The Conversion from the Dehydrogenase Type to the Oxidase Type of Rat Liver Xanthine Dehydrogenase by Modification of Cysteine Residues with Fluorodinitrobenzene*

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When rat liver xanthine dehydrogenase was incubated with fluorodinitrobenzene (FDNB) at pH 8.5, the total enzyme activity decreased gradually to a limited value of initial activity with modification of two lysine residues in a similar way to the modification of bovine milk xanthine oxidase with FDNB (Nishino, T., Tsushima, K., Hille, R. and Massey, V. (1982) J. Biol. Chem. 257, 7348–7353). After modification with FDNB, the two peptides containing dinitrophenyl-lysine were isolated from the molybdopterin domain after proteolytic digestion and were identified as Lys \(^\text{754}\) and Lys \(^\text{771}\) by sequencing the peptides. During the modification of these lysine residues, xanthine dehydrogenase was found to be converted to an oxidase form in the early stage of incubation. Incorporation of the \(^3\text{H}\)-dinitrophe- nyln group into enzyme cysteine residues was 0.96 mol per enzyme FAD for 68% conversion to the oxidase form. The modified enzyme was reconverted to the dehydrogenase form by incubation with dithiothreitol with concomitant release of \(^3\text{H}\)-dinitrophenyl compounds. After modification with \(^3\text{H}\)-FDNB followed by carboxymethyla- tion under denaturing conditions, the enzyme was digested with proteases. Three \(^3\text{H}\)-dinitrophenyl-labeled peptides were isolated and sequenced. The modified residues were identified to be Cys \(^\text{855}\), Cys \(^\text{987}\) and Cys \(^\text{1324}\). These residues are conserved among the all known mammalian enzymes, but Cys \(^\text{992}\) and Cys \(^\text{1324}\) are not conserved in the chicken enzyme. Cys \(^\text{1324}\) of the rat enzyme was found not to be involved in the conversion from the dehydrogenase to the oxidase by limited proteolysis experiments, but Cys \(^\text{855}\) and Cys \(^\text{987}\) which seemed to be modified alternatively with FDNB appear to be involved in the conversion.

Xanthine oxidase (XO) catalyzes the oxidation of xanthine using molecular oxygen as an electron acceptor. The enzyme is a dimer containing one FAD, two 2Fe/2S centers, and one molybdopterin cofactor per \(M_\text{r} = 150,000\) subunit (1, 2). Mammalian XO exists as the NAD-dependent dehydrogenase type (XDH) in freshly prepared samples, i.e. it exhibits low xanthine-O\(_2\) reduc- tase activity but high xanthine-NAD reductase activity even in the presence of O\(_2\) (3, 4). But during extraction or purification procedures, the enzyme can be easily converted to an O\(_2\)-depend- ent oxidase type (XO), i.e. it exhibits low xanthine-NAD activity but high xanthine-O\(_2\) reductase activity. This conversion occurs reversibly by oxidation of sulphhydryl groups or irreversibly through proteolysis (3–10). As H\(_2\)O\(_2\) and O\(_2\) are formed as products when molecular oxygen is used as an electron acceptor, the conversion from XDH to XO has been proposed as the basis of the mechanism of recirculation injury (11).

Although the enzyme is easily converted to XO, the enzyme can be purified as the dehydrogenase (XDH) form by rapid purification (6) or in the presence of thiol reagents such as mercaptoethanol or DTT (7, 8), or as an XO form which can be converted to XDH by incubation with thiols (9). The differences in structural, spectroscopic and kinetics properties between XDH and XO obtained by analyses of the purified enzymes were reviewed recently (12, 13). Upon modification of the protein molecule either by proteolysis or disulfide formation, sig- nificant conformational changes occur, particularly around the flavin (8, 14, 15). The enzyme from rat liver can be converted to XO by limited proteolysis, which results in nicking at two positions which were identified by sequencing the NH\(_2\) termini of three isolated peptides (10). However, the identification of the cysteine residues whose oxidation is responsible for XDH to XO was not easy because of the involvement of many cysteine residues being modified to form disulfide bridges during the conversion by ordinary sulfhydryl modifiers. Waud and Rajago- palan (16) observed that as many as 14 cysteine residues in rat liver enzyme were titrated with DTNB during conversion from XDH to XO. More recently rat liver and bovine milk enzymes can be converted to XO by 4,4’-dithiodipyrindine (4-PDS) as a modifier forming 4 disulfide bridges, i.e. eight cysteine residues were associated with this modification (8, 17). It is likely that parts of cysteine residues are close enough to readily form disulfide bridges within the enzyme because cysteine disulfide bridges were formed by reaction with 4-PDS rather than single cysteine residues being modified (8, 17). Although it has been reported that a single cysteine residue is involved in the conversion (18), the method employed in the report is not appro- priate for the determination of the number of residues in chem- ical modification studies, as discussed by Rakitzis (19).

In this work, the cysteine residues responsible for XDH to XO interconversion were determined by chemical modification using fluorodinitrobenzene (FDNB). Chemical modification of bovine milk XO by FDNB was reported previously to show modification of two lysine residues with a 6-fold decrease in xanthine-O\(_2\) oxidoreductase activity (20). XO of rat liver, re-
versely or irreversibly prepared, showed almost the same type of reactivity with FDNB as the milk enzyme with modification of two lysine residues near the xanthine binding site. During the modification with FDNB, however, it was found that the dehydrogenase form can be converted to the oxidase form prior to modification of lysine residues. The peptides containing modified cysteine residues were isolated and sequenced.

EXPERIMENTAL PROCEDURES

Material and Methods—Rat liver xanthine dehydrogenase was purified from livers of 8–10-week-old rats, by the methods previously reported (21). The ratio of absorbance at 280 nm to that at 450 nm of the purified enzyme was around 5.2. The enzyme activity was measured according to the method reported previously (9). XO activity was determined following absorbance change at 254 nm without NAD+. The activity-to-favin ratio at 25 °C (A_{\text{FR,25}}) was obtained by dividing the change in absorbance/minute at 295 nm in the presence of NAD under aerobic conditions by the absorbance at 450 nm of the enzyme used in the assay. The D/O ratio as defined by Waud and Rajagopalan (6) 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. The enzyme concentration was determined spectrophotometrically using a value of 35,800 M^{-1} cm^{-1} for the molar absorbance of enzyme-bound FAD. The freshly purified enzyme was converted to the XDH form, with a D/O ratio of 9–10, by incubation with 5 mM DTT. The completely reversible XO form was prepared by treatment with 4-PDS according to the methods described previously (9, 17). ^{3}H-FDNB was obtained from New England Nuclear Co. and was diluted with unlabeled FDNB to a specific activity of 259,000 dpm/nmol. Molar extinctions at 360 nm of 17,700 M^{-1} cm^{-1} for DNP-lysine and 17,500 M^{-1} cm^{-1} for average DNP-amino acids were used (22). A molar extinction of 16,000 M^{-1} cm^{-1} at 340 nm was used for DNP-cysteine (22) before and after DTT treatment.

Chemical Modification of Rat Liver XDH or XO with FDNB, and Purification and Sequence Analysis of Peptides Containing Labeled DNP-Amino Acids—XDH was prepared as described above and the first reaction was carried out with unlabeled 700 µM FDNB in 50 mM potassium phosphate buffer, pH 7.8, for 10 min at 25 °C to react with non-specific residues. The reaction was then quenched by cooling the solution quickly to 0 °C on ice followed by immediate passing through a Sephadex G-25 column at 4 °C. The second reaction was carried out with 700 µM ^{3}H-FDNB in 0.1 M pyrophosphate buffer pH 8.5 for strictly 10 min to convert XDH to XO. The labeled XDH was carboxymethylated under anaerobic conditions without prior DTT reduction and was then digested with a mixture of V8 protease Staphylococcus aureus (Boehringer Mannheim) and TPCK-treated trypsin (Sigma) for 3 h at 37 °C in 0.1 M ammonium bicarbonate buffer, pH 7.8. The peptides were purified by using three different C_{18} reverse phase HPLC columns from Capcellpak (Shiseido), Microsorb (Rainin), and µBondapak (Waters). Peptide detection was performed photometrically at 360 nm for specific absorbance of DNP-amino acids or at 226 nm.

XO, which was prepared either by spontaneous oxidation or by 4-PDS treatment (17), was incubated with various concentrations of FDNB in 0.1 M pyrophosphate buffer, pH 8.5, at 25 °C. XO modified with FDNB was partially digested with trypsin and was separated into three fragments under denatured conditions by the method described previously (10).

The finally purified labeled peptides were subjected to amino acid sequence analysis using an Applied-Biosystem gas-phase amino acid sequencer, model 477A, equipped with PTH analyzer 120A. Incorporation of radioactivity into the amino acid residue was confirmed by determination of radioactivity of the aliquots of each cycles of PTH-amino acids.

Partial Digestion of Native XDH with Endoproteinase Glu-C from Staphylococcus aureus V_{r} (Protease V_{r})—The partial digestion of native XDH with Protease V_{r} was carried out in 50 mM ammonium bicarbonate buffer, pH 7.8, containing 0.4 mM EDTA with or without 10 mM DTT at 25 °C with the ratio of substrate to enzyme of 25 to 1 (weight/weight). Aliquots were withdrawn at 2, 4, or 20 h of incubation, and activities were determined. To measure the intrinsic XDH activity, after proteolysis the enzyme was incubated with 10 mM DTT overnight to reduce disulfide bond that may have been formed by auto-oxidation during partial digestion. The samples withdrawn at 4 and 20 h were subjected to ultrafiltration using Centricon 10 microconcentrators, and a part of the filtrate was applied to a C_{18} reverse phase column equilibrated with 20% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min, which was monitored at 226 nm. The amino acid sequence of each peak was determined.

RESULTS

Xanthine Dehydrogenase Can Be Converted to an Oxidase by Modification with FDNB—When native XDH having a D/O ratio between 9 and 10 was incubated with FDNB at pH 8.5, the total enzyme activity was decreased gradually to a limiting value of the initial activity as shown in Fig. 1. This type of inactivation by prolonged incubation of enzyme with FDNB was also observed with rat liver XO as well as bovine milk XO (20) and is described below. During short time incubation with FDNB, however, XDH was found to be converted to XO, with 80% conversion within 10 min at pH 8.5. As the enzyme was not converted to XO without FDNB under these conditions and the conversion rate was much faster than the decrease of total activity, it was suggested that this conversion might be due to rapid modification of cysteine residues independently of modification of the residues responsible for irreversible inactivation of both enzyme forms by prolonged incubation with FDNB. When aliquots of enzyme, which had been converted to various degrees of XO activity, were incubated with 10 mM DTT overnight, it was found that while total activity declined steadily with time of treatment with FDNB, 80–90% of the residual activity was present as XDH after DTT treatment, indicating that the modification is reversible.

Although rapid conversion from XDH to XO suggests that the conversion is not due to spontaneous formation of a disulfide bridge but to modification of cysteine residues, it is still possible that the FDNB-modified cysteine residue might be attacked by another cysteine residue to result on the formation of disulfide bridge. Such cases are known to occur in the modification of rat liver (17) or bovine milk XDH (8) with 4-PDS. To check this possibility, the reconversion of the FDNB-modified as well as the 4-PDS-modified XDH was performed by incubation with DTT. The enzyme modified by 4-PDS was rapidly reconverted to XDH with 5 mM DTT and the conversion was almost complete within 30 min (data not shown). On the other hand, it took several hours for the XO enzyme modified by FDNB to reconvert to 80% of XDH with 10 mM DTT (data not shown).

Incorporation of ^{3}H-FDNB into Enzyme with Concomitant Changes of XDH to XO and Release of ^{3}H-DNP Compounds upon Reconversion from XDH to XO with Dithiothreitol—as it was noticed that the conversion from XDH to XO with FDNB...
was not rapid at pH 7.8 (data not shown), to identify the responsible cysteine residues for the D-O conversion, subsequent reactions were performed in two steps to minimize nonspecific modifications. In the first step reaction, the XDH (86.4% of dehydrogenase activity, and 13.6% of oxidase activity) was incubated with 700 µM unlabeled FDNB at 25 °C for 10 min at pH 7.8 in potassium phosphate buffer followed by gel filtration using Sephadex G-25 to remove excess unlabeled FDNB, as described under “Experimental Procedures.” After the first step reaction, the AFR value decreased from 100 to 86, suggesting some incorporation of DNP group into lysine residues. However, most of the enzyme was retained as XDH (75% dehydrogenase activity, 25% oxidase activity). During this modification, unlabeled nonspecific DNP-Cys and DNP-Lys residues were estimated to be 2.3 and 0.3 mol/mol of enzyme FAD, respectively, using absorbance at 360 nm and 340 nm before and after DTT treatment. In the second step reaction, the sample was reacted with 3H-FDNB at 25 °C for 10 min at pH 8.5; 4 (○●○●), the difference spectrum between 2 and 1; 5 (●●●●●), the difference spectrum between 3 and 2.

Comparison—During the course of FDNB modification and digestion experiments, it was noticed that protease V₈ showed very different patterns of partial digestion of XDH, compared with that of trypsin. Tryptic digestion resulted in proteolysis at two positions resulting in the formation of 20-, 40-, and 85-kDa fragments, with concomitant conversion from XDH to XO (10). If this trypsinic digestion was carried out in the presence of 5 mM DTT under the same conditions, however, XDH retaining 82% initial activity without conversion into XO was obtained with formation of only 20- and 125-kDa fragments as analyzed by SDS-PAGE (Fig. 4). This indicates that proteolysis at the interconnecting segment between 20 and 125 kDa was not responsible for the irreversible conversion, but the segment between 40 and 80 kDa was.

By protease V₈ treatment, most of the enzyme remained with apparently the same size as the native one, and the enzyme was partly digested by prolonged incubation at 25 °C resulting in only a small amount of formation of 85- and 62-kDa fragments, which were detected by SDS-PAGE (Fig. 4). The activity (initial

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AOR dg
bovine aldehyde oxidase (32); AOX h
XDH dp
28); XDH dm
chicken liver XDH (26);
with or without 5 to 10 mM DTT, with apparently no effect of DTT itself on V8 digestion. The lower XDH activity compared to the control enzyme (80–90%), which was incubated without DTT, might be due to minor nicking at the segment of interest. The peptide at each incubation time point was sequenced and was found to be the same, composed of the nine amino acid residues of the C-terminal peptide of XDH, containing Cys1324. (Asn 1323-Cys-Lys-Ser-Try-Ser-Val-Ile1331). Amino acids analysis indicated that the amount of peptides was nearly stoichiometric to the original enzyme, and the amino acid composition was coincident to the C-terminal nine-amino acid peptide (data not shown).

The Modification of XO with FDNB—As in the case of bovine milk XO, the oxidase type of rat liver xanthine dehydrogenase, which was prepared by the methods described under “Experimental Procedures,” was inactivated to a limited value by incubation with various concentrations of FDNB. As shown in the inset of Fig. 5, the enzyme activity decreased at a rate dependent on the FDNB concentration down to a level around 30% of the initial activity. The fact that this remaining activity is due to the modified enzyme, and not due to 30% of remaining native enzyme, was confirmed by the lack of any further decrease when more FDNB was added to the reaction mixture after the limiting activity level was reached (data not shown). The incorporation of FDNB into the enzyme was approximately 3.5 mol/enzyme FAD with 70% loss of activity calculated using an average molar extinction of 17,500 M⁻¹ cm⁻¹ of DNP amino acids. When inactivated enzyme was treated with DTT, some amount of DNP-compounds was released without reactivation of total catalytic activity; inactivated enzyme was still associated with 2–2.4 mol of DNP/mol of enzyme FAD. These results indicate that the cysteine residue of the C-terminal peptide is not involved in the conversion of XDH to XO.

Fig. 4. SDS-PAGE of protease-treated rat liver XDH. Rat liver XDH was digested with trypsin in the presence of DTT (lane 2, 5 μg; lane 3, 10 μg) or in the absence of DTT for 12 h at 30 °C (lane 5, 5 μg; lane 6, 10 μg). The enzyme digested with V8 protease for 3 h at 37 °C (lane 4). SDS-PAGE was performed according to the method of Weber and Osborn using 7.5% polyacrylamide gel (34). Marker proteins (Pharmacia Biotech Inc.) having molecular mass as indicated in kDa (lanes 1 and 7). Filter, almost the same amount of a single peptide was obtained, judged from the elution pattern of HPLC (data not shown), indicating that this segment was easily hydrolyzed by V8 protease. The peptide at each incubation time point was sequenced and was found to be the same, composed of the nine amino acid residues of the C-terminal peptide of XDH, containing Cys1324. (Asn 1323-Cys-Lys-Ser-Try-Ser-Val-Ile1331). Amino acids analysis indicated that the amount of peptides was nearly stoichiometric to the original enzyme, and the amino acid composition was coincident to the C-terminal nine-amino acid peptide (data not shown). The sample of 60–70% of XDH, which had lost its C-terminal peptide of nine amino acids, was again converted to XO when it was kept at 4 °C for several weeks without DTT under aerobic conditions. These results indicate that the cysteine residue of the C-terminal peptide is not involved in the conversion of XDH to XO.

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D/0 = 9) remained mostly (60–70%) as XDH after V8 digestion with or without 5 to 10 mM DTT, with apparently no effect of DTT itself on V8 digestion. The lower XDH activity compared with the control enzyme (80–90%), which was incubated without V8 protease, might be due to minor nicking at the segment between 40- and 85-kDa domains. During digestion, aliquots were withdrawn after 4 and 20 h and applied to Centricon 10 ultrafiltrators to separate the small fragments of digested peptides that could be missed in SDS-PAGE analysis. From each filtrate, almost the same amount of a single peptide was obtained, judged from the elution pattern of HPLC (data not shown), indicating that this segment was easily hydrolyzed by V8 protease. The peptide at each incubation time point was sequenced and was found to be the same, composed of the nine amino acid residues of the C-terminal peptide of XDH, containing Cys1324. (Asn 1323-Cys-Lys-Ser-Try-Ser-Val-Ile1331). Amino acids analysis indicated that the amount of peptides was nearly stoichiometric to the original enzyme, and the amino acid composition was coincident to the C-terminal nine-amino acid peptide (data not shown). The sample of 60–70% of XDH, which had lost its C-terminal peptide of nine amino acids, was again converted to XO when it was kept at 4 °C for several weeks without DTT under aerobic conditions. These results indicate that the cysteine residue of the C-terminal peptide is not involved in the conversion of XDH to XO.
site were suggested to be modified during incubation.

To identify the modified residues in the rat enzyme, isolation of peptide fragments modified with FDNB were performed after digestion of the modified enzyme by trypsin. Purified rat liver XO was incubated with 700 μM of FDNB in 0.1 M pyrophosphate buffer, pH 8.5, overnight to produce nicked DNP-enzyme (2.12 mol of DNP/mol of E-FAD). The nicked DNP-enzyme was digested by trypsin at 30 °C in 50 mM potassium phosphate buffer, pH 8.5, containing 0.4 mM EDTA at 25 °C for 30 min. The reaction was stopped by cooling on ice and immediately was subjected to gel filtration on a Sephadex G-25 column to remove excess FDNB. The eluted DNP-enzyme still retained 36% residual activity and was incubated with 10 mM DTT overnight. After DTT treatment, the number of DNP-residues associated with the protein decreased from 3.4 to 2.5 mol/mol of enzyme FAD and the 36% residual activity was unchanged. The DNP-enzyme was digested by trypsin at 30 °C in 50 mM potassium phosphate buffer, pH 7.8, overnight to produce nicked DNP-enzyme (2.12 mol of DNP/mol of E-FAD). The nicked DNP-XDH was subjected to gel filtration after denaturation using HPLC column and found to be dissociated into three fragments as described previously with native rat liver enzyme (10). The elution pattern of gel filtration using HPLC (10) is shown in Fig. 6 and recoveries of the peptides are summarized in Table I. Peak 1, which is considered to be an incompletely digested fragment, has only 10% retained DNP compounds as judged by the 360 nm absorbance. The peptide fragment of 85 kDa contained 76% of the total absorbance at 360 nm of all fragments. As the 85-kDa fragment is the molybdopterin-containing domain (13), this result is consistent with the results obtained from the experiments with bovine milk XO, that FDNB reacts with the residues near to the xanthine binding site. The spectrum of the 85-kDa fragment is typical of a DNP-peptide with absorption maximum at 360 nm. The 85-kDa fragment was further digested with trypsin and V8 protease and DNP-peptides were purified using Capcellpak and μBondapak of C_{18} reverse-phase HPLC columns and were sequenced. The two DNP-peptides were isolated and the sequences were as follows: 1) Thr^{746}-Asn-Cys-Thr-Ile-Ala-Val-Pro-DNP-Lys^{754}-Gly-Glu^{756} and 2) Leu^{762}-Phe-Val-Ser-Thr-Gln-Asn-Thr-Met-DNP-Lys^{772}. The elution pattern of HPLC gel filtration of the peptides derived from the partially digested DNP-enzyme and the absorption spectrum of DNP-peptide eluted from HPLC column. The molecular weights of the eluted peptide samples shown in Fig. 6 were estimated by SDS-PAGE using the standard proteins. The recovery of peptides from gel filtration was calculated by absorbance at 280 nm, and the recovery of DNP-residues content in each of the peptides was estimated by absorbance at 360 nm.

| Retention time (min) | MW (kDa) | (A280) Recovery (%) | (A360) Recovery (%) |
|---------------------|---------|---------------------|---------------------|
| 31.4                | 100     | 9.5                 | 10                  |
| 34.1                | 85      | 46.5                | 76                  |
| 40                  | 40      | 22.5                | 6                   |
| 46                  | 20      | 7                   | 1                   |
| Total percent       | 85.5    | 93                  |                     |

FIG. 5. Time course of inactivation of rat liver XO by FDNB treatment and absorption spectrum of FDNB treated XO. The FDNB-treated enzyme, which had been prepared by incubation with 650 μM FDNB for 40 min at 25 °C in 0.1 M pyrophosphate buffer, pH 8.5, containing 0.4 mM EDTA followed by gel filtration on Sephadex G-25 column (—). The same DNP-enzyme was partially digested by trypsin followed by gel filtration on ultragel AcA22 column (——). Inset, the time course of inactivation of rat XO by FDNB. 3 × 10^{-6} M enzyme in 0.1 M pyrophosphate buffer, pH 8.5, containing 0.4 mM EDTA was incubated with various concentrations of FDNB at 25 °C. Aliquots were withdrawn at different times and activities were determined in the standard assay system. (C, 0.5 mM; α, 1.15 mM; ●, 5.5 mM FDNB)

Are also conserved in the sequence of the bovine milk enzyme. These residues are well conserved in all known mammalian XOs (Fig. 3, panel d). As the mode of inhibition of the bovine milk enzyme was very similar to that of rat enzyme, the same lysine residues in milk enzyme could well be modified by FDNB.

DISCUSSION

In this study we modified cysteine residues using FDNB, a less specific thiol-modifying reagent. XDH pretreated with unlabeled FDNB at lower pH was modified with 3H-DTNB at pH 8.5, with modification of 1.36 mol cys 3H-DNP/FAD accompanying conversion of XDH to XO. It is unlikely that FDNB modification was due to disulfide bridge formation as in the case of 4-PDS (8, 17) but was due to a single cysteine modification since the rate of reactivation of modified enzyme by DTT was significantly slower than the XO formed by reaction with 4-PDS. The lack of disulfide formation in this modification may be due to lower reactivity of dinitrophenyl-cysteine than dinitropyridyl-cysteine toward other cysteine residues that may exist near the modified cysteine residue. Radioactivity was incorporated into the enzyme upon modification with concomitant conversion from XDH to XO, and furthermore, the incorporated 3H-DNP was slowly released by DTT treatment with reconversion to XDH. Although only 1.36 mol DNP-residues were incorporated and distributed between three cysteine residues, some
amount might be not involved in the XDH to XO conversion. Three DNP-modified peptides containing Cys1324 were obtained from the modified enzyme with the FDNB were quite similar to those of inactivated XDH, indicating that Cys1324 was nonspecifically modified. Non-involvement of Cys1324 for the conversion has been also confirmed by site-directed mutagenesis experiments using Baculovirus/insect cell system where Cys1324 was replaced by serine.2 The explanation for the fact that only one mol/FAD incorporation into two different cysteine residues might be that one of the two cysteine residues, which may form a disulfide bridge during reversible conversion of XDH to XO, is alternatively modified so that dinitrophenylation of either of the residues may disturb the further modification of the other cysteine residue by steric hindrance. Thus, it is likely that Cys535 and/or Cys992 are responsible for the conversion from XDH to XO. We have attempted to address this question by replacement of these residues with serine, but expression of the mutant enzymes has so far been unsuccessful.

The mammalian enzyme is known to be converted from XDH to XO (12, 13). Although it is not well characterized in XDHs from other sources, it is known that at least XDHs from chicken and probably Drosophila are not converted to XO. It is intriguing that the residues corresponding to both Cys535 and Cys992 are all conserved in mammalian XDHs (10, 23–25), whereas the residue corresponding to Cys535 is replaced by arginine in chicken XDH (26). In the chicken XDH, the residue corresponding to Cys992 is followed by glutamic acid (26) whereas it is followed by glycine in mammalian XDHs (10, 23–25). The lack of spontaneous formation of disulfide bridges in chicken XDH might be explained by such a replacement. The differences in surrounding residues might also explain that the residue corresponding to Cys592 in chicken XDH cannot be easily modified by sulfhydryl modifying reagents. Most probably the conformational change might occur particularly around the FAD moiety in the XDH to XO conversion by modification of these residues (8, 13, 14). The residue corresponding to Cys992 is not conserved in XDHs from sources other than vertebrates, suggesting that these enzymes might not be converted into XO forms by sulfhydryl oxidation.

The Cys593 residue is in the NAD (37) and FAD-binding domain (13), whereas Cys592 and Cys1324 are in the molybdopterin domain (10). These residues could be located at the interface of these domains close enough to form a disulfide bridge resulting in the conformational changes. The only available three dimensional structure of enzymes of the xanthine oxidase family is at the moment the structure of aldehyde oxidoreductase from Desulfovibrio gigas (38). As this enzyme lacks an FAD domain but possesses molybdopterin and iron-sulfur center domains (34), the residue corresponding to Cys593 is missing. The residue corresponding to Cys992 in this enzyme is also missing (34), but the residues corresponding to the neighboring residues of Cys992 such as Thr610, His611 or Lys612 are actually located at the surface of the molybdopterin domain (38). These residues might be supposed to be at the interface to the FAD domain of XDH. In other aldehyde oxidases the discussed cysteine residues are replaced by tyrosine residues. All of these enzyme are known to exist solely as an oxidase form.

As with bovine milk XO, rat liver XDH was also inactivated to a limited value by rather prolonged incubation with various concentrations of FDNB. These inactivation modes and changes of absorption spectrum of the rat enzyme by modification with FDNB were quite similar to those of inactivated Bovine milk XO forms by sulfhydryl oxidation. The residue corresponding to Cys992 in this enzyme is also conserved among all of the known mammalian XOs (10, 23–25).

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