In Vivo Glycation of Aldehyde Reductase, a Major 3-Deoxyglucosone Reducing Enzyme: Identification of Glycation Sites†

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ABSTRACT: We have reported that the enzyme which reduces 3-deoxyglucosone (3-DG), a major intermediate and a potent cross-linker in the Maillard reaction, is identical with aldehyde reductase [Takahashi, M., Fujii, J., Teshima, T., Suzuki, K., Shiba, T., & Taniguchi, N. (1993) *Gene 127*, 249−253]. The enzyme purified from normal rat liver was found to be partially glycated as judged by binding to a boronate column and reactivity to anti-ε-hexitol lysine IgG. Sites of *in vivo* glycation of rat liver aldehyde reductase were identified by sequencing of digested peptides labeled with $NaB[^3H]_4$ and by mass spectrometry. The major glycated sites were lysines 67, 84, and 140. The glycated enzyme had low catalytic efficiency (k_{cat}/K_m) as compared to the nonglycated form. In streptozotocin-induced diabetic rats, the glycated form was significantly increased in kidneys. Because the enzyme plays a role in detoxifying 3-DG formed through the Maillard reaction *in vivo*, glycation of aldehyde reductase and reduction of its activity may result in the metabolic imbalance under diabetic conditions.

The biochemical basis of the pleiotropic complications of diabetes mellitus is not clearly understood as yet. The glycation reaction is one of the most likely candidates for the cause of the complications (Monnier et al., 1984; Brownlee et al., 1988). Glucose- and fructose-derived metabolites are responsible for protein modification and cross-linking of proteins. The cross-linking of long-lived proteins such as collagen and lens crystallin correlates with aging and diabetes (Monnier & Cerami, 1981; Baynes & Monnier, 1989). Furthermore, glycation alters the activity of some enzymes such as Cu,Zn-superoxide dismutase (Arai et al., 1987; Ookawara et al., 1992), carbonic anhydrase (Kondo et al., 1987), alcohol dehydrogenase (Shilton & Walton, 1991), and Na,K-ATPase (Garner et al., 1990).

3-Deoxyglucosone (3-DG),[1] a 2-oxoaldehyde compound, is produced through the degradation of Amadori product in the advanced Maillard reaction and is a potent glycating agent (Kato et al., 1987a). Protein polymerization by 3-DG under physiological conditions has been reported (Kato et al., 1987b). Thus an enzyme that metabolizes 3-DG would play a crucial role in moderating aging and diabetic complications. We have reported that the 3-DG reducing enzyme was identical with aldehyde reductase as judged by substrate specificity and cDNA structure (Takahashi et al., 1993).

In this paper, we show that aldehyde reductase in normal rat livers was found to be partially glycated and its glycated form was less active as compared to nonglycated form, and the percentage of the glycated form was significantly increased in kidneys of streptozotocin-induced diabetic rats. We also identified the *in vivo* glycated sites by protein sequencing and by mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials. Aldehyde reductase was purified from rat liver as described previously (Takahashi et al., 1993). Glyco-Gel II boronate affinity gel was purchased from Pierce Chemical Co. $NaB[^3H]_4$ was obtained from New England Nuclear. $NaBH_4$ was purchased from Wako Pure Chemical Industries, Japan. PD-10 columns were products of Pharmacia-LKB, Sweden. All other chemicals used were of analytical grade.

Separation of Aldehyde Reductases by Boronate Affinity Column Chromatography. The purified aldehyde reductase (3.2 mg) was applied to a Glyco-Gel II boronate affinity column (1 × 7 cm) that had been equilibrated with 0.25 M ammonium acetate buffer, pH 8.5, containing 50 mM $MgCl_2$. After the column was washed with the buffer used for equilibration, the bound enzyme was eluted with 0.1 M potassium phosphate buffer, pH 8.5, containing 0.2 M sorbitol.

Enzyme Assay. 3-DG was chemically synthesized according to Khadem et al. (1971). The structure and purity of the synthesized 3-DG were confirmed using ^1H NMR and elemental analysis. Enzyme activity was measured by the rate of decrease in the absorbance at 340 nm using a Hitachi model 557 spectrophotometer. The standard assay mixture contained 100 mM sodium phosphate, pH 7.0, 0.1 mM NADPH, and 10 mM 3-DG. One activity unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH/min at 25 °C. Kinetic parameters were calculated from the data by least-squares linear regression analysis.

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[1] Abbreviations: 3-DG, 3-deoxyglucosone; NMR, nuclear magnetic resonance; SDS, sodium dodecylsulfate; PBS, 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4; RP-HPLC, reverse-phase high-performance liquid chromatography; PTH, phenylthiohydantoin.

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Amino Acid Analysis. The purified enzyme was hydrolyzed in 6 N HCl at 110 °C for 24 h *in vacuo*. Amino acid analysis was carried out with a Hitachi L-8500 automatic amino acid analyzer.

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting of the Protein. Proteins were fractionated on 10% SDS–polyacrylamide gel according to Laemmli (1970). Protein bands were visualized by Coomassie Blue staining. Transfer of proteins to nitrocellulose was performed using a Sartoblot IIS semidry electroblotter (Sartorius) (Kyhse-Andersen, 1984). After blocking in 4% bovine serum albumin for 30 min, the blots were incubated for 2 h at room temperature with a 1:1000 dilution of an affinity-purified anti-ε-hexitol lysine IgG (Miyata et al., 1993). After the nitrocellulose membranes were washed three times for 30 min each, the blots were incubated with 1:1000 diluted, affinity-purified, peroxidase-conjugated, goat anti-rabbit IgG (Dako). The immunoblots were again washed three times in PBS and then developed in PBS using 4-chloro-1-naphthol as substrate.

Experimental Diabetic Rats. Seven-week-old male Wistar rats weighing 200 ± 20 g were used. Diabetes was induced by injection of streptozotocin (60 mg/kg) in 0.1 M sodium citrate, pH 4.5. Animals were fed MM-5 chow (CLEA Japan Inc.) and water ad libitum. Blood glucose in cervical vein was determined by glucose oxidase technique using a Diacolor GC glucose analysis kit (Ono Phamaceutical Co., Ltd.) after an overnight fast. Rats were diagnosed as diabetic when their fasting blood glucose exceeded 16.7 mmol/L 1 week after injection of streptozotocin and remained ≥16.7 mmol/L at 17 weeks, the time of sacrifice. Mean plasma glucose levels were 35.1 ± 0.64 mmol/L and 6.1 ± 0.14 mmol/L in the diabetic groups and the nondiabetic controls, respectively.

Separation of Glycated and Nonglycated Proteins in Rat Kidney Homogenate by Boronate Affinity Column Chromatography. After homogenization of one gram of each kidney from normal and diabetic rats in 5 vols of 50 mM potassium phosphate buffer (pH 7.4), the homogenate was centrifuged for 30 min at 3000*g*, and the supernatant was again centrifuged for 1 h at 100000*g*. This supernatant fraction was dialyzed against 0.25 M ammonium acetate buffer, pH 8.5, containing 50 mM $MgCl_2$, and fractionated on a boronate column as described above. Glycated proteins were retained by the column and were subsequently eluted by 0.2 M sorbitol.

Enzyme Immunoassay for Rat Aldehyde Reductase. In order to determine the relative amounts of glycated aldehyde reductase of normal and diabetic rat kidneys, an enzyme-linked immunosorbent assay (ELISA) system for rat aldehyde reductase was developed. This was done as follows: A polyclonal antibody against rat aldehyde reductase was raised in rabbits and purified by precipitation with 50% saturated ammonium sulfate, DEAE-cellulose column chromatography, and immunoaffinity column chromatography using purified aldehyde reductase as an adsorbent. The purified antibody was dialyzed against 0.1 M $NaHCO_3$, pH 8.0, overnight at 4 °C. To 1 mL of the antibody solution (1 mg/mL) was added 200 μL of *N*-hydroxysuccinimidobiotin dissolved in dimethyl sulfoxide (1 mg/mL). After 4 h of incubation at room temperature the mixture was dialyzed against PBS, pH 7.4, overnight at 4 °C. This antibody–biotin conjugate was used as the second antibody of the ELISA. Aliquots (50 μL) of the antibody (5 μg/mL, dissolved in 50 mM $NaHCO_3$, pH 9.6) were added to the well of flat-bottomed, polystyrene microtiter plates and left at 4 °C overnight. The wells were then washed three times with PBS, filled with 0.1% bovine serum albumin in PBS, and then kept for 2 h at 37 °C. Unbound proteins were removed by washing with a buffer (20 mM sodium phosphate buffer saline, pH 7.4, containing 0.05% Tween 20). Samples and standard protein (purified rat aldehyde reductase) were diluted with PBS containing 0.1% bovine serum albumin, and then 100 μL aliquots of the diluted solutions were added to the antibody-coated wells. After incubation for 2 h at room temperature, unbound antigen was removed by washing three times with the same buffer. Then 50 μL aliquots of the second antibody were added to each well. After incubation for 2 h at room temperature, the second antibody was removed by washing, and 50 μL aliquots of horseradish peroxidase–avidin D solution (diluted 1:5000 with PBS) were added to each well. After incubation for 15 min at room temperature, unbound avidin D solution was removed. The substrate for horseradish peroxidase (50 μL of 0.003% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0, containing 0.6 mg of *o*-phenylenediamine/mL) was then added to the wells. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 50 mL of 2 N sulfuric acid. Absorbance was measured at 490 nm with an Immuno-Reader NJ-2000 (InterMed). The ELISA was able to measure $10-10^3$ ng/mL of rat aldehyde reductase.

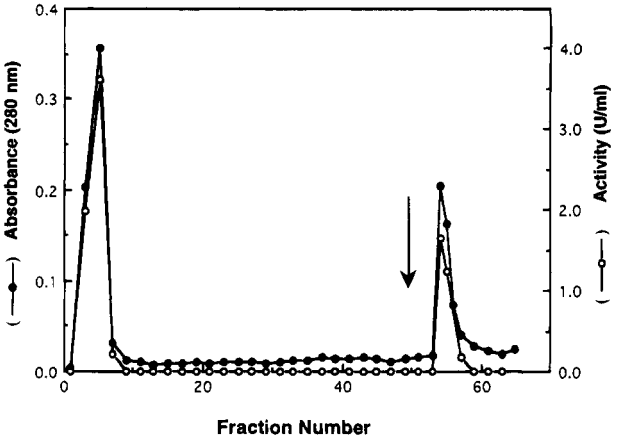

FIGURE 1: Glyco-Gel II boronate affinity column chromatography of purified aldehyde reductase from rat liver. Purified aldehyde reductase (3.2 mg) from normal rat liver was applied to the column. After being washed, the bound fraction was eluted with buffer containing 0.2 M sorbitol, beginning at the arrow.

$NaB[^3H]_4$ or $NaBH_4$ Reduction of Covalently Attached Hexose Groups. Hexoses attached to ε-amino groups of lysyl residues of rat liver aldehyde reductase were converted into 1-deoxyhexitolyl groups by reduction with $NaB[^3H]_4$, thus stabilizing carbohydrate-protein linkages and conferring a radioactive label on each site of glycation. This was carried out as follows: A solution of $NaB[^3H]_4$ (5 mCi; 15 μmol) was added to each 0.45 mL solution of glycated and nonglycated aldehyde reductases (1.2 mg; 33 nmol). After 4 h at room temperature, the solutions were adjusted to pH 4 with dilute acetic acid to terminate the reaction. The samples were then applied to PD-10 columns to remove free $NaB[^3H]_4$. To prepare the nonradioactive sample for amino acid sequencing, the same procedure was carried out using nonradioactive $NaBH_4$ instead of $NaB[^3H]_4$.

Table 1: Results of Glyco-Gel II Chromatography of Rat Aldehyde Reductase[a]

enzyme sample	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} min^{-1})	relative percent (%)
unbound fractions	96.6 ± 0.71	0.88 ± 0.017	$10.7 \times 10^4 \pm 0.14 \times 10^4$	100
bound fractions	87.2 ± 0.73[b]	1.20 ± 0.011[b]	$7.3 \times 10^4 \pm 0.03 \times 10^4$ [b]	68

[a] All data were obtained at pH 7.0, 25 °C; k_{cat} and apparent K_m values were obtained by least-squares linear regression analysis of the initial rate data that were obtained under nonsaturating conditions ($r > 0.999$). Values of the parameters are means ± SE ($n = 5$). Proteins were determined by qualitative amino acid analysis. [b] $p < 0.001$ compared to unbound fractions according to unpaired Student's t test.

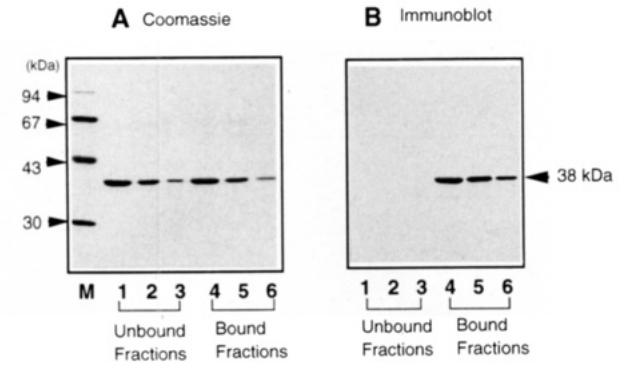

FIGURE 2: Immunoblot analysis of the bound and pass-through fractions. Aliquots from each pass-through (lanes 1−3) and binding (lanes 4−6) fractions were subjected to SDS−polyacrylamide gel electrophoresis. Each lane contained 2 (lanes 1 and 4), 1 (lanes 2 and 5), or 0.5 μg (lanes 3 and 6) of protein. Proteins were stained with Coomassie blue (A) or indirect immunostaining using anti-ϵ-hexitol lysine IgG as a primary antibody (B).

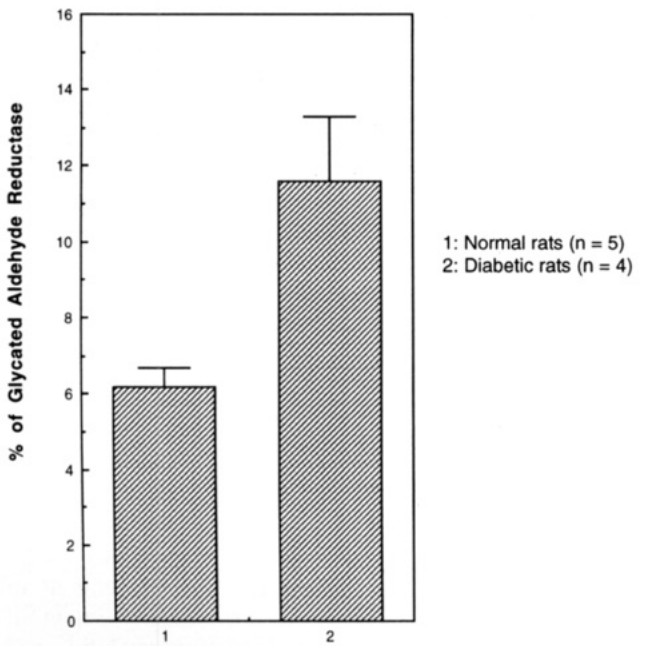

FIGURE 3: Ratio of glycated aldehyde reductase of normal and diabetic rats kidney. The extent of glycation of aldehyde reductase in normal ($n = 5$) and diabetic rats ($n = 4$) kidneys was determined according to the method described under Experimental Procedures. Values are means ± SD.

Preparation of Glycated, Lysyl-Endopeptidase-Treated Peptides. After reduction with $NaB[^3H]_4$, *in vivo* glycated and nonglycated aldehyde reducates were S-carboxymethylated and then digested with 0.4% (w/w) lysyl-endopeptidase (Wako Pure Chemicals) at room temperature for 6 h in 50 mM Tris-HCl, pH 9.0. The resulting peptides were subjected to HPLC (Shimadzu LC6A) on a TSKgel Boronate-5PW column (7.5 × 75 mm, Tosoh) equilibrated with 10 mM $MgCl_2$ and 20 mM HEPES (pH 8.5), and radioactive glycated

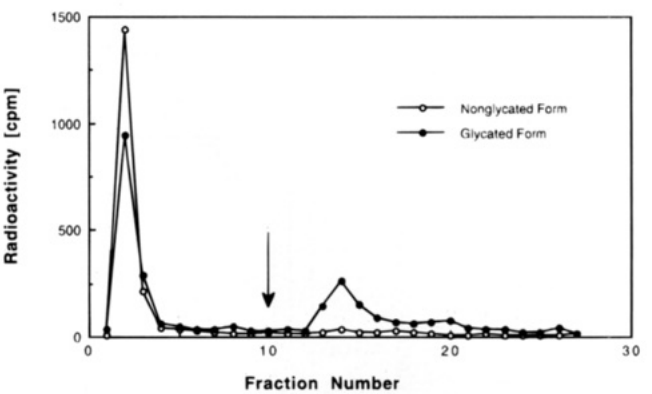

FIGURE 4: TSKgel Boronate-5PW chromatography of lysyl-endopeptidase-digested peptides from $NaB[^3H]_4$-reduced aldehyde reductase. Both nonglycated (○) and glycated (●) enzymes were reduced with $NaB[^3H]_4$, digested with lysyl-endopeptidase, and subjected to HPLC on a TSKgel-Boronate 5PW column. Bound fraction was eluted by the addition of sorbitol (arrow). The column was operated at a flow rate of 1.0 mL/min and fractions of 1 mL were collected. The radioactivity in 50 μL aliquots of each fraction was counted.

peptides were eluted with 0.1 M sorbitol in 20 mM HEPES (pH 8.5). The glycated peptides were then separated by RP-HPLC on a Cosmosil C_{18} column (4.6 × 250 mm, Nacalai Tesque), and fractions of 1 mL were collected. The column was operated at a flow rate of 1.0 mL/min. A gradient system formed between solvent A (0.1% trifluoroacetic acid in sterilized water) and solvent B (0.08% trifluoroacetic acid in 80% acetonitrile) was used. The program was as follows: 0 min, 0% solvent B; 80 min, 60% solvent B; 100 min, 100% solvent B; 115 min, 100% solvent B; 120 min, 0% solvent B; 135 min, 0% solvent B. Peptides were detected by their absorbance at 214 nm, and radioactivity was measured by liquid scintillation counting. The same procedure was used for the $NaBH_4$-reduced sample, and the peaks corresponding to the radioactive glycated peptides were collected and then subjected to amino acid analysis.

Amino Acid Sequencing. The isolated glycated peptides were subjected to protein sequencing in a gas-phase protein sequencer. PTH-amino acids were quantitated by RP-HPLC (Applied Biosystems).

Mass Spectrometry. In order to confirm the identification of the adducts of the peptides, mass spectrometry was carried out. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a JEOL JMS-LDI1700 mass spectrometer (Akishima, Japan) with a N_2 gas discharge laser (337 nm). Glycated peptides purified from the RP-HPLC were lyophilized, dissolved in 1 mL of 2% acetic acid, and then mixed with 1 mL of 100 mM sinapinic acid. Samples were deposited on a probe and air-dried prior to insertion into the instrument. Chemical mass was used for the expression of molecular weight in this study.

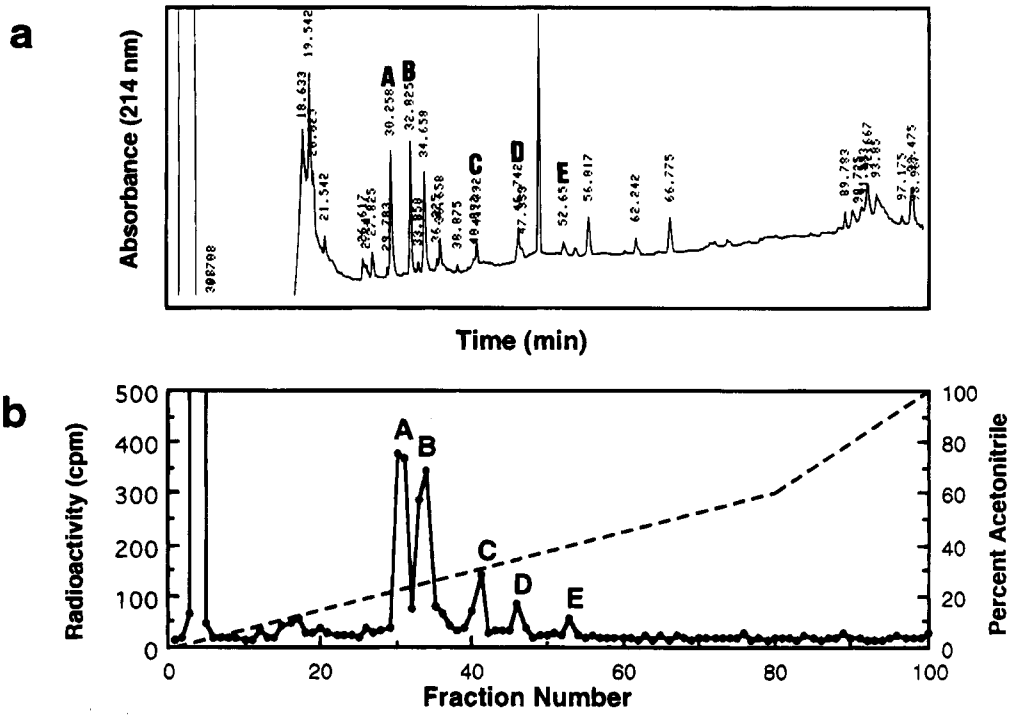

FIGURE 5: RP-HPLC of TSKgel Boronate-5PW-bound fractions. After chromatography on TSKgel Boronate-5PW, glycated and labeled peptides were isolated by RP-HPLC on a C_{18} column. The eluent was monitored by absorbance at 214 nm (a), and radioactivity was measured by liquid scintillation counting (b). A−E indicate the glycated peptides.

Table 2: Edman Degradation of Glycated Peptides[a]

	peptides				
cycle	A	B	C	D	E
1	Tyr (16.7)	Leu (11.2)	Glu (12.8)	Ala (12.6)	Met (11.0)
2	Asp (8.8)	Trp (4.8)	Ser (4.1)	Leu (8.5)	Pro (5.8)
3	Ser (7.8)	Asn (1.2)	Val (4.0)	Glu (4.1)	Leu (6.1)
4	Thr (0.9)	Thr (1.9)	Gly (3.5)	Ala (7.0)	Ile (6.5)
5	His (0.9)	*blank	Ala (2.7)	Leu (7.8)	Gly (5.1)
6	Tyr (2.2)	His (1.7)	Gly (2.9)	Val (8.6)	Leu (5.2)
7	*blank	His (1.8)	*blank	Ala (0.1)	Gly (4.0)
8	Glu (1.5)	Pro (1.8)	Ala (2.6)	*blank	Thr (5.3)
9	Thr (0.5)	Glu (1.8)	Val (1.8)	Gly (5.6)	Trp (0.2)
10	Trp (0.5)	Asp (3.6)	Pro (1.3)	Leu (5.1)	*blank
11	Lys (0.7)	Val (3.2)	Arg (0.9)	Val (5.3)	Ser (6.4)
12		Glu (1.7)	Glu (0.5)	Lys (2.5)	Glu (0.8)
13		Pro (1.5)	Glu (0.8)		Pro (0.6)
14		Ala (2.6)	Leu (0.9)		Gly (0.9)
15		Val (2.6)	Phe (0.6)		Gln (1.3)
16		Arg (0.8)	Val (1.1)		Val (0.5)
17		Lys (<0.1)			Lys (0.3)
18					
19					
20					
molecular mass	1622	2221	2169	1376	2005
calculated mass of peptide	(1458)	(2057)	(2005[b])	(1212)	(1841)

[a] Number of picomoles of PTH-derivatives are shown in parentheses. A "blank" result indicates that no PTH-derivative was detected. Mass spectrometric analysis of each peptide was carried out by a MALDI-TOF mass spectrometer with N_2 discharge laser. [b] A calculated value of 20 amino acids of corresponding peptide ending as Lys, which is deduced from cDNA sequence (Takahashi et al., 1993).

RESULTS

Separation of Aldehyde Reductase by Boronate Affinity Column Chromatography. When a freshly purified aldehyde reductase from normal rat liver was subjected to phenylboronate affinity chromatography, about 18% of the enzyme was retained by the gel and was subsequently eluted by 0.1 M potassium phosphate buffer containing 0.2 M sorbitol (Figure 1). Table 1 shows k_{cat} and apparent K_m values against 3-DG for pass-through and binding fractions. The catalytic efficiency (k_{cat}/K_m) of the binding fraction was found to be 68% as compared to that of the pass-through fraction.

Identification of the Retained Fraction as Glycated Enzyme Using Anti-ϵ-hexitol Lysine IgG. Immunoblot analysis using an affinity-purified antibody specific for ϵ-hexitol lysine revealed that the antibody specifically recognized the protein retained by the gel (Figure 2). This indicated that the retained enzyme was glycated. Taken together with the enzyme's behavior on the boronate column, these data indicated that a considerable amount of aldehyde reductase was glycated even in normal rat liver.

Glycation of Aldehyde Reductase in Experimental Diabetic Rats. Since kidney is a predominant organ which suffers diabetic complications and contains a large amount of aldehyde reductase, we tried to estimate the extent of glycation of the enzyme in kidneys of normal rats and experimental diabetic rats. Proteins in crude rat kidney homogenate were fractionated by a boronate-affinity column, and the amount of aldehyde reductase in bound and unbound fractions was quantified by ELISA. Figure 3 shows the percentage of the glycated form of aldehyde reductase with total aldehyde reductase as 100% in normal and diabetic rats. The ratio of glycated aldehyde reductase in kidneys was significantly increased in diabetic rats as compared to normal rats.

Isolation of Glycated, Lysyl-Endopeptidase-Treated Peptides from Rat Aldehyde Reductase. The glycated and nonglycated aldehyde reductase labeled with $NaB[^3H]_4$ were digested with lysyl-endopeptidase followed by separation by HPLC on TSKgel Boronate-5PW (Figure 4). All the

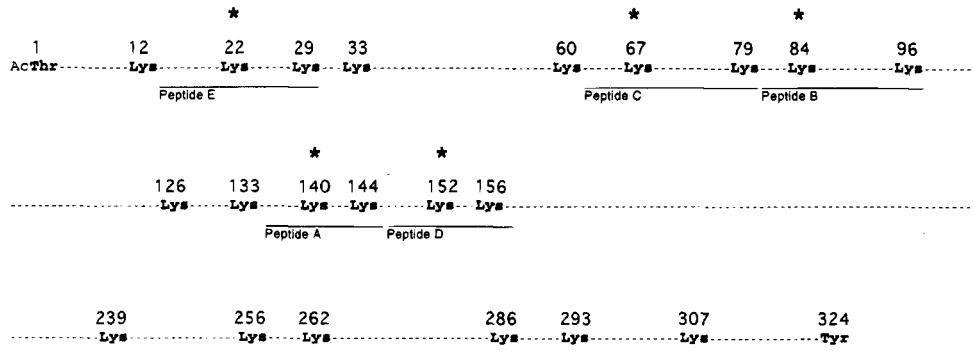

FIGURE 6: Glycation sites of rat aldehyde reductase. Portions of the sequence that have been identified as glycated peptides are underlined. Glycation sites are marked with asterisks.

radioactivity incorporated into the nonglycated aldehyde reductase and about 70% of the radioactivity in the glycated aldehyde reductase passed through the column. This radioactivity was considered to represent nonspecific incorporation, such as NaB[^{3}H]$_4$ reduction of disulfide bonds (Brown, 1960), certain peptide bonds (Crestfield et al., 1963; Paz et al., 1970), the indole group of tryptophan residues (Gribble et al., 1974), and carboxyl groups (Ishizumi et al., 1968). The bound fraction of glycated aldehyde reductase from the boronate column was further purified by RP-HPLC using a C_{18} column (Figure 5). Five independent peaks were found to be radioactive by scintillation counting and immunoreactive to anti-ϵ-hexitol lysine IgG by ELISA (data not shown) and were subjected to sequence analysis.

Sequence Analysis of the Glycated Peptide. The sequencing results (Table 2) obtained for each peptide, A, B, C, D, and E, were found to correspond to amino acid residues 134−144, 80−96, 61−79, 145−156, and 13−29 of rat aldehyde reductase (Takahashi et al., 1993), respectively, except for the absence of a signal for PTH-derivative at the position corresponding to lysine, which was assumed to be a glycation site (Figure 6). Mass spectrometric analysis of peptide A indicated a molecular mass of 1622 (Table 2), 164 higher than the calculated value from the sequence Tyr-134 to Lys-144 in rat aldehyde reductase. The difference of 164 corresponded to the molecular mass of a N^{ϵ}-(1-deoxyhextolyl) group on a lysine residue. This additional molecular mass of 164 was obtained for all the other glycated peptides (Table 2). The α-amino group at the N-terminus cannot be glycated, because the N-terminal Thr of rat aldehyde reductase is acetylated which was identified by mass spectrometry (data not shown). It was concluded, therefore, that glycation of rat aldehyde reductase had occurred mainly at the ϵ-amino groups of lysines 22, 67, 84, 140, and 152, which were in peptides E, C, B, A, and D, respectively (Figure 6). Judging from the relative radioactivities of the peaks in Figure 5, approximately 43% of the *in vivo* glycation had occurred at Lys-140, 37% of it had occurred at Lys-84, 13% at Lys-67, 5% at Lys-152, and 2% at Lys-22. Glycation appeared to be relatively specific to Lys-140 and Lys-84. Thus, the properties of glycation of rat aldehyde reductase resembled those of several other proteins, including hemoglobin (Shapiro et al., 1980), serum albumin (Garlick & Mazer, 1983), pancreatic RNase A (Watkins et al., 1985), osteocalcin (Gundberg et al., 1986), Cu-Zn-superoxide dismutase (Arai et al., 1987), alcohol dehydrogenase (Shilton & Walton, 1991), and type I collagen (Reiser et al., 1992), whose amino groups exhibit differential reactivity toward glucose.

DISCUSSION

In the glycation hypothesis of diabetes mellitus, metabolites of Amadori products as well as glycation of proteins itself cause irreversible, pleiotropic complications. Some enzymes are known to undergo reduction of activity by glycation of their Lys residues at the initial stage of the Maillard reaction (Arai et al., 1987; Kondo et al., 1987; Garner et al., 1990). 3-DG causes cross-linking of proteins and modification of their biological properties and is highly toxic to cells. 3-DG can, however, be detoxified by enzymes *in vivo* (Kato et al., 1990; Liang et al., 1991). Recently, we (Takahashi et al., 1993) and another group (Kanazu et al., 1991) demonstrated that aldehyde reductase was such an enzyme that detoxified 3-DG by reducing the aldehyde group to an alcohol. The importance of aldehyde reductase in diabetic conditions is supported by the suggestion that methylglyoxal, which is also 2-oxoaldehyde and is an excellent substrate for aldo-keto reductases including aldehyde reductase, takes part in glycation (Vander Jagt et al., 1992). Here we showed that a significant amount of aldehyde reductase was glycated (about 18%) even in normal rats and that glycated aldehyde reductase exhibited less activity than nonglycated enzyme (Table 1). The reduction of enzyme activity by glycation is 68%. The real significance *in vivo* situation remains to be solved. In the case of diabetes, however, as shown in Figure 3, an increase of glycation of aldehyde reductase was found.

Although a large number of proteins have been reported to be glycated and the glycated sites were identified in some of them, *in vivo* glycation sites have been identified for only a small number of proteins (Shapiro et al., 1980; Garlick & Mazer, 1983; Gundberg et al., 1986; Shilton & Walton, 1991; Reiser et al., 1992). We have identified the glycated sites of aldehyde reductase purified from normal rat liver by amino acid sequencing of glycated peptides isolated by TSKgel Boronate-5PW column chromatography and RP-HPLC (Figure 5). It was confirmed that each of them was gycated on the basis of (1) incorporation of a tritium label by NaB[^{3}H]$_4$ reduction, (2) affinity to the phenylboronate groups of TSKgel Boronate-5PW, (3) reactivity to anti-ϵ-hexitol lysine IgG, which was confirmed by ELISA analysis (data not shown), (4) resistance to digestion by lysyl-endopeptidase, (5) failure to detect any PTH-derivative at the expected position, and (6) mass-spectrometric analysis of the adduct (Table 2). The glycated Lys residues were concentrated in the N-terminal region of the enzyme (Figure 6). All five Lys residues are conserved in human aldehyde reductase (Wermuth et al., 1987), which indicates that the same

residues may be glycated in the human enzyme *in vivo*. These residues except Lys-22 are unique to aldehyde reductase, although intense homology can be seen in the aldo-keto reductase superfamily to which aldehyde reductase belongs (Bohren et al., 1989). Because glycation decreases the enzyme activity, at least one of the glycated Lys residues must be located in a region important for the catalytic reaction. Glycation of Lys residues identified here may affect enzymatic activity by influencing substrate binding or conformation of the enzyme but is unlikely to affect coenzyme binding since Lys-262, which is not glycated, is associated with coenzyme binding (Bohren et al., 1991).

The site specificity of glycation is dependent upon both the equilibrium Schiff base concentration and the rate of the Amadori rearrangement at each site. Imidazole groups are effective acid−base catalysts in a neutral solution and could function in the manner suggested by Walton's group (Shilton & Walton, 1991; Shilton et al., 1993). In aldehyde reductase, His residues are in proximity to the glycated Lys residues at positions 84 and 140. Hence, these His residues could function as acid−base catalysts for these sites. Because there is no information available about the tertiary structure of aldehyde reductase, we cannot speculate as to which residues are involved in catalysis at other glycation sites.

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REFERENCES

Arai, K., Maguchi, S., Fujii, S., Ishibashi, H., Oikawa, K., & Taniguchi, N. (1987) *J. Biol. Chem. 262*, 16969−16972.

Baynes, J. W., & Monnier, V. M. (1989) *Prog. Clin. Biol. Res. 304*, 1−410.

Bohren, K. M., Bullock, B., Wermuth, B., & Gabbay, K. H. (1989) *J. Biol. Chem. 264*, 9547−9551.

Bohren, K. M., Page, J. L., Shankar, R., Henry, S. P., & Gabbay, K. H. (1991) *J. Biol. Chem. 266*, 24031−24037.

Brown, W. D. (1960) *Biochim. Biophys. Acta 44*, 365−367.

Brownlee, M., Cerami, A., & Vlassara, H. (1988) *N. Engl. J. Med. 318*, 1315−1321

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622−627.

Garner, M. H., Bahador, A., & Sachs, G. (1990) *J. Biol. Chem. 265*, 15058−15066.

Garlick, R. L., & Mazer, J. S. (1983) *J. Biol. Chem. 258*, 6142−6146.

Gribble, G. W., Lord, P. D., Skotnicki, J., Dietz, S. E., Eaton, J. T., & Johnson, J. L. (1974) *J. Am. Chem. Soc. 96*, 7812−7814.

Gundberg, C. M., Anderson, M., Dickson, I., & Gallop, P. M. (1986) *J. Biol. Chem. 261*, 14557−14561.

Ishizumi, K., Koga, K., & Yamada, S. (1968) *Chem. Pharm. Bull. (Tokyo) 16*, 492−497.

Kanazu, T., Shinoda, M., Nakayama, T., Deyashiki, Y., Hara, A., & Sawada, H. (1991) *Biochem. J. 279*, 903−906.

Kato, H., Cho, R. K., Okitani, A., & Hayase, F. (1987a) *Agric. Biol. Chem. 51*, 683−689.

Kato, H., Shin, D. B., & Hayase, F. (1987b) *Agric. Biol. Chem. 51*, 2009−2011.

Kato, H., van Chuyen, N., Shinoda, T., Sekiya, F., & Hayase, F. (1990) *Biochim. Biophys. Acta 1035*, 71−76.

Khadem, H. E., Horton, D., Meshreki, M. H., & Nashed, M. A. (1971) *Carbohydr. Res. 17*, 183−192.

Kondo, T., Murakami, K., Ohtsuka, Y., Tsuji, M., Gasa, S., Taniguchi, N., & Kawakami, Y. (1987) *Clin. Chim. Acta 166*, 227−236.

Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods 10*, 203−209.

Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.

Liang, Z.-Q., Hayase, F., & Kato, H. (1991) *Eur. J. Biochem. 197*, 373−379.

Miyata, T., Oda, O., Inagi, R., Iida, Y., Araki, N., Yamada, N., Horiuchi, S., Taniguchi, N., Maeda, K., & Kinoshita, T. (1993) *J. Clin. Invest. 92*, 1243−1252.

Monnier, V. M., & Cerami, A. (1981) *Science 211*, 491−493.

Monnier, V. M., Kohn, R. R., & Cerami, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 583−587.

Ookawara, T., Kawamura, N., Kitagawa, Y., & Taniguchi, N. (1992) *J. Biol. Chem. 267*, 18505−18510.

Paz, M. A., Henson, E., Rombauer, R., Abrash, L., Blumenfeld, O. O., & Gallop, P. M. (1970) *Biochemistry 9*, 2123−2127.

Reiser, K. M., Amigable, M., & Last, J. A. (1992) *J. Biol. Chem. 267*, 24207−24216.

Shapiro, R., McManus, M. J., Zalut C., & Bunn, H. F. (1980) *J. Biol. Chem. 255*, 3120−3127.

Shilton, B. H., & Walton, D. J. (1991) *J. Biol. Chem. 266*, 5587−5592.

Shilton, B. H., Campbell, R. L., & Walton, D. J. (1993) *Eur. J. Biochem. 215*, 567−572.

Takahashi, M., Fujii, J., Teshima, T., Suzuki, K., Shiba, T., & Taniguchi, N. (1993) *Gene 127*, 249−253.

Vander Jagt, D. L., Robinson, B., Taylor, K. K., & Hunsaker, L. A. (1992) *J. Biol. Chem. 267*, 4364−4369.

Watkins, N. G., Thorpe, S. R., & Baynes, J. W. (1985) *J. Biol. Chem. 260*, 10629−10636.

Wermuth, B., Omar, A., Forster, A., di Franceco, C., Wolf, M., von Wartburg, J.-P., Bullock, B., & Gabbay, K. H. (1987) *Prog. Clin. Biol. Res. 232*, 297−307.

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