Molecular Characterization of Mammalian Dicarbonyl/L-Xylulose Reductase and Its Localization in Kidney*

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In this report, we first cloned a cDNA for a protein that is highly expressed in mouse kidney and then isolated its counterparts in human, rat hamster, and guinea pig by polymerase chain reaction-based cloning. The cDNAs of the five species encoded polypeptides of 244 amino acids, which shared more than 85% identity with each other and showed high identity with a human sperm 34-kDa protein, P34H, as well as a murine lung-specific carbonyl reductase of the short-chain dehydrogenase/reductase superfamily. In particular, the human protein is identical to P34H, except for one amino acid substitution. The purified recombinant proteins of the five species were about 100-kDa homotetramers with NADPH-linked reductase activity for α-dicarbonyl compounds, catalyzed the oxidation-reduction between xylitol and L-xylulose, and were inhibited competitively by n-butyril acid. Therefore, the proteins are designated as dicarbonyl/L-xylulose reductases (DCXRs). The substrate specificity and kinetic constants of DCXRs for dicarbonyl compounds and sugars are similar to those of mammalian diacetyl reductase and L-xylulose reductase, respectively, and the identity of the DCXRs with these two enzymes was demonstrated by their co-purification from hamster and guinea pig livers and by protein sequencing of the hepatic enzymes. Both DCXR and its mRNA are highly expressed in kidney and liver of human and rodent tissues, and the protein was localized primarily to the inner membranes of the proximal renal tubules in murine kidneys. The results imply that P34H and diacetyl reductase (EC 1.1.1.5) are identical to L-xylulose reductase (EC 1.1.1.10), which is involved in the uronate cycle of glucose metabolism, and the unique localization of the enzyme in kidney suggests that it has a role other than in general carbohydrate metabolism.

Carbonyl compounds are routinely generated in the course of metabolic reactions and by oxidative stresses in a variety of biological systems. When two carbonyl groups juxtapose on a carbon chain, the reactivity of each carbonyl group tends to be elevated, and those compounds with such α-dicarbonyl groups are known to be prone to conversion into advanced glycation end-products (AGEs). 1 AGEs are a group of insoluble complex compounds that frequently accumulate in the plasma proteins and tissues of diabetic subjects and are also associated with renal failure regardless of diabetic background (1–3). Advanced aging also accounts for the triggering of AGE accumulation (4). The starting compounds are assumed to originate from carbohydrates such as glucose and fructose, or from lipid compounds, which then undergo a non-enzymatic Maillard reaction followed by a series of as yet unidentified non-enzymatic and enzymatic reactions, via major intermediate compounds harboring an α-dicarbonyl group in their molecules (1, 3–5). The AGEs subsequently stimulate a group of scavenger receptors called RAGEs, leading to an aberrant production of inflammatory cytokines (6, 7). In addition, by cross-linking proteins, especially those with long lives such as collagen, laminin, and other extracellular matrix proteins, AGEs may cause sclerotic disorders in the blood vessels and in tissues (8). This may eventually lead to the progression of diabetic retinopathy (9).

The potential relevance of aldose reductase and aldehyde reductase in detoxifying such dicarbonyl compounds has been documented (10, 11); however, a question remained of whether there exists one or more reductases working specifically in the renal system. Supporting this notion, it is widely noted that renal failure is one of the major causes accounting for the accumulation of AGEs on plasma proteins and tissues, most prominently in the tubules of the nephron (12). Accordingly, it has been postulated that the function of the renal tubules is pivotal to the clearance of AGEs and their precursors, dicarbonyl compounds (13).

In the course of screening potential renal therapeutic target genes, we have constructed a 3′-directed cDNA library from materials enriched with renal tubules and glomeruli of mouse kidney to generate a gene expression profile of the kidney. Following large-scale sequencing of about 1000 random cDNA clones, a non-biased representation of the mRNA population in the kidney was obtained. A data base survey of the sequences revealed that one of these candidate clones displayed significant sequence homology with mouse lung carbonyl reductase (MLCR (14)), which is also the short-chain dehydrogenase/reductase (SDR) superfamily (15). This inspired us to further investigate its possible involvement in renal carbonyl detoxification. Subsequent PCR-based homologue cloning...  

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) D89656, AB061719, AB061720, AB045204, and AB013846.

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1 The abbreviations used are: AGE, advanced glycation endproduct; MLCR, mouse lung carbonyl reductase; SDR, short-chain dehydrogenase/reductase; DCXR, dicarbonyl/L-xylulose reductase; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; RT, room temperature; P26h, hamster sperm 26-kDa protein; P34H, human sperm 34-kDa protein; PBS, phosphate-buffered saline.

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yielded the isolation of human, hamster, rat, and guinea pig counterparts of the murine cDNA. The enzymatic characterization of the recombinant proteins expressed from the cDNAs of the five species shows that they turn out to be reductases that are specific for dicarbonyl compounds, i.e. diacetyl reductase (EC 1.1.1.5). Surprisingly, the recombinant proteins also displayed significant reductase activity toward L-xylulose, and the identity of diacetyl reductase with L-xylulose reductase (EC 1.1.1.10) has been demonstrated by co-purification of the two enzyme activities from guinea pig liver, in which L-xylulose reductase was first identified (16). Based on these findings, we designated the protein encoded in the isolated cDNA as dicarbonyl-L-xylulose reductase (DCXR). Furthermore, we report the distribution of DCXR in the tissues of the five mammals investigated as well as its immunohistochemical localization in rat and mouse kidneys.

**EXPERIMENTAL PROCEDURES**

**Library Construction and cDNA Cloning—**Fractions enriched with glomeruli and renal tubules were prepared from male BALB/c mice using magnetic beads (Dynabeads M280 and M450, Dynal A.S., Oslo, Norway). 10 mg of the cell nuclei units/100 µl was injected into the tail vein of a mouse, and 0.2 ml of a Dynabeads M280 suspension (6 x 10^6/ml), followed by 0.2 ml of a Dynabeads M450 suspension (4 x 10^6/ml), was injected through the renal afferent arteriole after a ventral opening operation. The kidney was removed and minced, and then the glomerular fraction was collected following the sieving selection (17). The tissue fraction containing the Dynabeads was separated from the other tissues by magnetic force to obtain the fraction enriched with glomeruli and renal tubules. The collected fragments were examined under a light microscope to confirm the enrichment of glomeruli (roughly 7.5 x 10^3 glomeruli and 4 x 10^5 renal tubule fragments in a 0.1-ml suspension). The extraction of RNA, the synthesis of cDNA followed by its MboI digestion, the construction of the cDNA library, and the subsequent body mapping analysis were performed as described previously (18, 19), and other miscellaneous handling of DNA and protein was performed according to general procedures (20). The short cDNA fragments (average size of 256 bp) generated by the MboI digestion were sequenced using a PRIM 377 DNA sequence (Applied Biosystems, Foster City, CA). A cDNA clone that showed high expression frequency (3 out of 958 clones) was subjected to further cloning of the cDNA containing a full-length open reading frame. The oligonucleotide primers were obtained from Takara and Toyobo (Osaka, Japan). A pair of primers, hu-4f and hu-5r, was used in the PCR using a forward primer (5'-TGCTGGCGAGAACGCAGAGAT-3') in the CapFinder DNA library construction kit (CLONTECH, Palo Alto, CA) and a reverse primer (5'-AGTTGTACATGCCACCTGGTTGCTAG-3'), which was designed based on the sequence of one DCXR cDNA fragment containing a putative 3'-untranslated sequence. The sequence of this clone was determined by repeating PCR using a set of primers hu-3r (5'-GCTCAG-3') and the gene-specific primer, hu-3r. The hu-3r primers were also used to amplify the coding regions of this clone that was ligated into a cDNA library as the gene-specific primers to PCR and subsequently RACE (rapid amplification of cDNA ends). The amplification of the cDNAs for the DCXRs of the three species by PCR was achieved with a set of primers, Expf and Expr, with the following modifications were made. 1) The enzyme was eluted from the DEAE-Sephacel column with 30 mM NaCl in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 2 mM 2-mercaptoethanol after washing with the column buffer containing 10 mM NaCl. 2) The Blue-Sepharose column was washed with buffer A (10 mM Tris-HCl, pH 8.5, containing 2 mM 2-mercaptoethanol), and then the enzyme was eluted with buffer A containing 0.5 mM NADP^+. The enzyme activity was measured using 1 mM potassium phosphate buffer pH 7.0, 0.1 mM NADPH, substrate and enzyme, in a total volume of 2.0 ml. "Cold enzyme"—The standard reaction mixture for the reductase activity consisted of 0.1 mM potassium phosphate buffer, pH 6.0, 0.1 mM NADP^+, substrate and enzyme, in a total volume of 2.0 ml. "Cold enzyme" was employed as the substrate for DCXR and hamster liver diacetyl reductase, and 1 mM L-xylulose was used as the substrate for guinea pig liver L-xylulose reductase. The dehydrogenase activities of the enzyme were measured in 0.1 mM potassium phosphate buffer, pH 7.0, containing 0.25 mM NADP^+ and alcohol substrate. Sugars were obtained from Sigma-Aldrich (St. Louis, U.S.A.) and Fluka Chemie (Buchs, Switzerland), except that 3-deoxyglucosone was a gift from Dr. V. Monnier (Case Western Reserve University). One unit of enzymatic activity was defined as the amount of enzyme that catalyzes the reduction and formation of 1 µmol of NADPH per min at 25°C (1 unit). The enzyme reaction was carried out in 0.1 mM potassium phosphate buffer, pH 7.0, at five different concentrations of the substrate or coenzyme to obtain apparent K_m and V_max values. Kinetic studies in the presence of inhibitors were carried out in a similar manner. In addition to the kinetic constants, the inhibition constant, K_i, was calculated by using the appropriate programs of the MacFITTER (Biosoft, Cambridge, UK). All standard errors of fits were less than 15%.

Cold inactivation experiments were carried out as follows. The enzyme (0.4 mg/ml) was dialyzed against 0.1 mM potassium phosphate buffer, pH 7.0, at 25°C for 8 h, and then diluted with 9 volumes of the 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 taken at different times and analyzed for the diacetyl reductase activity as described above.

**Tissue Distribution Analyses—**First-strand cDNAs were prepared from the total RNAs (1 µg samples) of mouse, rat, hamster, guinea pig and human tissues (Sawady, Tokyo, Japan) as described above. The cDNAs were subjected to PCR in a 20-µl reaction mixture containing Taq DNA polymerase (1 unit) and the following primers (1 µM each). The reverse primers were designed based on the amplification of rodent DCXR cDNAs were Expf and Expr, respectively, which did not amplify the cDNAs for MLCR and P26h, and the respective primers for that of human DCXR cDNA were hu-Nf and hu-Kr. cDNAs for human, mouse and rat β-actins were also amplified as internal controls with the specific primers that were obtained from Takara and Toyobo (Osaka, Japan).
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Antibody Production and Western Blot Analysis—To prepare an antibody against murine DCXR, a peptide corresponding to positions 38–51 of the enzyme sequence was synthesized, conjugated to Keyhole Limpet Hemocyanin, and injected subcutaneously into rabbits (20). In addition, a polyclonal antibody against recombinant His-tag human DCXR was also raised in rabbits. To prepare the (His)_6-tagged human DCXR, we amplified the cDNA insert using the primers, hu-4f and hu-5r (see Table I). The obtained PCR fragments were digested with the restriction enzymes, then ligated into expression plasmids, pTrcA (Invitrogen). The recombinant enzyme was expressed in E. coli BL21(DE3) cells, and purified using nickel-nitritotriacetic acid-agarose (Qia- gen, Valencia, CA) according to the manufacturer’s instructions. The antibodies in the antiserum were purified first on a protein-A column and subsequently with an affinity column cross-linked with the corresponding peptide or recombinant protein (20). The anti-mouse DCXR antibody specifically reacted with the mouse and rat DCXRs, whereas the anti-human DCXR antibody cross-reacted with the DCXRs of human and all the rodent species.

The tissues of mice, rats, hamsters and guinea pigs were homogenized with 4 volumes of 0.25 M sucrose at 4 °C, and the homogenates were centrifuged at 105,000 × g for 1 h. The supernatant fractions (each comprising 20 μg of protein) and the other human tissue samples (each comprising 40 μg of protein, CLONTECH) were subjected to Western blot analysis (20) using the above antibodies.

Immunohistochemical Analysis—The kidneys removed from Sprague-Dawley rats (20 weeks, male) and BALB/c mice (10 weeks, female) were fixed in neutralized 10% (v/v) formalin, embedded in paraffin, and sectioned at 3 μm. For immunohistochemistry, these sections were stained by the avidin-biotin-peroxidase complex method (23). The primary and secondary biotinylated antibodies for DCXR staining were the same as those used in the immunohistochemistry. Subsequently the sections were incubated with Cy2-labeled streptavidin (100X, Amersham Biosciences, Inc., Buckinghamshire, UK) for 1 h. For double immunofluorescence microscopy, the tissue fractions enriched in glomeruli and renal tubules of C57BL/6 mouse kidney. The library was composed of relatively short 3'-directed DNA fragments (theoretical average size, 256 base pairs) generated by MboI digestion of the cDNA to ensure its non-biased complexity. Following large-scale DNA sequencing of about 1,000 cDNAs, the library was found to contain 958 clones, three of which were from a cDNA species that has not been reported. The cDNA contained a 735-bp open reading frame, in which a polypeptide of 244 amino acids with a molecular weight of 25,744 Da is encoded (Fig. 1). We designated the protein as DCXR based on its enzymatic characteristics described below.

To investigate whether other mammalian kidneys contain DCXR, we analyzed sequences deposited in the EST division of the GenBank™ data base, and found several human (AF113123) and rat (AI69856; Rat (Ra), AB061719; hamster (Ha), AB045204; guinea pig (Gp), AB061720; human (Hu), AB138346).
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Nucleotide sequences of the primers used in this study

| Primer | Sequence | Position | Restriction enzyme |
|--------|----------|----------|-------------------|
| hu-1f  | TACGCGTCGAGAAGAGCGACAGAG | 49-70    | KpnI              |
| hu-2f  | TACGCGTCGAGAAGAGCGACAGAG | 49-70    | KpnI              |
| hu-3r  | CCAGATTCTTACTGAGAAGCGAGCCCTTC | 401-749  | BamHI             |
| hu-4f  | CAGTGTCTGAAGCGAAGCGACAGAG | 401-749  | BamHI             |
| hu-5r  | CAGTGTCTGAAGCGAAGCGACAGAG | 401-749  | BamHI             |
| Expf   | CCGGATCCATGGACCTGGGCTTGGCA | 1-18     | EcoRI             |
| Expr   | CCGGATCCATGGACCTGGGCTTGGCA | 1-18     | EcoRI             |
| ra-Nf  | AAAATGAGCTGGCTTGCAG | 1-19     | HindIII           |
| hu-Kr  | CCCAGCTTACGAGCCAGAAGGGCC | 720-735  | KpnI              |
| hu-Nf  | GGCATACGAGGTGGCTTCTGGGCGGCGC | 1-23     | NdeI              |
| hu-Kr  | AAGGTACCTTAGGCGACGAGGAGG | 720-735  | KpnI              |
| Race-1r | CAGTGCTGCGCCCGATGCTTTT | 49-70    | KpnI              |
| Race-2r | CCACCGTCCGCACTGCTTTA | 212-232  | KpnI              |
| Race-3r | CAGTGACCTCAGGAAGGA | 274-288  | KpnI              |
| Race-4r | CAGGTGACCTCCAGGAA | 164-183  | KpnI              |
| Race-5r | CAGTTCTCTGGGCTTCCCACTG | 188-208  | KpnI              |
| Race-2f | GGTGCTCTGAGCATGCTGAC | 460-479  | KpnI              |
| Race-4f | AAGATGATGGCCTCGGAGC | 481-499  | KpnI              |
| Race-5f | CGAGATCGAATCCACTTGG | 601-620  | KpnI              |

The restriction enzyme cutting site in each sequence is shown in italic, and the initiation and stop codons are indicated in bold. The nucleotide positions correspond to those of the cDNA species isolated, and Race-1 through -5r and Race-2f through -5f were the primers used in the 5’-RACE and 3’-RACE, respectively.

An homology of over 90% was observed among the DCXRs of the rodent species, and a homology of about 85% was observed between the rodent and human proteins. A homology search against entries in the protein data bases provided by the GenomeNet data base service (Kyoto University, Kyoto, Japan) sorted out large numbers of SDR family proteins, of which human sperm 34-kDa protein, P34H (27), MLCR (14), and 183 units/mg for hamster DCXR; and 33% (9.0 mg) and 112 units/mg for guinea pig DCXR. For the five DCXR preparations, SDS-PAGE revealed a single protein band (Fig. 2A), and gel exclusion chromatography on a Superdex 200 HR column resulted in a single peak with an apparent Mr of ~100 kDa, demonstrating a tetrameric structure for DCXR.

The reductase activities of the recombinant DCXRs, except for human DCXR, were gradually lost when they were incubated at 0 °C in 0.1 M phosphate buffer, pH 7.0 (Fig. 3A). This cold inactivation was reversible as shown in a representative result with mouse DCXR (Fig. 3B), in which the enzyme activity was almost completely recovered by incubation of the inactivated DCXR at above 15 °C. The Superdex 200 HR gel exclusion analysis at 4 °C revealed that most of the hamster DCXR molecules inactivated at 0 °C for 1 h were converted into the dimer form (data not shown). The addition of 2 mM glycerol, 1 mM propylene glycol, or 0.1 mM NADP⁺ resulted in almost complete protection against cold inactivation. The enzymes could be stored without any loss of activity for 3 months at −20 °C by adding 20% glycerol.

Table I shows the pH dependence of the diacetyl reductase activities of the DCXRs of the five species. These activities decreased by increasing the pH from 5.8 to 9.0 and exhibited about 40% of their maximum at pH 7.0. The enzymes efficiently reduced other aliphatic and alicyclic compounds with conjugated dicarbonyl (α-dicarbonyl) groups, which are listed in Table II, but were inactive toward 1 mM 2,4-pentanedione, 2,5-hexanedione, and 1,4-cyclohexanone that do not have α-dicarbonyl groups. The enzymes slowly reduced acetone and propionate, but the reduced products of diacetyl and 3,4-hexanedione, respectively, were not significant. This activity was observed for 1 mM concentrations of other monocarbonyl compounds (acetone, cyclohexanone, indan-1-one, pyridine-3-aldehyde, and pyridine-4-aldehyde), 50 μM 3-oxoandrostanes, and 0.1 mM menadione, most of which are good substrates for MLCR and P26h (22). The Kcat/Km values for the substrates were similar among the four rodent DCXRs, and the Kcat/Km values for most substrates of the human DCXR were decreased by increasing the pH from 5.8 to 9.0 and exhibited about 40% of their maximum at pH 7.0. The enzymes efficiently reduced other aliphatic and alicyclic compounds with conjugated dicarbonyl (α-dicarbonyl) groups, which are listed in Table II, but were inactive toward 1 mM 2,4-pentanedione, 2,5-hexanedione, and 1,4-cyclohexanone that do not have α-dicarbonyl groups. The enzymes slowly reduced acetone and propionate, but the reduced products of diacetyl and 3,4-hexanedione, respectively, were not significant. This activity was observed for 1 mM concentrations of other monocarbonyl compounds (acetone, cyclohexanone, indan-1-one, pyridine-3-aldehyde, and pyridine-4-aldehyde), 50 μM 3-oxoandrostanes, and 0.1 mM menadione, most of which are good substrates for MLCR and P26h (22). The Kcat/Km values for the substrates were similar among the four rodent DCXRs, and the Kcat/Km values for most substrates of the human DCXR were...
Characterization of Dicarbonyl/L-xylulose Reductase

The reductase activity of DCXR was inhibited by short-chain fatty acids (Table IV), of which n-butyric acid showed the highest inhibitory potency. The inhibition patterns of n-butyric acid examined with hamster DCXR were uncompetitive with respect to both NADPH and diacetyl in the forward reaction and were uncompetitive with respect to NADP$^+$ and competitive with respect to xylitol ($K_v = 25 \mu M$) in the reverse reaction. n-Butyric acid (1 mM) did not inhibit the reductase activity of MLCR and P26h, suggesting that it is a specific DCXR inhibitor, which binds to the enzyme-NADP$^+$-alcohol complex.

The above substrate specificity for α-dicarbonyl compounds and the ability to catalyze the oxidoreduction between xyitol and L-xylulose suggested that DCXR is identical with both diacetyl reductase and L-xylulose reductase. To test this hypothesis, the activities of diacetyl reductase and L-xylulose reductase were co-purified from liver cytosols of hamster and guinea pig, from which the two enzymes were previously purified (21, 26). As shown in Table V, the two enzyme activities were detected in the cytosols of the two animal livers, and more than 90% of the activities were inhibited by 1 mM n-butyric acid. In the first column chromatography on Sephadex G-100, a minor diacetyl reductase activity of about 35 kDa, which is attributed to aldehyde and carbonyl reductases (21), was separated from the major enzyme peak (of about 100 kDa) that was co-eluted with an L-xylulose reductase activity. The two enzyme activities in the high molecular weight activity peaks of the two animal livers were also co-migrated in the subsequent purification steps, in which their activity ratios were essentially constant. The final enzyme preparations from the two animal livers were homogeneous on SDS-PAGE, showing the same molecular weights as the respective recombinant enzymes (Fig. 2A), and reacted with the anti-human DCXR antibody on Western blot analysis (Fig. 2B).

The relative activity is expressed as the percentage of the activity before cooling.

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characterization of dicarbonyl/l-xylulose reductase

the kinetic constants were determined at ph 7.0. the kcat value was calculated with the subunit molecular weight of 26,000.

| Substrate                  | Mouse   | Rat    | Hamster | Guinea pig | Human |
|----------------------------|---------|--------|---------|------------|-------|
|                            | kcat    | kcat/Km| kcat/Km | kcat/Km    | kcat/Km|
| Diacetyl                   | 0.67    | 61     | 1.1     | 25         | 23    |
|                            | 0.005   | 21     | 0.15    | 36         | 3.8   |
|                            | 0.015   | 21     | 0.15    | 36         | 3.8   |
|                            | 1      | 0.23   | 0.14    |            |       |
| 1,4-dibromo-2,3-butanediene| 0.018   | 42     | 0.002   | 4400       | 2140  |
|                            | 0.005   | 15     | 0.001   | 3250       | 1320  |
|                            | 0.005   | 15     | 0.001   | 3250       | 1320  |
|                            | 0.005   | 15     | 0.001   | 3250       | 1320  |
| 2,3-pentanediene           | 0.32    | 94     | 0.32    | 26         | 41    |
|                            | 0.015   | 94     | 0.015   | 26         | 41    |
|                            | 0.015   | 94     | 0.015   | 26         | 41    |
|                            | 0.015   | 94     | 0.015   | 26         | 41    |
| 2,5-hexanediene            | 0.25    | 80     | 0.13    | 77         | 268   |
|                            | 0.015   | 80     | 0.015   | 268        | 11    |
|                            | 0.015   | 80     | 0.015   | 268        | 11    |
|                            | 0.015   | 80     | 0.015   | 268        | 11    |
| 3,4-hexanediene            | 0.099   | 270    | 0.10    | 560        | 699   |
|                            | 0.015   | 270    | 0.015   | 699        | 11    |
|                            | 0.015   | 270    | 0.015   | 699        | 11    |
|                            | 0.015   | 270    | 0.015   | 699        | 11    |
| 2,3-heptanediene           | 0.21    | 114    | 0.12    | 14         | 117   |
|                            | 0.015   | 114    | 0.015   | 117        | 11    |
|                            | 0.015   | 114    | 0.015   | 117        | 11    |
|                            | 0.015   | 114    | 0.015   | 117        | 11    |
| Isatin                     | 0.21    | 95     | 0.18    | 170        | 170   |
|                            | 0.015   | 95     | 0.015   | 170        | 170   |
|                            | 0.015   | 95     | 0.015   | 170        | 170   |
|                            | 0.015   | 95     | 0.015   | 170        | 170   |
| 1-phenyl-1,2-propandione   | 0.43    | 41     | 0.39    | 41         | 325   |
|                            | 0.015   | 41     | 0.015   | 325        | 11    |
|                            | 0.015   | 41     | 0.015   | 325        | 11    |
|                            | 0.015   | 41     | 0.015   | 325        | 11    |
| Pyruvic acid               | 0.13    | 22     | 0.19    | 41         | 325   |
|                            | 0.015   | 22     | 0.015   | 325        | 11    |
|                            | 0.015   | 22     | 0.015   | 325        | 11    |
|                            | 0.015   | 22     | 0.015   | 325        | 11    |
| Acetoin                    | 9.0     | 3.0    | 0.33    | 6.9        | 21.3  |
|                            | 0.015   | 3.0    | 0.015   | 21.3       | 1.3   |
|                            | 0.015   | 3.0    | 0.015   | 21.3       | 1.3   |
|                            | 0.015   | 3.0    | 0.015   | 21.3       | 1.3   |
| Methyglyxal                | 8.1     | 11     | 1.4     | 7.6        | 6.9   |
|                            | 0.015   | 11     | 0.015   | 6.9        | 2.1   |
|                            | 0.015   | 11     | 0.015   | 6.9        | 2.1   |
|                            | 0.015   | 11     | 0.015   | 6.9        | 2.1   |
| DADPH                      | 0.005   | 54     | 0.002   | 3570       | 9000  |
|                            | 0.015   | 54     | 0.015   | 9000       | 233   |
|                            | 0.015   | 54     | 0.015   | 9000       | 233   |
|                            | 0.015   | 54     | 0.015   | 9000       | 233   |
| NADPH                      | 0.22    | 41     | 0.36    | 23         | 64    |
|                            | 0.015   | 41     | 0.015   | 64         | 17    |
|                            | 0.015   | 41     | 0.015   | 64         | 17    |
|                            | 0.015   | 41     | 0.015   | 64         | 17    |


diacetyl reductase and l-xylulose reductase.

tissue distribution—the tissue distribution of the dcrx rna was initially assessed by rt-pcr. as illustrated in fig. 4a, we detected the expression of dcrx transcript in various tissues of human and four rodent species. the high level of the transcript in kidney was common to all these species, although the expression levels in the other tissues seemed to be different depending on the species. the tissue distribution patterns of

for carbonyl compounds as those of the respective recombinant dcrxs. for example, the km values for 1,4-dibromo-2,3-butanediene, 2,3-hexanediene, and l-xylulose were 5.6, 91, and 220 μm, respectively, for the hamster liver enzyme, and the same values for the guinea pig liver enzyme were 9.2, 180, and 270 μm. the l-xylulose reductase activities in the cytosols of mouse and rat livers were 4.5 and 28 milliunits/mg, respectively, and were inhibited by 1 mM n-butyric acid, although the enzymes were not purified. the data clearly indicate that dcrx is identical with both diacetyl reductase and l-xylulose reductase.
the DCXR transcript almost correlated with the results of Western blot analysis using anti-human DCXR polyclonal antibody. Western blot analysis using the anti-mouse DCXR antibody for the mouse and rat tissues or with the anti-human DCXR antibody for the other rodent and human tissues. Tissues: Br, brain; Lu, lung; He, heart; Li, liver; K, kidney; Sp, spleen; Te, testis; and Ep, epididymis. PC (positive control).

![Fig. 4. Tissue distribution of DCXR and its mRNA.](image)

**FIG. 4. Tissue distribution of DCXR and its mRNA.** a, RT-PCR analysis for expression of mRNAs for rodent and human DCXRs. b, RT-PCR analysis for expression of mRNAs for mouse, rat, and human β-actins. c, Western blot analysis using the anti-mouse DCXR antibody for the mouse and rat tissues or with the anti-human DCXR antibody for the other rodent and human tissues. Tissues: Br, brain; Lu, lung; He, heart; Li, liver; K, kidney; Sp, spleen; Te, testis; and Ep, epididymis. PC (positive control).

**FIG. 5. Immunohistochemical staining of DCXR in kidneys of mouse and rat.** The BALB/c mouse kidney sections were stained with the anti-mouse DCXR antibody (a and c) and the preimmune rabbit IgG (b). The Sprague-Dawley rat kidney sections were stained with the antibody (d) and the monoclonal anti-AGE antibody, 6D12 (e), and the two stained sections are superimposed (f). The immunoreactive staining colors are pink (a), green (c), red (d), and green (e). PT, proximal tubule; DT, distal tubule; CD, collecting duct; G, glomerulus. Bars = 10 μm.

**FIG. 5.** Immunohistochemical and cytochemical localization of DCXR in kidney—The localization of DCXR in mouse and rat kidneys was examined by immunohistochemical staining with the anti-mouse DCXR antibody, which cross-reacted with rat DCXR. In a BALB/c mouse kidney section, the immunostaining was predominant at the brush border of the proximal tubules and was not evident in the distal tubules and in the collecting duct and staining was absent in the glomeruli (Fig. 5, a and c). In a Sprague-Dawley rat kidney section, the immunopositive reaction was mainly observed in the distal tubules and the collecting duct (Fig. 5d) but again was not evident in the glomeruli (data not shown). The sequential rat kidney section was also stained using the monoclonal antibody 6D12 that is known to react with carboxymethyllysine, one of the major AGEs found in diabetic patients (36), to test the possibility of whether carboxymethyllysine and DCXR are co-distributed. The immunostaining for carboxymethyllysine was positive at the collecting duct (Fig. 5e), and its co-localization with DCXR was demonstrated by the double staining (Fig. 5f).

Finally, the mouse kidney sections were stained with the anti-mouse DCXR antibody and the anti-rabbit IgG gold complex, and the immunohistochemically stained preparations were then analyzed by electron microscopy. Extensive labeling by gold particles was seen in the microvilli of the inner wall of the proximal tubules (Fig. 6) and in the distal tubules with less intensity (data not shown).

**DISCUSSION**

The present cloning and expression of a cDNA encoding DCXR, which is enriched in mouse kidney, together with the molecular characterization of human, rat, hamster, and guinea pig counterparts, indicate that the protein belongs to the SDR superfamily and exhibits both diacetyl reductase and L-xylulose reductase activities, which are distinct from the substrate specificity of MLCR and P26h (22). The sequences of the rodent DCXRs are the first report of these to our knowledge, but the sequence of our human DCXR is identical to that of P34H (27), which is thought to be involved in sperm-zona pellucida interaction, except for one amino acid substitution at position 239. Because the amino acid substituted in P34H is outside the substrate-binding site in our ongoing structural determination of human DCXR crystal2 and may not cause large changes of its

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2 O. El-Kabbani, S. Ishikura, and A. Hara, unpublished observation.
enzymatic properties, human DCXR and P34H are probably the same protein. The tissue distribution of DCXR and its mRNA suggests general roles for P34H or DCXR in the metabolism of dicarbonyl compounds and glucose rather than its epididymis-specific function (27). In fact, no abnormality in fertilization was observed in mice lacking the dxxr gene. Furthermore, the deficiency of L-xylulose reductase, which is demonstrated here to be identical with DCXR, has been known to result in essential pentosuria, and individuals with this disorder are normal at least in their reproductive function, as has been demonstrated in a family study of the pentosuria allele (37, 38). In terms of a genetic disorder, the identification of the variant gene resulting in essential pentosuria will be an important study in the future.

Dicarbonyl compounds are substrates for monomeric aldoketo reductases (10, 11, 39) and carbonyl reductase (40), as well as dimeric dihydrodiol dehydrogenase (11) and sepiapterine reductase (41), in addition to tetrameric diacetyl reductase (21, 42, 43) purified from animal tissues. Of these enzymes, diacetyl reductase resembles DCXR with respect to the tetrameric structure, pH optimum, and substrate specificity, including the kinetic constants for various carbonyl compounds. The cold inactivation of recombinant rodent DCXRs is also similar to the reports that diacetyl reductases of bovine and pigeon are unstable at 4°C (42, 43). In fact, we demonstrate here the identity of DCXR and diacetyl reductase by their co-purification from hamster and guinea pig livers. In addition, the results of the present study emphasize that diacetyl reductase and L-xylulose reductase, which have been classified as distinct enzymes in the Enzyme Nomenclature system, are identical. The recombinant DCXRs, i.e. diacetyl reductases, efficiently reduced L-xylulose, and catalyzed its reverse reaction, xylitol dehydrogenation, showing the $K_m$ values for the sugars similar to those of L-xylulose reductases partially purified from guinea pig liver (26, 44, 45) and from human erythrocytes of normal individuals without the pentosuria allele (38). The identity of the two enzymes was here demonstrated by their co-purification from hamster and guinea pig livers. The cold inactivation of guinea pig DCXR may account for the instability of guinea pig liver L-xylulose reductase reported in previous studies (44, 45). The inhibition of L-xylulose reductase activity in the liver cytosol of mouse and rat by the specific DCXR inhibitor, n-butryric acid, also suggests the identity of the two enzymes in the two animal species. In aqueous solutions, between 8 and 20% of the L-xylulose probably exists in the free ketose/chain form based on the percentage of its enantiomer that is in the chain form (46).

Because DCXR reduced aliphatic dicarbonyl compounds and oxidized xylitol that does not cyclize, the enzyme acts on the chain form of L-xylulose in the forward direction, and the true $K_m$ values for L-xylulose may be much lower than the apparent values estimated.

DCXRs oxidized d-threitol, as well as xylitol, of the sugar alcohols tested. Based on the chemical reaction between L-xylulose and xylitol, which was catalyzed by the enzymes, the oxidized product of d-threitol is d-threose or d-erythrulose. However, neither the d- nor the l-form of threose serves as the best substrate for the DCXRs. Although d-erythrulose, which is not commercially available, was not tested as a substrate in this study, it is structurally similar to L-xylulose and would most likely be a good substrate for the enzyme. The enzyme that catalyzes the reversible reaction between d-erythrulose and d-threitol is called d-erythrulose reductase (EC 1.1.1.162), and the enzyme purified from chicken liver (47) has been reported to show substrate specificity for aliphatic $\alpha$-dicarbonyl compounds similar to those of animal diacetyl reductases (21, 42, 43) and the mammalian DCXRs characterized in this study. In addition, d-erythrulose reductase of bovine liver shows cold inactivation and reduces some other tetroses and pentoses at low rates (48). Furthermore, the amino acid sequence of the chicken liver enzyme is similar to those of the mammalian DCXRs (about 78% sequence identity). Thus, diacetyl reductase, L-xylulose reductase, and, possibly, d-erythrulose reductase are the same enzyme. Although the metabolic pathways for forming diacetyl and d-erythrulose in mammals are not known, L-xylulose reductase is an enzyme of the uronate cycle, which accounts for about 5% of the total glucose catabolism per day in man (49). In this respect, the three enzymes should be named L-xylulose reductase.

The tissue distribution analysis revealed that DCXR is a ubiquitous protein, although it is highly expressed in liver and kidney. This supports the general function of the enzyme in the uronate cycle. As previously suggested for diacetyl reductase (21), DCXR also acts as a detoxification enzyme toward reactive dicarbonyl compounds, which are formed in the tissue or ingested as components of foods and beverages (32). In addition, the co-distribution of DCXR and carboxymethyllysine in the renal tubules led us to speculate that DCXR may interact with the dicarbonyl precursors of AGEs. DCXR moderately reduced methylglyoxal and threoses, which have been shown to be involved in AGE formation (34) and the generation of superoxide anions (50), respectively. Alternatively, DCXR may play a role in the production of xylitol, an intracellular organic osmolyte, in the cells of the renal tubules and collecting ducts. In the kidney, several intracellular organic osmolites, including sorbitol are known, and aldose reductase, the enzyme that produces sorbitol from glucose, is abundant in the renal medulla (51) and implicated in osmoregulation in this tissue (52). Glucose is metabolized by the uronate cycle that includes DCXR more abundantly than by the polyol pathway, in which aldose reductase constitutes the first and rate-limiting step because of its high $K_m$ value for glucose. Xylitol has been shown to have a low transepithelial permeability in the airway surface of the lung and acts as an osmolyte (53). The present finding that DCXR is localized in the brush-border membranes of renal tubular cells suggests that the local accumulation of the osmolyte xylitol in the membrane is responsible for water reabsorption in the proximal tubules. In addition, xylitol formation by the enzyme would contribute to cellular osmoregulation against osmolytic stress in the distal tubules and collecting.

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3 Y. Uematsu, N. Tada, and J. Nakagawa, unpublished observation.

4 M. Maeda, personal communication.
ducts. Although pentosuric individuals are clinically healthy apart from their chronic excretion of L-xylulose (37), some disorders in the metabolism of dicarbonyl compounds and renal function may be gleaned from the proposed roles of DCXR, which will be tested by generating transgenic and knockout animals of the dcxr gene.

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Molecular Characterization of Mammalian Dicarbonyl/l-Xylulose Reductase and Its Localization in Kidney

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