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Structural determinant for cold inactivation of rodent L-xylulose reductase

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Abstract

L-Xylulose reductase (XR) is a homotetramer belonging to the short-chain dehydrogenase/reductase family. Human XR is stable at low temperature, whereas the enzymes of mouse, rat, guinea pig, and hamster are rapidly dissociated into their inactive dimeric forms. In order to identify amino acid residues that cause cold inactivation of the rodent XRs, we have here selected Asp238, Leu242, and Thr244 in the C-terminal regions of rodent XRs and performed site-directed mutagenesis of the residues of mouse XR to the corresponding residues (Glu, Trp, and Cys) of the human enzyme. Cold inactivation was prevented partially by the single mutation of L242W and the double mutation of L242W/T244C, and completely by the double mutation of D238E/L242W. The L242W and L242W/T244C mutants existed in both tetrameric and dimeric forms at low temperature and the D238E/L242W mutant retained its tetrameric structure. No preventive effect was exerted by the mutations of D238E and T244C, which were dissociated into their dimeric forms upon cooling. Crystallographic analysis of human XR revealed that Glu238 and Trp242 contribute to proper orientation of the guanidino group of Arg203 of the same subunit to the C-terminal carboxylate group of Cys244 of another subunit through the neighboring residues, Gln137 and Phe241. Thus, the determinants for cold inactivation of rodent XRs are Asp238 and Leu242 with small side chains, which weaken the salt bridges between Arg203 and the C-terminal carboxylate group, and lead to cold inactivation.

Keywords: L-Xylulose reductase; Cold inactivation; Short-chain dehydrogenase/reductase family; Site-directed mutagenesis; Subunit interaction

L-Xylulose reductase (XR, EC 1.1.1.10) catalyzes the NADPH-dependent reduction of L-xylulose to xylitol in the uronate cycle of glucose metabolism [1,2]. Recently, XRs of human, mouse, rat, guinea pig, and hamster have been cloned and shown to be identical with diacetyl reductase (EC 1.1.1.5) that reduces various α-dicarbonyl compounds [3,4]. The enzyme is distributed in many mammalian tissues, of which liver and kidney show high expression of the enzyme. In mouse and rat kidney, the enzyme is localized in brush-border membranes of proximal tubular cells [4]. Thus, XR plays roles in detoxification of toxic α-dicarbonyl compounds and intracellular osmoregulation in renal tubules as well as in the sugar metabolism.

XRs of human and the four rodents share more than 84% homology with each other and belong to the short-chain dehydrogenase/reductase (SDR) superfamily [5]. A distinguishing feature among the mammalian XRs is that the rodent enzymes are unstable near 0 °C in contrast to the human enzyme which does not show such cold inactivation. Cold inactivation has been reported for many other oligomeric enzymes [6,7], including human 17β-hydroxysteroid dehydrogenase type 1 [8] in the SDR family. Most of the enzymes undergo reversible dissociation of their oligomeric forms to smaller units at low temperature, which decreases hydrophobic interactions that are attributed to a combined action of the van der Waals interactions between nonpolar groups and hydration of these groups of the enzymes [7]. However, the amino acid residues responsible for cold inactivation of the enzymes remain unknown.

The loss of catalytic activity of the rodent XRs by cold treatment is also reversible and accompanied by dissociation of their tetrameric forms into dimeric forms [4]. The finding suggests that cold lability of the rodent

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XRs is attributed to amino acid residues, which are different from those of cold-stable human XR and located at the subunit interfaces. In crystal structures of the tetrameric SDR enzymes, the four subunits are related by three mutually perpendicular dyad axes (*P*, *Q*, and *R*). The *P*- and *Q*-axis interfaces are similar among the tetrameric SDR enzymes and involve many hydrogen bonds and salt bridges between the subunits, whereas there are few and weak interactions between the *R*-axis related subunits [9,10]. The residues, that are different between the four rodent XRs and the human enzyme, are 36, of which three residues at positions 238, 242, and 244 are located near the *R*-axis related interface in a model structure of human XR constructed on the basis of crystal structure of mouse lung carbonyl reductase (MLCR) [11], which shows a high sequence identity of about 65% with XR. In this study, we replaced the three residues (Asp238, Leu242, and Thr244) in the C-terminal region of mouse XR independently or simultaneously with the corresponding residues (Glu, Trp, and Cys) of the human enzyme by site-directed mutagenesis. The mutant proteins were then expressed in *Escherichia coli* and the effects of the mutations on cold inactivation were examined.

Materials and methods

Plasmid construction and production of recombinant enzymes. Site-directed mutagenesis was performed by PCR using *Pfu* DNA polymerase (Stratagene) and a set of the following primers. The forward primer, 5′-aacatatggacctgggtcttgcag-3′ corresponds to nucleotides 1–19 of the cDNA for mouse XR [4] and includes an underlined *Nde*I site. The mutagenic 30- and 33-mer reverse primers are complementary to nucleotides 712–735 and 709–735, respectively, of the cDNA and contain a *Hin*dIII site at the 5′-terminus of the sequences. These reverse primers contain mutated codon(s), in which the respective amino acid codons for the positions of mouse XR were replaced with those of human XR. For example, the sequence of the primer for double mutation of L242W/T244C is 5′-tt*aagct***ta**GCAagcCCAgaagcccccatc-3′, in which the *Hin*dIII site, mutated codon, and stop codon are shown in italic, capital, and bold letters, respectively. The PCR product was inserted at the restriction sites of pRset vector (Invitrogen) and the coding regions were sequenced using a CEQ2000XL DNA sequencer (Beckman Coulter) to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

The mutant XRs and the wild-type (WT) enzymes of mouse and human were expressed in *E. coli* BL21(DE3), and the resulting proteins were purified at room temperature using the buffers containing 20% glycerol as described previously [4]. Purity was confirmed by SDS–PAGE and protein concentration was determined by the method of Bradford [12] using bovine serum albumin as a standard.

Assay of enzyme activity. The activity of XR was assayed by measuring the change of NADPH absorbance (at 340 nm). The standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 6.0, 0.1 mM NADPH, 5 mM diacetyl, and enzyme, in a total volume of 2.0 ml. The kinetic constant for the substrate was determined using 0.1 M potassium phosphate buffer, pH 7.0, instead of the buffer, pH 6.0. The values represent means of three determinations. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute at 25 °C.

Cold inactivation experiments. The enzymes (each 0.1 mg/ml) were dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C for 8 h to remove the components in the solution of the stored enzyme. The dialyzed solutions were diluted with 9 volumes of the ice-cold phosphate buffer containing 1 mg/ml bovine serum albumin. The enzyme solution was incubated in an ice bath or at various temperatures and 50 μl-aliquots of the solution were then taken for the activity analysis at different times. For D238E and T244C mutants, the enzyme solutions of 3.6 mg/ml were treated similarly, because the diluted solutions (less than 0.3 mg/ml) of two mutants were slightly unstable even at 25 °C. The effect of pH on cold inactivation of mouse XR was examined using 0.1 M phosphate buffers (pH 6.0–9.0). The inactivation was expressed as relative activity, $(1 - v/v_0) \times 100$, in which v_0 and v are the activities at 0 min and a specific time, respectively, after the incubation. To examine protective effects of compounds on cold inactivation, the dialyzed enzyme solution was diluted with the buffer containing the compounds tested and the activity was assayed 15 min after the incubation in the ice bath.

Molecular weight analysis and circular dichroism spectroscopy. The molecular weights of the enzymes were analyzed at 4 or 25 °C by gel-filtration on a Superdex 200 HR 10/30 column (1 × 30 cm, Amersham Bioscience) which was equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl. Molecular mass standards consisting of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were obtained from Sigma Chemicals. The elution of the proteins was monitored at 280 nm. Circular dichroism (CD) spectrum of enzyme (0.1 mg/ml in 0.1 M potassium phosphate, pH 7.0) was measured between 180 and 350 nm at 4 or 25 °C in a Jasco-720WI spectropolarimeter using a 1-cm path cell.

Structure determination of human XR. The model structure of human XR was constructed on the basis of the crystal structure of MLCR [11] using Insight-II/Homology (Molecular Simulations, San Diego, CA). The wild-type human XR was crystallized [13] and its structure was determined by X-ray crystallography to a final resolution of 1.96 Å. X-ray analysis, model building, and refinement have been described in detail elsewhere (El-Kabbani et al., submitted) and the coordinates obtained were deposited in the Protein Database under PDB Accession Code 1PR9.

Results

Cold inactivation of mouse XR was time-dependent and followed pseudo-first-order kinetics within 15 min after the pre-incubation of the enzyme at 0 °C. The time required for 50% inactivation was 5 min. When cold lability was determined in the buffers ranging from pH 6.0 to 9.0, no significant change in the inactivation rate of the enzyme was observed (data not shown). Mouse XR was dissociated into the dimeric form at low temperature (Fig. 1A). When the inactive dimeric form was incubated at 25 °C and its molecular weight was analyzed by gel-filtration, the enzyme appeared as the active tetrameric form, indicating that the enzyme undergoes a reversible conformational transition between a tetrameric and a dimeric form. In contrast to mouse XR, human XR was not inactivated at 0 °C and eluted as a tetramer on the gel-filtration at 4 °C (data not shown). The CD spectra of the active and cold-inactivated mouse XRs were measured at 4 or 25 °C, but no apparent spectral difference was observed between the

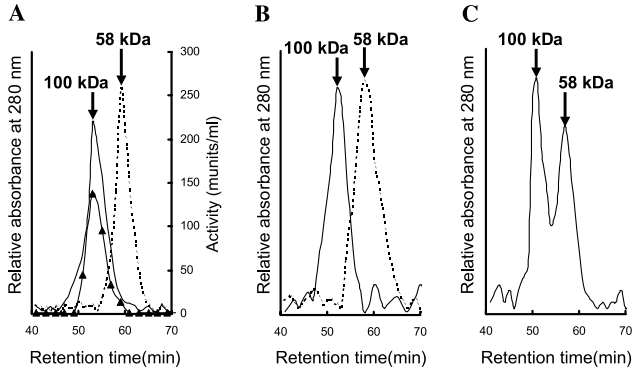

Fig. 1. Gel-filtration of recombinant mouse XR and C-terminal mutants on the Superdex 200 HR column. (A) The inactivated wild-type enzyme was filtered on the column at 4 °C (- - -). The inactive dimeric enzyme (retention time 56–63 min) was concentrated, incubated at 25 °C for 30 min, and reapplied to the column at 25 °C. The elute was monitored at 280 nm (——) and the activity (▲) was assayed. (B) Elution patterns of the mutant enzymes of D238E (- - -) and D238E/L242W (——) at 4 °C. (C) Result of the L242W mutant at 4 °C.

two enzymes (data not shown), suggesting that cold inactivation did not affect the secondary and tertiary structures of monomeric subunits of the enzyme, despite dissociation into the dimeric form.

To assess amino acid residues that are responsible for the difference in the stability at low temperature between mouse and human XRs, we first searched the residues of human XR that are located at or near the subunit interfaces in its model structure and are not conserved in the cold-labile rodent XRs. Of such residues, three residues at positions 238, 242, and 244 were then selected and the three residues (Asp238, Leu242, and Thr244) of mouse XR were independently or simultaneously replaced with the corresponding residues (Glu, Trp, and Cys) of the human enzyme. Three single mutants (D238E, L242W, and T244C), two double mutants (D238E/L242W and L242W/T244C), and a triple mutant (D238E/L242W/T244C) were expressed in *E. coli* cells and purified to homogeneity (about 9–15 mg/liter culture). All the mutant enzymes showed the same CD spectra as the wild-type enzyme (data not shown). Since residues in the C-terminal regions of some members of the SDR family have been reported to be involved in substrate binding [14,15], the effect of the mutations on kinetic constants for a representative substrate, diacetyl, was examined. The kinetic constants of the mutant enzymes were similar to those of the wild-type enzyme, except that subtle alteration in the K_m or V_{max} values was observed in the D238E, L242W/T244C, and D238E/L242W/T244C mutants (Table 1).

Of the single mutations, only that of L242W moderately improved the instability of mouse XR at 0 °C (Fig. 2A). Although the stability of the L242W/T244C mutant at 0 °C was almost the same as the single mutant of L242W, the mutations of D238E/L242W and D238E/L242W/T244C completely protected the cold inactivation (Fig. 2B), indicating that the replacement of both Asp238 and Leu242 of mouse XR with Glu and Trp, respectively, is required to convert the cold-labile mouse XR into the cold-stable enzyme form that is similar to human XR. The activities of the mutant enzymes, which had been inactivated significantly (D238E and T244C) and moderately (L242W and L242W/T244C), were almost completely recovered by incubation at 25 °C for 30 min.

Table 1
Effect of the mutations on the kinetic constants for diacetyl

Enzyme	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg/mM)
D238E	1.1	121	110
L242W	0.78	76	97
T244C	0.73	59	81
D238E/L242W	0.74	94	127
L242W/T244C	0.48	43	90
D238E/L242W/T244C	0.49	30	61
Mouse XR[a]	0.67	95	142

Kinetic constants were determined at pH 7.0.
[a] The values were taken from [4].

When the change in molecular weights of the mutant enzymes due to cold inactivation (at 0 °C for 1 h) was examined by gel-filtration at 4 °C, the D238E and T244C mutants appeared as the dimeric forms, and the cold-stable D238E/L242W and D238E/L242W/T244C mutants remained as the tetrameric forms, as the representative elution patterns of D238E and D238E/L242W mutants are shown in Fig. 1B. In addition, the moderately inactivated mutants of L242W (Fig. 1C) and L242W/T244C showed two protein peaks at positions, corresponding to the tetrameric and dimeric forms. These results clearly indicated that the interactions between the subunits of mouse XR are tightened by the replacement of Asp238 and Leu242 with the corresponding residues, Glu and Trp, of human XR.

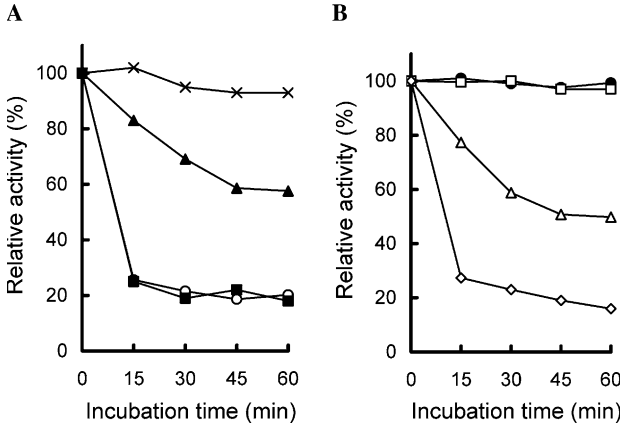

Fig. 2. Effect of the mutations on cold inactivation. The wild-type XRs of mouse (◇) and human (×) and mutant enzymes of D238E (○), L242W (▲), T244C (■), D238E/L242W (●), L242W/T244C (△), and D238E/L242W/T244C (□) were incubated in an ice bath and 50-μl aliquots of the solutions were taken for the enzyme assay at indicated time.

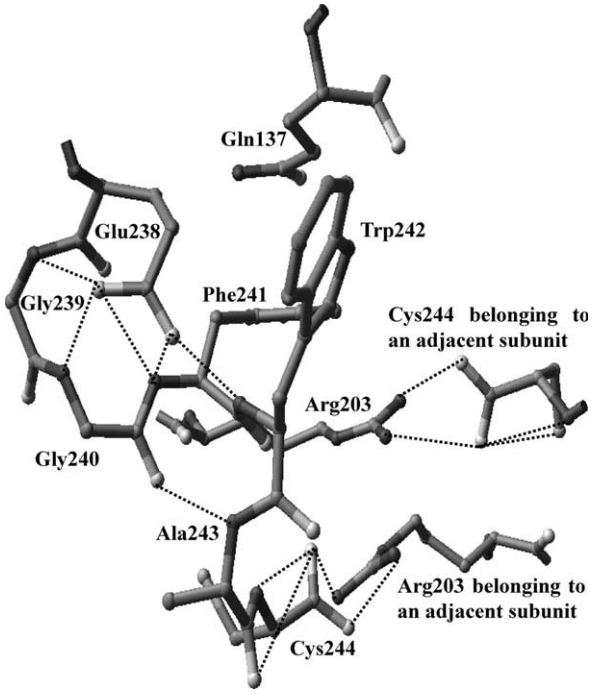

Fig. 3. Close-up view of the interface between *R*-axis related subunits as determined by crystallography of wild-type human XR. The C-terminal carboxylate group of Cys244 forms a salt bridge with the guanidino group of Arg203 belonging to an adjacent subunit. Possible hydrogen bonding interactions in the C-terminal region are depicted. The figure was created with Swiss-PdbViewer [19].

Analysis of the structure of wild-type human XR-NADPH complex by crystallography revealed that Glu238 and Trp242 are located near the *R*-axis interface of the two subunits, which are linked by salt bridges between the guanidino group of Arg203 of one subunit and the C-terminal carboxylate group of Cys244 of the adjacent subunit (Fig. 3). The side chain of Trp242 closes (2.9 Å) to the main-chain carbonyl of Gln137 and that of Glu238 forms hydrogen bond with the main-chain atoms of the residues at positions 239–242. The complete prevention against cold stability of mouse XR by D238E/L242W points out to the importance of the presence of the bulky side chain of Trp 242 and the hydrogen bond interactions between Glu238 and the residues for the proper orientation of the side chain of Arg203 to the C-terminal carboxylate group of Cys244.

Discussion

The present results of the mutations revealed that the cause of cold inactivation of mouse XR is due to the presence of Asp238 and Leu242 instead of the corresponding residues, Glu and Trp, of the human XR. In the crystal structure of the human enzyme, Glu238 forms hydrogen bond interactions with the main-chain atoms of Gly239–Trp242 and Trp242 closes to Glu137 (Fig. 3). The residues 239–241 and Gln137 are conserved in both cold-sensitive rodent XRs and cold-stable human XR. The involvement of Gln137 and Phe241 in the *R*-axis related subunit interaction has been demonstrated by our previous mutagenesis studies, in which the replacement of Gln137 with Met attenuates cold inactivation of mouse and rat XRs, and the introduction of an additional mutation of F241L to this Q137M mutant again converts it into the cold-sensitive form [16]. In human XR, the side chains of Gln137 and Phe241 are stacked up on the side-chain of Arg203 (Fig. 3), and the spatial configuration of the three residues leads to the proper orientation of Arg203 of the same subunit to the C-terminal carboxylate group of Cys244 of the adjacent subunit so that the formed salt bridges in the *R*-axis interface are stable at low temperature. In rodent XRs, the small side chain of Asp238 probably interferes its hydrogen bonding network with the main-chain atoms of residues 239–242, and the non-bulky and small side-chain of Leu242 compared to that of Trp also affects the configuration of Gln137 because the mutation of L242W improved the stability of mouse XR at low temperature. These changes caused by the presence of Asp and Leu at positions 238 and 242, respectively, impair the orientation of the side chain of Arg203 to the C-terminal carboxylate group of the adjacent subunit and then weaken the salt bridge.

Asp238 and Leu242, the structural determinants for cold inactivation of rodent XRs, are conserved in MLCR that shows quite a similar quaternary structure to the crystal structure of human XR (El-Kabbani et al., submitted). However, MLCR is stable at low temperature. The stability of MLCR at low temperature may result from the presence of Met at position 137 instead of Gln137 of XR, because of the abolishment of cold inactivation of mouse and rat XRs by the mutation of Q137M [16]. In the crystal structure of MLCR, the side chains of Met137 and Tyr241 are stacked on that of Arg203 at similar intervals (3.4–3.6 Å) to the case of the corresponding residues of human XR. Met137, Arg203, Asp238, and Leu242 are also conserved in cold-stable hamster NAD^+-dependent 3α-hydroxysteroid dehydrogenase (P26h), which is suggested to have similar quaternary structure to that of MLCR because of high sequence identity of 87% with MLCR [17]. In addition, the conversion of the cold-stable Q137M mutant of rat XR into the cold-sensitive form by the additional mutation of F241L [16] indicates the critical role of the residues with an aromatic ring at position 241 in the stability at cold temperature. Thus, the residues at positions 137, 238, 241, and 242 play important roles in proper orientation of Arg203 that tighten the salt bridges in the *R*-axis interface of XR and its homologous enzymes.

Cold inactivation of tetrameric mouse XR is due to dissociation in dimers by disruption of the salt bridges in the *R*-axis interface of the subunit interaction. The

formed dimers did not show apparent alteration in its tertiary structure (as evidenced by the CD spectral analysis) such that they must retain the *Q*-axis interface. The *Q*-axis dimer interface has been suggested to be critical to the structural integrity of the active sites of the dimeric and tetrameric SDR family enzymes [18]. The dissociation into the inactive dimers suggests that the subunit association in the *R*-axis interface, together with the *Q*-axis interface, is critical to function of XR.

Taken together, we identified two residues determining the stability of XR at low temperature and elucidated the mechanism of its cold inactivation. Although the physiological significance of cold lability of the enzyme is obscure, the present results would contribute to understanding not only the structure-based mechanism of cold inactivation of the other enzymes but the general principles of protein structure.

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