19] Tight interactions among the amino acid residues of the cluster are crucial for the stabilization of the XDH form of the enzyme. These interactions are disrupted, however, when Phe549 is removed from the cluster, either by a change in conformation induced by disulfide formation between Cys535 and Cys992,or by release of the linker peptide through proteolysis. It is highly likely that this disruption is the trigger for all subsequent rearrangements.[17–19] In this paper we show that treatment with a relatively low concentration of guanidine-HCl or a high concentration of urea resulted in the conversion of XDH to XO. Based on these results, we discuss the transition mechanism between the two forms.

EXPERIMENTAL PROCEDURES

Freshly purified milk XOR[20] or rat mutant C535A/C992R/C1316S XDH (17) was incubated with 5 mM dithiothreitol (DTT) in 50 mM Tris-HCl buffer at pH 7.8, 25°C for 1 hour. The treatment of XDH with guanidine-HCl or urea was performed on ice in 67 mM Tris-HCl, pH 7.8, containing 5 mM DTT, 0.2 mM EDTA, and 1 mM salicylate. The enzyme activity was measured according to the method reported previously.[14] NAD-dependent activity was determined by following the absorbance change at 340 nm and O_2-dependent activity was determined at 295 nm with NAD^+ using a Hitachi 3300 spectrophotometer equipped with a temperature-controller.

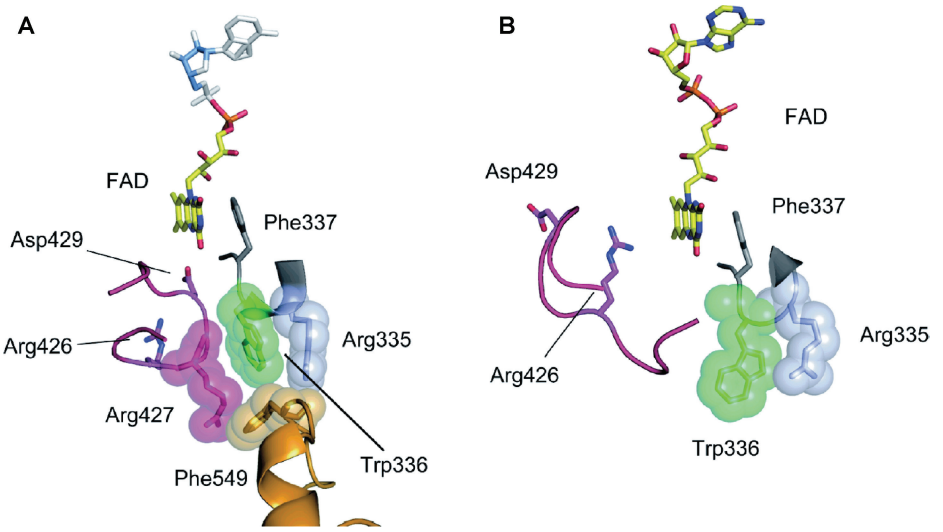

FIGURE 1 Crystal structure of XDH (A) and XO (B) around the unique active site amino acid cluster (space-filling of important amino acid residues), FAD cofactor (yellow-colored stick model), and active site loop (red-colored stick model) (from Enroth et al.[6]).

RESULTS AND DISCUSSION

Guanidine-HCl or Urea Treatment of Bovine Milk XOR

As described previously, the amino acid cluster consisting of two arginine residues (R335 and R427 in bovine XOR), one tryptophan residue (W336 in bovine XOR), and one phenylalanine residue (F549 in bovine XOR) plays a crucial role in the transition from XDH to XO. As shown in Figure 1, the cluster is tightly packed in the XDH form (A) in the crystal structure of the bovine milk enzyme,[19] while it is disrupted in the XO form (B), where only limited electron density of the amino acid residues could be observed. As the cluster is likely formed by π-cation interaction, it is expected to be disrupted by treatment with guanidine-HCl or urea. We found that the incubation of DTT-treated XDH from bovine milk with up to 2 M guanidine-HCl on ice resulted in a concentration-dependent loss of XDH activity (Figure 2A). These activity changes were independent of incubation time up to one hour (data not shown). As uric acid formation activity was not decreased, XO activity appeared to be increased by guanidine-HCl treatment. The above results indicate that the XOR-XO equilibrium is shifted in favor of XO in the presence of low concentrations of guanidine-HCl, presumably due to partial disruption of the cluster. However, even XO activity was decreased drastically at guanidine-HCl concentrations above 3 M, presumably because of extensive protein unfolding in the presence of high concentrations of the reagent. During the treatment of XOR with guanidine-HCl, it was also confirmed that the transition was neither due to disulfide formation nor proteolysis,

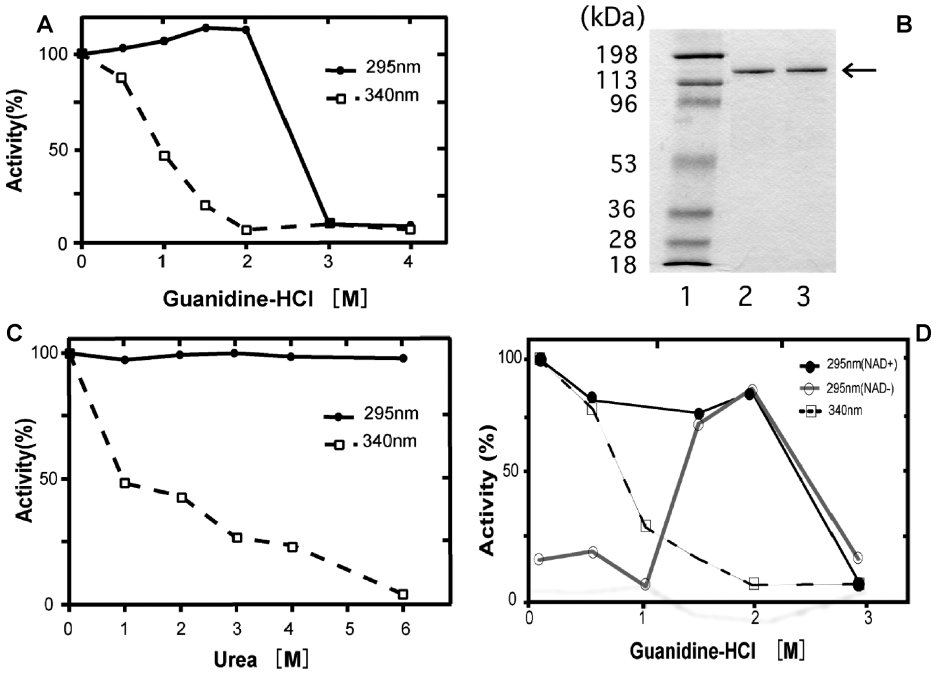

FIGURE 2 (A) Changes of uric acid formation activity (determined at 295 nm) in the presence of NAD^+ and NADH formation activity (determined at 340 nm) of bovine milk XOR after treatment with various concentrations of guanidine-HCl for 1 hour (see Experimental Procedures). (B) SDS-PAGE after guanidine-HCl treatment of bovine milk XOR (lane 2) and after dialysis (lane 3) of the lane 2 sample. Lane 1, Marker proteins from Bio-Rad (C) Change of uric acid formation activity (determined at 295 nm) in the presence of NAD^+ and NADH formation activity (determined at 340 nm) of bovine milk XOR after treatment with various concentrations of urea for 1 hour. (D) Changes of uric acid formation activity (determined at 295 nm) in the presence and absence of NAD^+ and NADH formation activity (determined at 340 nm) after treatment of rat C535A/C992R/C1316S mutant XOR with various concentrations of guanidine-HCl.

since the incubation mixture contained DTT, and no proteolytic cleavage was observed in SDS-PAGE (Figure 2B). Furthermore, the XO form treated with 1.5 M could be completely reconverted into XDH by dialysis against the same buffer without guanidine-HCl. Such transition from XDH to XO forms was also observed in the presence of urea, although a higher concentration of the reagent was necessary for complete conversion (Figure 2C).

Guanidine-HCl or Urea Treatment of Rat Liver Triple Mutant XDH

If the transition is mainly due to disruption of the amino acid cluster, the rat triple mutant, C535A/C992R/C1316S, that was reported previously as an XDH non-convertible by SH modifiers,[17] should be converted by guanidine-HCl treatment. As shown in Figure 2D even the rat triple mutant could be converted to XO.

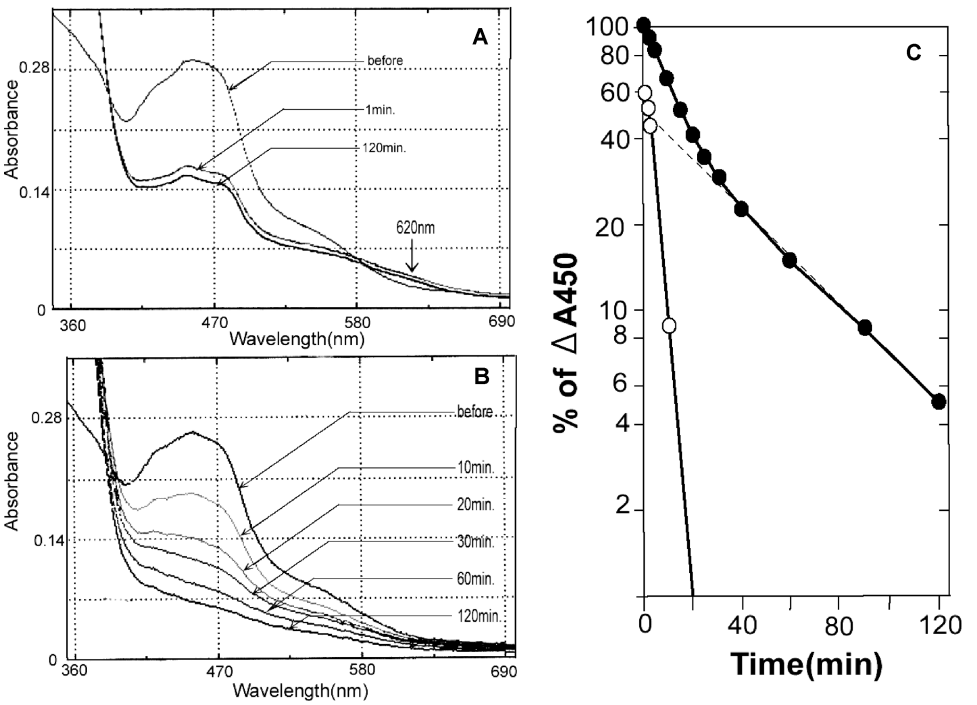

FIGURE 3 Spectral change upon addition of 0.2 mM NADH to guanidine-HCl untreated (A) and treated (2 M guanidine-HCl for 1 hour at 0°C before addition with NADH) (B) bovine milk XOR under anaerobic conditions at room temperature. Selected spectra from the time course are shown; the others are omitted for clarity. The arrow shows the position of the 620 nm wavelength (A). (C) Semilogarithmic plot of percentage absorbance change at 450 nm against incubation time. The corrected first phase was obtained graphically by subtracting absorbance changes due to the slow phase (—) from the overall change.

Reduction of XOR by NADH before and after Guanidine Treatment

As reported previously, when the XDH or XO form was mixed with NADH,[14,15,21] XDH was reduced readily by NADH to form $FADH_2/NAD^+$, FADH*/NAD^+ and FAD/NADH complexes, but XO was not reduced readily with NADH.[19] The rate constants of biphasic reduction of native bovine milk XDH with NADH were reported to be 6,000 and 1080 min^{-1}.[21] When the guanidine-HCl-treated enzyme was mixed with NADH (Figure 3B), the observed rate of this reaction was significantly slower than that in the case of XDH (Figure 3A), and no increase of the absorbance at 620 nm, which reflects the formation of flavin semiquinone, was observed. The reduction process was apparently a biphasic first-order reaction, and the almost fully reduced state was reached after 120 min (Figure 3B), in contrast with the case of native XDH, where only partial reduction was seen even after 120 min (Figure 3A). The apparent rate constants for the first and second phases were obtained as 0.07 and 0.005 min^{-1}, respectively, and each ab-

sorbance change corresponded to approximately 50% of the overall change (Figure 3C). The different rates and levels of reduction could be attributed to different affinities of XDH and XO for NADH/ NAD^+; XDH-FAD is known to form tight complexes of $FADH_2/NAD^+$, $FADH^*/NAD^+$ and FAD/NADH at equilibrium,[14,15,21] while XO-FAD might be reduced only slightly by addition of NADH due to its low affinity, and then NAD^+could be dissociated from partially reduced $FADH_2$and remaining oxidized FAD could be reduced partially again with NADH. That is, the reduction process of XO seemed to be due to an equilibrium shift of the oxidized/reduced XO ratio by gradual reduction owing to the low affinity of excess NADH, that is, a different process from that of XDH form. The biphasic process in the case of XO might be due to non-equivalence of the two subunits.

Mechanism of Transition Between XDH and XO

As suggested previously, the XDH form is stabilized by the tightly packed amino acid cluster. Disruption of the cluster is accompanied with movement of the active site loop (loop A: Gln423-Lys433) and extrusion of the C-terminal peptide that is supposed to support NAD^+ binding.[17] This movement not only replaces Asp429 adjacent to the FAD cofactor with Arg426, which might alter the electrostatic environment of the FAD cofactor in a manner that would destabilize FAD semiquinone, but also blocks the approach of NAD^+ cofactor to the reduced FAD. The present results suggest that the XDH and XO forms are in thermodynamic equilibrium and this equilibrium can be shifted by disruption of the unique amino acid cluster with a denaturant such as guanidine-HCl or urea, in a concentration-dependent manner. Interestingly, recent crystallographic analyses suggest that the conformational changes of the two subunits are not concerted during the XDH-XO transition,[18] in accordance with the biphasic reduction process with NADH of the 2 M guanidine-treated XO type enzyme (Figure 3C).

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