Xanthine oxidoreductase (XOR) is a 300-kDa homodimer that can exist as an NAD\(^+\)-dependent dehydrogenase (XD) or as an O\(_2\)-dependent oxidase (XO) depending on the oxidation state of its cysteine thiols. Both XD and XO undergo limited cleavage by chymotrypsin and trypsin. Trypsin selectively cleaved both enzyme forms at Lys\(^{184}\), while chymotrypsin cleaved XD primarily at Met\(^{181}\) and at Phe\(^{560}\). Chymotrypsin, but not trypsin, cleavage also prevented the reductive conversion of XO to XD; thus the region surrounding Phe\(^{560}\) appears to be important in the interconversion of the two forms. Size exclusion chromatography showed that disulfide bond formation reduced the hydrodynamic volume of the enzyme, and two-dimensional gel electrophoresis of chymotrypsin-digested XO showed significant, disulfide bond-mediated, conformational heterogeneity in the N-terminal third of the enzyme but no evidence of disulfide bonds between the N-terminal and C-terminal regions or between XOR subunits. These results indicate that intrasubunit disulfide bond formation leads to a global conformational change in XOR that results in the exposure of the region surrounding Phe\(^{560}\). Conformational changes within this region in turn appear to play a critical role in the interconversion between the XD and XO forms of the enzyme.

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Xanthine oxidoreductase (XOR)\(^1\) plays an important function in vertebrate metabolism by catalyzing the oxidation of xanthine to uric acid, the rate-limiting step in purine degradation (1). Native XOR is a 300,000-dalton dimer composed of identical and catalytically independent subunits. Each subunit consists of 1333–1358 amino acids, depending on the species, and contains binding sites for molybdopterin, iron, and flavin co-factors (2, 3). The genes and full-length coding regions for mammalian enzymes exhibit, respectively, 70 and 52% identity with the identical and catalytically independent subunits. Each subunit consists of 1333–1358 amino acids, depending on the species, and contains binding sites for molybdopterin, iron, and flavin co-factors (2, 3). The genes and full-length coding regions for mammalian enzymes exhibit, respectively, 70 and 52% identity with XOR. In addition, it is unclear to what extent conformational changes within the flavin-binding site reflect larger changes in the global conformation of XOR and whether such changes are necessary for the conversion of XD to XO. XD can also be irreversibly converted to XO by proteolysis (6, 11, 17). Recent crystallographic studies have shown that proteolysis of XD leads to structural alterations near the flavin cofactor domain (22). However, the crystallographic structure of intact XO generated by cysteine oxidation has not been solved, and thus it is unclear how similar the structure of disulfide-bonded XO is to proteolysed XO. In the present study we have used peptide mapping, chromatography, and two-dimensional gel electrophoresis to identify differences in the solution conformations of XD and XO and to investigate the role conformational changes play in the XD to XO conversion.

EXPERIMENTAL PROCEDURES

Materials—XOR (2.5–3.1 IU/mg) isolated from bovine milk in the oxidase form (XO) according to Waud et al. (23) was a generous gift from Dr. Joseph McCord (Webb-Waring Antioxidant Research Institute, University of Colorado Health Sciences Center). Sequencing grade trypsin...
and chymotrypsin were purchased from Boehringer-Ingelheim Inc. BS3 (bis(sulfosuccinimidyl) suberate) was purchased from Pierce. Polyvi-
nylidene difluoride membranes (Hyperbond) were purchased from Beck-
man Instruments Inc. Centricon ultrafiltration membranes were
purchased from Amicon Inc. Other chemicals and reagents were ob-
tained from either Sigma or Fischer Scientific.

Preparation of XD and Determination of XD and XO Isoforms—

Purified XO was converted to XD by incubation with 5–10 mM DTT at
room temperature for 1–2 h (17). The enzyme was then cooled to 4 °C
and subjected to ultracentrifugation at 4 °C using Centricon 10,000-Da cut-
off membranes to remove DTT. Enzymatic activity specifically associ-
ated with either XD (xanthine:NAD⁺ oxidoreductase activity) or XO
(xanthine:O₂ oxidoreductase activity) was calculated as the difference-
sensitive rates of aerobic formation of uric acid from xanthine in the
presence (XD) or absence (XO) of 0.6 mM NAD⁺, and the ratio of XD to
XO activities (D/O) was calculated according to Waud and Rajagopolan
(12) as described previously (24). Total XOR activity (both XD and XO
activities) was routinely assayed by the reduction of dichloroindophenol
using xanthine as a substrate (xanthine/dichloroindophenol assay) as
described previously (24). Total protein was quantified using the bicine-
nchonic acid method (25).

Protease Digestion—XOR (5–20 μg) in the XD or XO form was incu-
bated at room temperature (25 °C) with 1/20 (w/w) chymotrypsin or
trypsin in 50 mM Tris, 150 mM NaCl, pH 8, for 90 min. The reaction was
stopped by the addition of phenylmethylsulfonyl fluoride. The samples
were then assayed for enzyme activity and/or processed for SDS-PAGE
analysis as described below.

Electrophoretic Analyses in One and Two Dimensions—Electrophore-
sis was performed using a mini-Protean II gel apparatus (Bio-Rad) with
0.75-mm spacers. For one-dimensional electrophoresis samples were
diluted with an equal volumes of 2 × concentrated SDS-PAGE sample
buffer (0.125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH
6.5), heated for 3 min at 95 °C, and separated by electrophoresis in 10%
acrylamide gels containing 0.1% SDS at room temperature. For two-
dimensional electrophoresis samples were diluted with equal volumes
of 2 × concentrated SDS-PAGE sample buffer without 2-mercaptoetha-
hol, heated for 3 min at 95 °C, and subjected to electrophoresis in 10%
acrylamide gels as described above. When the tracking dye reached the
bottom of the gel, electrophoresis was stopped and the protein-contain-
ing lanes were excised and prepared for electrophoresis in the second
dimension by incubation in 3 ml of SDS-PAGE sample buffer containing
5% 2-mercaptoethanol for 1 h at 60 °C. After reduction the lanes were
layered on top of the second dimension gel (10% acrylamide, 0.1% SDS)
perpendicular to the direction of electrophoresis. Gaps were sealed with
melted agarose (1%) containing 2% SDS, 50 mM DTT. After electro-
phoresis protein bands were detected by silver staining (26).

Chemical Cross-linking Studies—XD was cross-linked by reaction
with a 50-fold molar excess of BS3 in phosphate-buffered saline, pH 7.5,
according to the manufacturer’s instructions.

Protein Sequence Determination—N-terminal amino acid sequence
analyses were performed by automated Edman degradation of proteins
electroblotted to polyvinylidene difluoride (27) using an Applied Biosys-
tems Inc. model 477A automated protein sequencing instrument
equipped with an in-line model 120 HPLC.

Size Exclusion-HPLC Analysis—HPLC-size exclusion chromatogra-
phy (HPLC-SEC) analysis was performed on a Beckman System Gold
Instrument using a 7.8 × 600-mm SEC-3000 column (Phenomenex Inc.)
equilibrated in phosphate-buffered saline, pH 7.5. Chromatography
was performed at room temperature at a flow rate of 1 ml/min. The
elution of protein and enzyme activity were monitored at 220 nm using
an online UV detector and (where indicated by collecting fractions)
using an electronically activated fraction collector (Gilson, FC 203B)
that was synchronously integrated to sample injection. Determination
of XD and XO retention times was performed using a 25-μl injection
loop and sample protein concentrations of 1 mg/ml. XO was converted to
XD by incubation with 10 mM DTT at room temperature for at least 1 h
prior to analysis. Mean retention times of each enzyme form were
determined from a minimum of five HPLC-SEC analyses. The SEC
properties of cross-linked XO were determined using a 100-μl injection
loop. Eluted protein was collected into 0.3-ml fractions. XO activity was
determined using the xanthine/dichloroindophenol assay. The electro-
phoretic characteristics of the cross-linked protein were determined by
SDS-PAGE analysis and silver staining as described above.

RESULTS

XD and XO Have Different Proteolytic Digestion Patterns—

Differences in the environment surrounding the flavin co-factor
binding regions of XD and XO have been reported previously
(20, 21). To determine whether these differences reflect broader
structural changes we investigated the trypsin and chymotryp-
in digestion patterns of both catalytic forms (Fig. 1). Chymo-
trypsin cleavage of XO (D/O = 0.1; 90% XO form) generated
three major cleavage products with an estimated molecular
weights on SDS-PAGE of 85,000 (band 1), 64,000 (band 2), and
44,000 fragments (band 3). In contrast, chymotrypsin cleavage of
XD (D/O = 7.1; 88% XD form) yielded a single major product
with an estimated molecular weight of 125,000 (band 5) and
minor amounts of a 44,000 fragment (band 6). Trypsin cleavage
of either isozyme of XOR generated a single 125-kDa fragment
(Fig. 1B, bands 7 and 8). In agreement with earlier studies (6,
11, 17) neither chymotrypsin nor trypsin digestion affected
total XOR activity or altered the apparent molecular weights of
the isozymes (data not shown).

The identities of the cleavage sites were determined by N-
terminal sequencing and comparing the experimentally deter-

FIG. 1. Trypsin and chymotrypsin digestion patterns of XD
and XO. XD (D/O = 7) and XO (D/O < 0.1) were incubated with trypsin
or chymotrypsin at 25 °C at an enzyme to protease ratio of 20/1 for the
indicated times. The reaction was stopped with phenylmethylsulfonyl
fluoride, and the digestion pattern was analyzed by SDS-PAGE using a
10% acrylamide gel. A shows the chymotrypsin digestion patterns of XD
and XO. B shows the trypsin digestion patterns of XD and XO. The
migration positions of molecular weight marker proteins (Kulidoscope
standards, Bio-Rad) are shown on the right in each panel.
mined sequences to the deduced sequence of bovine XD cDNA (7). Table I shows the sequences of the first 7 amino acids of the major XD and XO cleavage products, the position of these sequences within the enzyme, and the corresponding cleavage site. The N-terminal sequences of the 125-kDa fragments (bands 7 and 8) generated by trypsin digestion of XD or XO correspond to Lys185–Leu191 and are consistent with cleavage of both enzyme forms at Lys 184. The sequence of the 125-kDa fragment (band 5) generated by chymotrypsin digestion of XD corresponds to Asn182–Thr188 and is consistent with cleavage at Met181. Based on the deduced amino acid sequence of bovine XD the calculated molecular mass values for peptide fragments from Asn182 to Val1331 (the C terminus of bovine XD) and Lys185–Val1331 are 127,225 and 126,854 kDa, respectively (28). These values are in good agreement with SDS-PAGE size estimates (74 kDa) for the major fragments generated by trypsin digestion of XD or XO and chymotrypsin digestion of XD, and indicate that these fragments contain the C terminus of XOR.

The N-terminal sequences of the 85-kDa fragment (band 1) generated by chymotrypsin digestion of XO corresponds to Gln561–Gln567 and is consistent with cleavage at Phe560. The sequence of the 64-kDa fragment (band 2) corresponds to the N terminus of purified XOR (Thr2–Phe5). The sizes of the 64- and 85-kDa fragments by SDS-PAGE size estimates (125 kDa) for the major fragments generated by trypsin digestion of XD or XO and chymotrypsin digestion of XD, and indicate that these fragments contain the C terminus of XOR.

The N-terminal sequence of the 44-kDa fragment (band 3) corresponds to Asn182–Thr188 and is consistent with cleavage at Met181. The sequence of the first 7 amino acids of the 44-kDa fragment (band 3) corresponds to Asn182–Thr188 and is consistent with cleavage at Met181. The size of this fragment agrees with that predicted for a peptide fragment from Asn182 to Phe560 (42,413 daltons) and suggests that the 44-kDa fragment is generated by cleavage at the 64-kDa fragment at Met181. Hydropathy analysis (28) of the amino acid sequences surrounding the cleavage sites (Fig. 2) shows that Met181 and Lys184 lie in a hydrophilic region, while Phe560 is in a relatively hydrophobic region of XOR. These results demonstrate that native XD and XO possess distinct solution conformations characterized, in part, by exposure of a hydrophobic region containing Phe560 in the XO form.

**XD and XO Possess Different Hydrodynamic Properties**—To determine whether the conformational differences in XD and XO represented global changes in their conformations we investigated their hydrodynamic properties by size exclusion chromatography. Fig. 3 shows the elution properties of XD (D/O = 6.2) and XO (D/O < 0.1) during HPLC-SEC at pH 7.5. Both forms of the enzyme eluted as single peaks (the small amount of absorbance at later retention times in the XD sample is due to DTT used to prepare XD) and appeared to be homogeneous in composition. However, the average retention time of XO (17.52 ± 0.11 min; n = 6) was longer than that of XD (17.13 ± 0.07 min; n = 5). These results indicate that disulfide bond reduction leads to a significant change in the frictional coefficient of XOR and is consistent with an increase in the hydrodynamic volume of the enzyme. The nature of the disulfide bonds within XO was investigated by two-dimensional peptide mapping of chymotrypsin-digested XO under nonreduced and reduced conditions.

**TABLE I**

| Enzyme form | Protease | Fragment size | N-terminal sequence | Corresponding position in XOR | Cleavage position |
|-------------|----------|---------------|---------------------|-------------------------------|------------------|
| XO          | Trypsin  | 125 kDa (Band 7) | KDHTVTLL | Lys185–Leu191 | Lys185 |
| XO          | Chymotrypsin | 85 kDa (Band 1) | QEVPNGQ | Gln561–Gln567 | Phe560 |
| XO          | Chymotrypsin | 64 kDa (Band 2) | TADELVPF | Thr2–Phe5 | NA |
| XO          | Chymotrypsin | 44 kDa (Band 3) | NQKKDHT | Asn182–Thr188 | Met181 |
| XD          | Trypsin  | 125 kDa (Band 8) | KDHTVTLL | Lys185–Leu191 | Lys184 |
| XD          | Chymotrypsin | 125 kDa (Band 5) | NQKKDHT | Asn182–Thr188 | Met181 |

**FIG. 2. Hydrophathy profile of regions surrounding Met181 and Phe560 of bovine XOR.** Kyte and Doolittle hydropathy profiles of bovine XOR from Val101–Phe201 (A) and from Gly502–Asn601 (B). The hydrophathy profiles are based on the amino acid sequence deduced from bovine XOR cDNA (7). The locations of trypsin and chymotrypsin cleavage sites are indicated in each panel.
Organization of Disulfide Bonds in XO—A representative two-dimensional peptide map of chymotrypsin-digested XO is shown in Fig. 4A. The digested enzyme was denatured in SDS in the absence of reducing agents and subjected to SDS-PAGE under nonreducing (first dimension) and reducing (second dimension) conditions. In addition to residual intact XO at 145 kDa, prominent bands at 85, 64, and 44 kDa were observed to lie on the diagonal indicated by arrowheads. Additional bands were also observed to the right of the diagonal at 64 and 44 kDa. The lack of significant staining to the left of the diagonal indicates that there are few, if any, disulfide bridges between undigested XO subunits or between the 85-, 64-, and 44-kDa fragments generated by chymotrypsin digestion. The presence of groups of bands spreading horizontally to the right of the 64- and 44-kDa bands indicates the existence of electrophoretic heterogeneity within these fragments. Since the first dimension was carried out in SDS under nonreducing conditions and fragments within each group have the same size, this observation suggests that this heterogeneity is due to disulfide bond-mediated conformational differences within these fragments. To verify that the electrophoretic heterogeneity within the 64- and 44-kDa bands indicates the existence of electrophoretic heterogeneity within these fragments. Since the first dimension was carried out in SDS under nonreducing conditions and fragments within each group have the same size, this observation suggests that this heterogeneity is due to disulfide bond-mediated conformational differences within these fragments.

Cross-linking Prevents Thiol-mediated Conversion of XO to XD—To determine whether a conformational switch is required for the conversion of XO to XD we investigate the effects of cross-linking on the conversion process. Table II shows the effects of cross-linking XO with the amine-specific reagent BS3 on the ability of DTT to convert XO to XD as indicated by an increase in the D/O ratio. The initial D/O values of cross-linked and non-cross-linked (control) enzyme were <0.1. After incubating with 10 mM DTT for 2 h the D/O of control XO increased to nearly 4, whereas the D/O value of the cross-linked enzyme was still less than 0.1. BS3 did not react with cysteine thiols or interfere with the ability of DTT to reduce XO cysteine thiols (data not shown). Moreover it did not interfere with the XD activity of enzyme that was reduced prior to adding cross-linking reagent (Table II). Thus, the inability of DTT to convert BS3 cross-linked XO to XD does not appear to be due to a specific interference of BS3 with XD enzymatic activity or its reaction with cysteine thiols.

The nature of the BS3 cross-linked enzyme was characterized by HPLC-SEC and SDS-PAGE. The majority of the cross-linked enzyme eluted with the same retention time as native

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**Fig. 3.** Size exclusion chromatography analysis of XD and XO. Samples of XD (D/O = 6.2) and XO (D/O < 0.1), each containing 20 μg of protein, were analyzed by HPLC-SEC in phosphate-buffered saline, pH 7.5, using a 7.8 × 600-mm SEC-3000 column (Phenomenex, Inc.). Eluting material was monitored at 220 nm. The dashed and solid lines show representative chromatograms of XD and XO, respectively. The mean retention times of XD (17.13 ± 0.07 min; n = 5) and XO (17.52 ± 0.11 min; n = 6) were significantly different; p < 0.01.

**Fig. 4.** Two-dimensional electrophoresis of chymotrypsin-digested XO. XO was digested with chymotrypsin (20/1) at room temperature and then analyzed by two-dimensional electrophoresis under nonreducing conditions (first dimension) and reducing conditions (second dimension). A, shows the two-dimensional electrophoresis pattern of XO incubated with chymotrypsin for 1 h. B, two-dimensional electrophoresis of XO incubated with chymotrypsin for 2 h and then with 5 mM DTT for 2 h. Protein fragments were detected by silver staining. The orientation of electrophoresis in each dimension is shown at the top and right side of each gel. The size of each band is shown on its right. The arrowheads indicate the position of the diagonal.
XO (Fig. 5). SDS-PAGE analysis of cross-linked XO (Fig. 5, inset) showed that it was composed of bands between 180 and 200 kDa as well as bands near the top of the gel and at ~150 kDa. The results demonstrate that cross-linking primarily occurs within, and/or between, individual XO subunits and that there is relatively little cross-linking between enzyme molecules or formation of large cross-linked enzyme aggregates. Therefore, cross-linking also does not appear to influence the assembly state of the enzyme. Together these results suggest that cross-linking with BS3 prevents a conformational change that is necessary for the reductive conversion of XO to XD.

Cleavage of XO at Phe 560 Prevents Conversion to XD—Since exposure of the region containing Phe 560 is a distinct feature of the XO conformation we tested the possibility that alterations to this region by proteolytic cleavage would interfere with conversion of XO to XD by thiol reduction. Fig. 6 shows the effects of trypsin and chymotrypsin digestion on the ability of DTT to convert XO to XD (Fig. 6A) and on the conversion of XD to XO (Fig. 6B). XO digested with trypsin for up to 2 h could still be converted to XD by incubation with 5 mM DTT for 1 h; however, digestion with chymotrypsin for as little as 30 min completely prevented conversion. In contrast, neither chymotrypsin nor trypsin digestion led to significant conversion of XD to XO.

DISCUSSION

It is well established that mammalian XOR can be reversibly converted from an NADH-dependent dehydrogenase to an O2-dependent oxidase by oxidation of cysteine thiol to disulfide bonds (11, 12). The present study demonstrates that the conversion of XD to XO involves a global conformational change that is coupled to a reduction in the apparent hydrodynamic size of XOR. The conformational change was mapped to a specific region surrounding Phe 560 and was shown to be a necessary step in the conversion process.

Although the specific activities of our preparations indicate that 30–50% of the purified enzyme is catalytically inactive (2), evidence from the literature and from the present studies suggests that active and inactive XOR molecules possess similar structures and undergo equivalent conformational changes. First, catalytically inactive forms are present in many highly purified and physically homogenous XOR preparations (2). The inactive forms appear to result from specific alterations of the
were then incubated with 5 mM DTT for 1 h. The total XOR activity (D + O) of duplicate samples are shown as a function of percentage of the enzyme in the XD form. In addition, the digestion was carried out at 37 °C and protease-digested samples averaged 15.4 ± 2 μmol of uric acid/ml/min. The pattern of chymotrypsin digestion of bovine milk XD is different from that previously reported for rat liver XD (11). In this earlier study, chymotrypsin cleavage of XD generated significant amounts of 64- and 44-kDa fragments. However, the D/O value of enzyme used in these studies was 1.3; thus their preparation appeared to contain significant amounts of the XO form. In addition, the digestion was carried out at 37 °C, which potentially leads to local unfolding (30) and has been reported to result in the conversion of XD to XO (17). Therefore, the basis for the difference in chymotrypsin cleavage pattern may reside in the relative XD/XO composition of the sample and/or temperature-dependent conformational changes, rather than actual differences in the conformations of the rat and bovine enzymes. This conclusion is supported by the similarity of the chymotrypsin cleavage pattern of chicken XDH at 25 °C (33) to that reported here for bovine XDH. Since avian XOR exists only as the XDH form, the Phe560 site appears to be in a relatively hydrophobic region of the enzyme that would be expected to exhibit reduced solvent exposure under physiological conditions. The observation that this site becomes accessible to chymotrypsin only after XD is converted to XO therefore suggests that disulfide bond formation produces significant structural changes within this region of XOR. Based on sequence alignment comparisons of XOR family members (32) and crystallographic studies of bovine XD (22), the Phe560 site appears to be in an unstructured region near the boundary of the flavin and molybdopterin domains. Thus, disulfide bond formation appears to produce conformational changes that delineate the extent of the flavin-binding region of XOR. Furthermore, size exclusion analysis indicates that disulfide bond formation reduces the hydrodynamic size of XOR. These results extend previous observations (20, 21) of conformational changes within the flavin-binding site following thiol oxidation-dependent conversion of XD to XO by demonstrating that the conversion involves specific structural alterations and global changes in the hydrodynamic properties of XOR.

The pattern of chymotrypsin digestion of bovine milk XD is different from that previously reported for rat liver XD (11). In this earlier study, chymotrypsin cleavage of XD generated significant amounts of 64- and 44-kDa fragments. However, the D/O value of enzyme used in these studies was 1.3; thus their preparation appeared to contain significant amounts of the XO form. In addition, the digestion was carried out at 37 °C, which potentially leads to local unfolding (30) and has been reported to result in the conversion of XD to XO (17). Therefore, the basis for the difference in chymotrypsin cleavage pattern may reside in the relative XD/XO composition of the sample and/or temperature-dependent conformational changes, rather than actual differences in the conformations of the rat and bovine enzymes. This conclusion is supported by the similarity of the chymotrypsin cleavage pattern of chicken XDH at 25 °C (33) to that reported here for bovine XDH. Since avian XOR exists only as the XDH form (8) and contains a region that is highly homologous to that surrounding Phe560 of bovine XOR (8), it is likely that the inaccessibility of the Phe560 region to chymotrypsin is a general feature of the XDH isoform.

Initial studies of the XD to XO conversion suggested that the cysteine residues affecting the dehydrogenase activity of XOR were located in the cysteine-rich N-terminal region (6, 17).
However, recent studies of rat liver XOR have led to the proposal that Cys^535 and Cys^892 are involved in the conversion through the formation of disulfide bonds between these residues (34). Our two-dimensional electrophoresis results provide evidence of significant disulfide bond formation within the N-terminal region of XOR (the 44- and 64-kDa fragments) but not between residues located in the C-terminal fragment (the 85-kDa band, which contains Cys^892) and N-terminal fragment (the 64-kDa band, which contains Cys^535). Since these residues are conserved in all mammalian enzymes studied to date, our data suggest the Cys^535 and Cys^892 form disulfide bonds with as yet unidentified Cys residues in the 64- and 85-kDa regions, respectively.

The basis of the disulfide bond heterogeneity in the N-terminal region of XO is unknown. However, milk XO appears to be formed by the action of a membrane-bound sulfhydryl oxidase during the secretion process (13, 35). If the conformation of XD leads to clustering of cysteine residues it is possible that this enzyme might catalyze formation of disulfide bonds within groups of closely associated cysteine residues resulting from the generation of XO molecules with different disulfide bond combinations and consequently different conformations and electrophoretic mobilities. The formation and rearrangement of disulfide bond isomers by folding catalysts such as protein-disulfide isomerase are known to occur during the folding of nascent proteins and are part of the process of generating proper native conformations (36). A similar process may occur in the conversion of XD to XO during milk secretion and lead to the formation of disulfide bond isomers of XO.

Two lines of evidence from the present study demonstrate that the conversion of XO to XD is dependent on specific structural changes associated with disulfide bond reduction. The first is that cross-linking XO prevents the reductive conversion of XO to XD. Cross-linking did not interfere with XOR activity, and the majority of the cross-linking was intramolecular and occurred without major changes in the conformation of XO. Thus it appears that the inability of DTT to convert cross-linked XO to XD is not due to alterations in substrate utilization or artificial intermolecular interactions between cross-linked XOR dimers. The second is that selective cleavage at Phe^560 prevents conversion to XD by DTT. Because trypsin and chymotrypsin cleave XO at nearby sites in the N-terminal region but only chymotrypsin cleavage prevents conversion to XD, it appears that structural changes within the region around Phe^560 are critical for the conversion process. As yet it is unclear if cleavage at Phe^560 alone or a combination of cleavage at Phe^560 and Met^683 is required to prevent reductive conversion of XO to XD. However, time course studies indicate that Phe^560 cleavage occurs first and that cleavage at this site correlates with the inhibition of the XO to XD conversion. Support for the importance of the region surrounding Phe^560 comes from observations that modification of Cys^535 of rat liver XOR converts XD to XO (34) and from crystallographic data that show that this region is disordered in intact bovine XD and protoxidized bovine XO and constitutes a linking segment between the flavin and molybdopterin domains (22). Our results do not support earlier conclusions, based on proteolytic digestion of rat liver XOR, that alterations within the cysteine-rich N-terminal region by cleavage at Lys^885 are responsible for the XD to XO conversion (6, 17). As indicated above, technical differences, especially the use of elevated temperatures for proteolytic digestion experiments, may be the basis for this discrepancy. In fact, more recent studies of rat liver XOR have shown that cleavage at Lys^885 does not induce the D to O conversion of rat XOR (34). Together with evidence from recent investigations of the role of cysteine residues in the XD to XO conversion (34), the results from our study indicate that the XD-XO interconversion requires a conformational switch in the region surrounding Phe^560. Alterations that affect the structure of this region, such as proteolysis, decouple this switch and interfere with the interconversion process.

Although the conversion of XD to XO represents a pathologically important event due to increased production of cytotoxic reactive oxygen species by the XO form (37), the biological importance of the conformational change associated with the conversion is less established. A potential physiological function of this conformational change is in milk lipid secretion. It has been known for nearly a century that XOR is highly enriched in cow’s milk (2). Numerous studies have documented that the primary form of the milk enzyme is XO (11, 13, 38), and it has been demonstrated that XOR is a major protein constituent of the membrane that surrounds lipid globules in milk (39). Immunofluorescence studies have shown that XOR is selectively associated with the apical plasma membrane in lactating cattle (40), and histochemical evidence suggests that the membrane-associated form is XO (41). We recently demonstrated that the primary form of XOR in mouse mammary tissue is XD, that rapid conversion of the XD form to the XO form occurs during milk secretion, and that membranes surrounding milk lipid globules contain an enzyme capable of converting XD to XO (13). Thus, it is possible that the conformational changes associated with the XD to XO conversion, in particular the exposure of the hydrophobic region around Phe^560, are important in the association of XOR with the apical membrane in mammary epithelial cells and may play a role in milk lipid secretion. Electron microscopic studies have shown that the proteins on the inner surface of milk fat globule membranes are structurally organized into highly ordered hexagonal arrays (42). It will be interesting to determine whether changes in the conformation of XOR contribute to the organization of these structures.

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