Isolation, Purification, and Characterization of Amadoriase Isoenzymes (Fructosyl Amine-oxygen Oxidoreductase EC 1.5.3) from Aspergillus sp.*

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Four “amadoriase” enzyme fractions, which oxidatively degrade glycated low molecular weight amines and amino acids under formation of hydrogen peroxide and glucosone, were isolated from an Aspergillus sp. soil strain selected on fructosyl adamantanamine as sole carbon source. The enzymes were purified to homogeneity using a combination of ion exchange, hydroxyapatite, gel filtration, and Mono Q column chromatography. Molecular masses of amadoriase enzymes Ia, Ib, and Ic were 51 kDa, and 49 kDa for amadoriase II. Apparent kinetic constants for N^--fructosyl N^--e-butoxy carbonyl lysine and fructosyl adamantanamine were almost identical for enzymes Ia, Ib, and Ic, but corresponding values for enzyme II were significantly different. FAD was identified in all enzymes based on its typical absorption spectrum. N-terminal sequence was identical for enzymes Ia and Ib (Ala-Pro-Ser-Ile-Leu-Ser-Thr-Glu-Ser-Ile-Ile-Val-Ile-Gly-Ala-Gly-Thr-Trp-Gly-) and Ic except that the first 5 amino acids were truncated. The sequence of enzyme II was different (Ala-Val-Thr-Lys-Ser-Ser-Leu-Leu-Ile-Val-Gly-Ala-Gly-Thr-Trp-Gly-Thr-Ser-Thr-). All enzymes had the FAD co-factor-binding consensus sequence Gly-X-Gly-X-Gly within the N-terminal sequence. In summary, these data show the presence of two distinct amadoriase enzymes in the Aspergillus sp. soil strain selected on fructosyl adamantanamine and induced by fructosyl propylamine. In contrast to previous described enzymes, these novel amadoriase enzymes can deglycate both glycated amines and amino acids.

Nonenzymatic glycation of proteins has been implicated in the pathogenesis of diabetic complications as one of several mechanisms by which chronic elevation of glycemia may be responsible for the early cellular and extracellular dysfunctions triggered by diabetes. The Amadori product, which forms in the initial stages of the Maillard reaction in vitro, has been shown to be recognized by receptors at the surface of monocytes, aortic endothelial, and mesangial cells (1–3), and treatment of diabetic mice with antibodies to glycated albumin decreases the rate of progression of diabetic nephropathy (4). Amadori products of glucose are precursors of the glycoxidation products pentosidine and N^--(carboxymethyl)lysine (5–7), which are elevated in diabetes and the levels of which increase with the severity of diabetic complications (8–10). Furthermore, N^--(carboxymethyl)lysine has now been identified as the major advanced glycation end product (AGE) epitope (11) and is also likely to be responsible for the major negative charge in AGE proteins which appears to play a role for recognition by AGE receptors in macrophages or endothelial cells (12, 13). Amadori products and glycated proteins are able to generate free radicals, which may oxidize low density lipoproteins and form covalent cross-links with other proteins (14, 15).

Collectively, these data suggest that increased levels of Amadori product in diabetes may be responsible for much of the glucotoxicity underlying the pathogenesis of diabetic complications. Based on this premise, we began a few years ago to search for novel ways to selectively prevent the effects of protein glycation by attempting to deglycate proteins enzymatically (16), with the ultimate goal of utilizing such enzymes in transgenic models of hyperglycemia. We found and partially purified a deglycating enzyme in a Pseudomonas sp. soil strain selected for growth with glycated e-aminocaproic acid as sole carbon source (17). Surprisingly, however, the enzyme obtained cleaved glycated substrates at the N-alkyl instead of the ketoamine bond and released free fructosamine, while the deamined alkyl residue spontaneously oxidized to the acid.

We synthesized a highly sterically inhibited Amadori product using adamantanamine, and utilized this substrate as sole carbon source to select soil organisms. Two molecularly distinct isoenzymes were obtained from an Aspergillus sp., which can degrade both glycated low molecular weight amines and amino acids. The work described below provides a basis for the molecular analysis of deglycating enzymes and their biological mechanism of regulation.

EXPERIMENTAL PROCEDURES

Materials

N,O-Bis(trimethylsilyl)acetamide (BtmSA) was obtained from Fluka. Silica gel-coated aluminum thin layer chromatography plates (0.2 mm thick) were obtained from EM Separations (Gibbstown, NJ). o-Glucosone was a gift from Dr. Milton Feather (Department of Biochemistry, University of Missouri) or synthesized from the phenylosazone deriva-

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Fructosyl adamantanamine was synthesized according to a modified protocol of Hodge and Rist (20). 5 g of adamantanamine, 3 g of glucose, and 10 ml of acetic anhydride were heated in 100 ml of ethanol for 2 h under reflux. The solution was evaporated, and the residue was purified by silica gel column chromatography (17 × 2.5 cm, eluent: n-butanol/110; 10:1; monitored by TLC, eluent: ethanol/7H2O, 10:1; detected with 0.2% ninhydrin in ethanol or 2% TTC in methanol, 1 N NaOH, 1:1). Fractions containing the product were pooled, evaporated, redissolved in water, and freeze-dried. The product identity was confirmed by 1H NMR: 1.36–1.70 (12H), 2.05 (3H), 3.31 (2H), and 3.52–3.56 (5H). Glycated BSA, poly-o-lysine (M, 150,000–300,000), and poly-o-lysine (M, 30,000–70,000) were prepared as follows: 15 g/ml BSA, poly-o-lysine or poly-o-lysine was incubated in 50 mM HEPES (pH 7.4) containing 1 mM glucose, 1 mM DTPA, and 1 mM chloroform and tolune for 5 days at 37°C. The pH was monitored during incubation and adjusted by NaOH. The solution was filtered three times against water and freeze-dried. Glycated aminopropyl glass (Sigma) and Sphorisorb-NH2 (Phenomenex) were prepared as follows: 50 mg/ml aminopropyl glass or Sphorisorb was incubated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH2PO4, 8.06 mM Na2HPO4) containing 1 mM glucose, 1 mM DTPA, and 1 mM chloroform and tolune for 24 h at 60°C. The suspension was filtered and the supernatant was washed by sterile water. N′-fructosyl N′-t-Boc-lysine and N′-fructosyl N′-acetyllysine were prepared according to Finot and Mauron (21). Fructosyl propylamine was prepared as follows: 30 g of glucose and 30 ml of propylamine were heated at 70°C until the sugar was dissolved. The mixture was diluted in 200 ml of isopropanol and poured into 800 ml of diethyl ether. The precipitate was filtered and dissolved in 500 ml of 0.03% fluorescamine in dioxane and centrifuged. The fluorescence was measured at 37°C for 15 min. The precipitate was crystallized from dioxane/methanol (1:1), and oxalate was exchanged by chloride by anion exchange chromatography. Fructosyl propylamine in reduced form was prepared as follows: 1.2 g of NaBH4 was added to 200 ml of fructosyl propylamine dissolved in 50 ml of water and incubated for 12 h at room temperature. The suspension was filtered and evaporated to dry. The residue was purified by Sephadex G-15 gel filtration column chromatography (eluent: PBS, detected with 0.2% ninhydrin in ethanol).

Identification of Enzymatic Degradation Products

The reaction products of the enzyme-rich fraction and 2 mg of N′-fructosyl N′-t-Boc-lysine or fructosyl propylamine were incubated at 37°C. After 1–4 days a sample was tested on TLC plates (eluent: n-butanol/acetate acid/water, 5:3:1, detection: 0.2% ninhydrin in ethanol or 2% TTC in methanol, 1 N NaOH, 1:1). As standards N′-fructosyl N′-t-Boc-lysine, fructosyl propylamine, glucosone, N′-fructosyl N′-Boc-lysine, and Boc-propylamine were used. For GC/MS 8 µl of enzyme-rich fraction was incubated in 1 ml of PBS containing 15 mM fructosyl propylamine and 50 mM aminoguanidine for 24 h at 37°C. The solution was filtered through a Microcon-10 (Amicon), and the filtrate was dried under vacuum (Savant, Farmingdale, NY). The residue was derivatized in 100 µl of pyridine/BmSA (1:1) for 1 h at room temperature. Coupled gas chromatography and mass spectroscopy (GC/MS) were performed on a Hewlett-Packard 5890 series II gas chromatograph using helium as the carrier gas (flow rate 26.3 cm/s) on an Ultra 2 capillary column (25 m, 0.2 mm (inner diameter), film thickness 0.33 µm (Hewlett-Packard). Methane was used for positive chemical ionization. Injector and interface temperatures were 270°C and 280°C, respectively. Temperature program was 100–200°C at 5°C min−1, 200–270°C at 10°C min−1, and isothermal at 270°C for 10 min. Glucose triazine generally provided by Dr. Marcus Glomb was derivatized as above and used as a standard.

Four different assays were developed to measure the activity of the enzyme.

Glucosone Formation—For purification, the enzyme activity was monitored by the release of glucosone measured by a colorimetric reaction with OPD (22) using fructosyl propylamine as a substrate. This assay is based on the end point measurement of glucosone formed after 120 min of reaction time. The reaction mixture contained 20 mM sodium phosphate, pH 7.4, 10 mM OPD, 10 mM fructosyl propylamine, and enzyme protein in a volume of 1 ml. After incubation at 37°C for 2 h, the absorbance at 320 nm was measured. The reaction was linear to 240 min in a dose-dependent manner under these conditions. One unit of enzyme activity was defined as the amount of the enzyme that produces 1 µmol of glucosone/min. Synthesized glucosone was used as a standard.

Free Amine Assay—To assay the release of free amine, fluorescence was measured after reaction with fluorescamine. 25 µl of a solution of pure enzyme or enzyme-rich fraction, 15 µl of 20% fructosyl propylamine in water, and 250 µl of PBS were incubated at 37°C for different time periods. The assay was stopped by filtration through a Microcon-10 (Amicon, Beverly, MA) at 4°C. 1 µl of the pure or 1:10 diluted filtrate was added to 1.5 ml of 50 mM phosphate buffer pH 8.0. Under vigorous vortexing 0.5 ml of 0.03% fluorescamine in dioxane was rapidly added. After 5 min fluorescence was measured (λex = 390 nm, λem = 475 nm). A standard plot was made with 6–150 ng of propylamine.

H2O2 Assay—Hydrogen peroxide was quantitated by the quinone dye following the absorbance at 505 nm (ex, 200–270°C at 10°C/min, 200–300°C at 10°C/min, and isothermal at 270°C for 10 min. Glucose triazine generally provided by Dr. Marcus Glomb was derivatized as above and used as a standard.

Oxygen Consumption—Oxygen consumption was determined with a YSI-Beckman glomometer II equipped with a Clarke type oxygen electrode as described before (17). Briefly, enzyme (50 µl) was added to the chamber containing 750 µl of PBS and 650 µl of water. The reaction was started by addition of 50 µl of 300 mM fructosyl propylamine (final concentration 10 µM).

Purification of the Enzyme

All purification steps were carried out at 4°C. Washed mycelia (180 g as wet weight) were suspended and homogenized in 900 ml of homogenizing buffer (20 mM sodium phosphate buffer, pH 7.4, 1 mM PMSF, 1 mM EDTA, 2 mM DTT) using a Polytron homogenizer (Kinematica, Littau, Switzerland) and Potter-Elvehjem Teflon-glass homogenizer. The homogenate was squeezed through the gauze and centrifuged at 20,000 × g for 30 min to remove unbroken cell and cell debris. The pellet was discarded, and solid ammonium sulfate was added to the supernatant to 45% saturation. The solution was centrifuged at 6,000 × g for 30 min. The supernatant was adjusted to 70% saturation by solid ammonium sulfate. After stirring for 30 min, the precipitate was obtained by centrifugation at 6,000 × g for 30 min and dissolved in a minimum volume of buffer A (20 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, 2 mM DTT) followed by dialysis against the same buffer. Insoluble material formed was removed by centrifugation at
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20,000 × g for 15 min. The supernatant was applied to a DEAE Sephadex column (5 × 16 cm) equilibrated with buffer A. The column was washed extensively with buffer A, and the bound protein was eluted with a 3000-ml linear gradient of NaCl of 0–0.2 M in the same buffer. Fractions containing enzyme activity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 2 mM DTT and applied to a hydroxyapatite column (1.5 × 4 cm) equilibrated with the same buffer. After washing the column extensively with the equilibration buffer, elution was carried out with 140-ml linear gradient of sodium phosphate buffer, pH 7.4, over 10–100 mM, containing 1 mM EDTA and 2 mM DTT. Fractions with enzyme activity were pooled and concentrated with an Amicon Diaflow Ultrafiltration using PM 10 membrane (Amicon), and applied to a Sephacryl S-200 column (1 × 90 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 2 mM DTT, and eluted with the same buffer. The enzyme-active fractions were pooled, dialyzed against buffer A, and applied onto a Mono Q column (0.5 × 5 cm) equilibrated with buffer A. The column was washed with 4 ml of buffer A, and elution was carried out with a 20-ml linear gradient of 0–0.5 M NaCl in buffer A. Fractions containing enzyme activity were used as the purified preparation of amadorases.

Protein Determination

Protein concentration was determined by the method of Bradford (24) with a Bio-Rad protein assay kit using BSA as a standard. Protein analysis by SDS-PAGE was carried out according to Laemmli (25). The molecular weight of native enzyme was determined by gel filtration on a Sephacryl S-200 column equilibrated with PBS containing 2 mM DTT using molecular weight markers (Sigma).

Kinetic Constants

Kinetic constants were calculated from the data by least-squares linear regression analysis.

Determination of pH Profiles and Isoelectric Points

pH profiles were determined over the range pH 5.8–9.5 using the following buffers: MES (pH 5.8–6.5), MOPS (pH 6.5–7.5), POPSO (pH 7.5–8.5), and CHES (pH 8.5–9.5). Overlaps were used in all cases, and checks were made to ensure that none of the buffers were inhibitory and that the enzymes were stable during the time needed for the measurements. Isoelectric points were determined by isoelectric focusing using PhastGel IEF 5–8 (Pharmacia) and pI maker (IEF Mix 3.6–9.3, Sigma) in PhastSystem (Pharmacia).

Amino Acid Sequencing

N-terminal amino acid sequencing was performed at the Molecular Biology Core laboratory (Department of Biochemistry, Case Western Reserve University) with an Applied Biosystems 477A Protein Sequencer (Foster City, CA).

RESULTS

Search for an Amadori Product Degrading Microorganism and Identification of Deglycase Activity—Soil specimens were screened for a microorganism that can degrade Amadori products by using culture media with glycated substrates as sole carbon source. In order to increase the chances that the enzyme would also have activity against glycated proteins, Amadori products were chosen with a maximal steric hindrance at the amino side. As carbon sources glycated aminopropyl glass, glycated poly-D-lysine, and glycated aminopropyl silica gel, glycated aminopropyl silica gel, glycated poly-D-lysine, and glycated amadanapalmamine were tested, but no organism could be isolated in presence of these substrates except fructosyl amadanapalmamine.

From the medium enriched with 5.5 mg/ml fructosyl amadanapalmamine, a microorganism was isolated and identified as an Aspergillus sp. strain. To ascertain the presence of Amadori product degrading activity, an extract of the fungus was produced and incubated with N'fructosyl N'-t-Boc-lysine. The reaction was monitored by TLC (Fig. 1) and compared with that of the heat-inactivated extract. In the course of the incubation the amount of the Amadori product in the extract was decreasing, whereas two new spots appeared on TLC. A ninhydrin-positive spot could be identified by comparison of the RF with an authentic standard as free N'-t-Boc-lysine. The other spot reacted with TTC and co-migrated with synthetic glucosone. In the same way, free propylamine and glucosone derived from the reaction mixture of enzyme-rich fraction and fructosyl propylamine were observed (data not shown).

Identification of the Reaction Products—In order to confirm the molecular identity of the products, GC/MS analysis was performed after preparing the triazine derivatives with aminoguanidine and the trimethylsilyl derivatives with BtmSA. In the sample two major peaks appeared at retention times of 26.13 and 26.60 min, respectively (Fig. 2, top panel). The mass spectrum of both compounds showed characteristic peaks at m/z 342, 270, and 217 (Fig. 2A). A sample of authentic glucosone was derivatized in the same way with aminoguanidine and BtmSA and subjected to GC/MS analysis. The chromatogram showed peaks at 26.13 and 26.60 min for the two triazine stereomers, and the fragmentation patterns were identical to those of the degradation products (Fig. 2B). From the TLC and GC/MS data, it can be concluded that the enzyme releases glucosone and free amine from the Amadori product. Oxygen consumption during the reaction was detected polarographically using an oxygen electrode, and H2O2 generation was detected by the quinone dye assay (data not shown). Thus, all the data confirm that the Amadori product is degraded by an oxidative cleavage of the ketoamine bond, whereby oxygen is used as an electron acceptor and free amine and glucosone are released (Fig. 3).

Enzyme Assays and Time and Dose Dependence—Time-course experiments on the formation of glucosone detected as quinonoid derivative by the OPD method were carried out with an enzyme-rich fraction incubated with fructosyl propylamine (Fig. 4). The amount of glucosone formed increased in the course of the incubation and was increased by enzyme concentration, proving thereby that an enzyme was responsible for the activity. A time-dependent release of free amine could be also found by free amine assay using fluorescamine (data not shown).
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cation. Purification was performed as described under “Experimental Procedures.” The extract was first fractionated by DEAE-Sepharose (Fig. 5A). Two peaks of amadoriase activity were observed. They were pooled into “pool 1” and “pool 2” and separately subjected to further purification. Fig. 5B shows the profile of the hydroxyapatite column chromatography of pool 1. Three peaks of activity were observed and purified individually by gel filtration and Mono Q column chromatography. Obtained enzymes were named amadoriase Ia, Ib, and II according to the order of elution from the hydroxyapatite column chromatography. Pool 2 was also subjected to hydroxyapatite column chromatography (Fig. 5C), and an active peak obtained was purified by gel filtration and Mono Q column chromatography. The enzyme obtained from pool 2 was named amadoriase Ic. Altogether four amadoriase enzyme fractions were isolated from Aspergillus sp. that had been selected with fructosyl adamantanamine and grown in the presence of fructosyl propylamine. The results of each purification of amadoriases are summarized in Table I. Each enzyme was purified to homogeneity as judged by Coomassie-stained SDS-PAGE (Fig. 6).

From the results of Sephacryl S-200 gel filtration, the apparent molecular masses of native amadoriases Ia, Ib, and Ic were calculated to be 40 kDa and II was 55 kDa (data not shown). The results
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TABLE I
Purification of amadoriases Ia, Ib, II, and Ic

Activity was measured by glucose formation assay as described under “Experimental Procedures.” 10 mM fructosyl propylamine was used as a substrate.

| Step          | Total protein | Total activity | Specific activity | Yield | Purification |
|---------------|---------------|----------------|-------------------|-------|--------------|
|               | mg            | milliunits    | milliunits/mg     |       | -fold        |
| Amadoriase Ia |               |                |                   |       |              |
| Cell extract  | 846           | 160,000        | 190               | 100   | 1.0          |
| (NH₄)₂SO₄     | 370           | 107,000        | 290               | 67    | 1.5          |
| DEAE-Sepharose (pool 1) | 13.0 | 25,000        | 1900              | 16    | 10.0         |
| Hydroxyapatite 1 (peak Ia) | 0.80 | 3080         | 3900              | 1.9   | 20.5         |
| Gel filtration | 0.42          | 1850           | 4400              | 1.2   | 23.2         |
| Mono Q        | 0.14          | 659            | 4700              | 0.4   | 24.7         |
| Amadoriase Ib |               |                |                   |       |              |
| DEAE-Sepharose (pool 1) | 13.0 | 25,000        | 1900              | 16    | 10.0         |
| Hydroxyapatite 1 (peak Ib) | 1.4 | 5430         | 3900              | 3.4   | 20.5         |
| Gel filtration | 0.92          | 3860           | 4200              | 2.4   | 22.1         |
| Mono Q        | 0.42          | 1980           | 4700              | 1.2   | 24.7         |
| Amadoriase II |               |                |                   |       |              |
| DEAE-Sepharose (pool 1) | 13.0 | 25,000        | 1900              | 16    | 10.0         |
| Hydroxyapatite 1 (peak II) | 2.8 | 5220         | 1900              | 3.3   | 10.0         |
| Gel filtration | 2.2           | 4150           | 1900              | 2.6   | 10.0         |
| Mono Q        | 0.46          | 1140           | 2500              | 0.7   | 13.2         |
| Amadoriase Ic |               |                |                   |       |              |
| DEAE-Sepharose (pool 2) | 10.0 | 7600        | 780               | 4.9   | 4.1          |
| Hydroxyapatite 2 | 1.2 | 2100         | 1800              | 1.3   | 9.5          |
| Gel filtration | 0.38          | 946            | 2500              | 0.6   | 13.2         |
| Mono Q        | 0.18          | 772            | 4300              | 0.5   | 22.6         |

FIG. 6. SDS-PAGE of purified amadoriases Ia, Ib, II, and Ic. Denatured protein was applied to a 10% homogeneous SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Each lane contained 0.3 mg of protein. The molecular mass of the marker proteins (M) were indicated in kDa.

indicated that all four enzymes were found to be monomers.

Substrate Specificity and Kinetic Constants of the Purified Amadoriases Ia, Ib, II, and I—Substrate specificity was examined for each purified enzyme (Table II). All four enzymes were active toward N'-fructosyl N'-t-Boc-lysine as well as fructosyl propylamine. However, the relative activity of amadoriases Ia, Ib, and Ic toward fructosyl adamantamamine was low compared with that for fructosyl propylamine. They had no activity against N'-fructosyl N'-acetyllysine. In contrast, amadoriase II was active toward fructosyl adamantamamine and N'-fructosyl N'-acetyllysine. None of them were active toward reduced fructosyl propylamine (10 mM), glycated BSA (2 mg/ml), glycated poly-L-lysine (0.02%) or glucose (10 mM). Apparent kinetic constants for N'-fructosyl N'-t-Boc-lysine and fructosyl adamantamamine are shown in Table III. The stoichiometry of the reaction catalyzed by the enzymes was determined using fructosyl propylamine as a substrate. Stoichiometric consumption of O₂ and formation of H₂O₂ and glucosone was observed for each enzyme (data not shown).

Prosthetic Group—In order to obtain information on the nature of the cofactor, the absorption spectrum of amadoriase enzymes was recorded. Fig. 7 shows the absorption spectra of amadoriases Ib and II. Two major absorption bands were detected at 362 and 452 nm with a shoulder at about 474 nm in the visible region, indicating the presence of flavine as prosthetic group. The spectra of the other enzymes were essentially the same.

pH Optimum and Isoelectric Points—The pH optima for amadoriases Ia, Ib, and Ic were pH 8.0 and for amadoriase II was pH 8.5. The isoelectric points of enzymes Ia, Ib, Ic, and II were 5.5, 5.5, 5.7, and 6.7, respectively.

Amino Acid Sequencing—To identify the proteins, N-terminal sequencing was carried out using a gas-phase protein sequencer. The sequence of all four enzymes are shown in Table IV.

DISCUSSION

We have isolated and purified four amadoriase enzyme fractions from Aspergillus sp. One of the four enzymes described above (amadoriase II) was different from the other three based on its N-terminal amino acid sequence and enzymatic properties. Amadoriase Ia and Ib appear identical, and amadoriase Ic have Michaelis-Menten properties and substrate specificities similar to amadoriase Ia and Ib. The N-terminal amino acid sequence of enzyme Ic is similar to that of enzymes Ia and Ib, except for the truncation of the first five N-terminal amino acids, which may have resulted from partial proteolytic breakdown during isolation. Thus, taken together, the data suggest the presence of two distinct amadoriase enzymes in the isolated Aspergillus strain. These enzymes cleave glycated substrates under consumption of oxygen and release of H₂O₂ and glucosone in a reaction involving FAD as a cofactor. Thus, they should be classified as fructosyl amine-oxygen oxido-reductases (EC 1.5.3). We propose the trivial name “amadoriase.”

Search for sequence homology with other known proteins using the combined GenBank CDS translations/PDB/Protein Update/PIR data base with 220,000 sequences revealed that none of these proteins had been described before. However, partial identity with D-amino acid oxidase from porcine (26), a human kidney p35 stress-inducible protein from F. oxysporum (28), human p21 protein (29), and hypothetical 44.4-kDa protein from Schizosaccharomyces pombe is apparent, especially for amadoriase Ic (Table IV). On close inspection, it turns out that all the enzymes have the consensus sequence for the ADP-binding βαβ-fold common to all FAD and NAD enzymes (30). All have the hydrophobic residues that form the hydrophilic core between the β-strands and the α-helix.
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Activity was measured by glucosone formation assay as described under “Experimental Procedures” except that the reaction mixtures contained various kinds of substrates instead of fructosyl propylamine.

Table II
Substrate specificity of amadoriases

| Substrates (10 mM) | Amadoriase Ia | Amadoriase Ib | Amadoriase Ic | Amadoriase II |
|-------------------|---------------|---------------|---------------|--------------|
| Fructosyl propylamine | 4.7 (100)* | 4.7 (100) | 4.3 (100) | 2.5 (100) |
| Fructosyl adamantanamine | 0.49 (10) | 0.49 (10) | 0.49 (11) | 3.0 (121) |
| N'-Fructosyl N'-t-Boc-lysine | 4.3 (92) | 4.4 (94) | 4.5 (104) | 4.8 (193) |
| N'-Fructosyl N'-acetylylysine | ND | 0.16 (3) | 0.12 (3) | 3.7 (151) |

* Activity is given as percentage in parentheses. The activity against fructosyl propylamine was taken as 100% for each enzyme.

Table III
Kinetic constants of amadoriases

| Amadoriase | N'-Fructosyl N'-t-Boc-lysine | Fructosyl adamantanamine |
|-----------|----------------------------|-------------------------|
|           | K_m  | k_cat | k_cat/K_m | K_m  | k_cat | k_cat/K_m |
| Ia        | 3.0 ± 0.3 | 250 ± 13 | 0.8 × 10^4 | 14.4 ± 0.3 | 48 ± 1.3 | 3.3 × 10^2 |
| Ib        | 3.1 ± 0.5 | 320 ± 32 | 1.0 × 10^4 | 14.7 ± 0.9 | 52 ± 1.7 | 3.5 × 10^3 |
| Ic        | 3.3 ± 0.2 | 320 ± 12 | 1.0 × 10^4 | 14.7 ± 0.9 | 52 ± 1.7 | 3.5 × 10^3 |
| II        | 1.6 ± 0.1 | 330 ± 58 | 2.0 × 10^4 | 3.4 ± 0.4 | 135 ± 6.5 | 4.0 × 10^4 |

The novel amadoriase enzymes described in this work join the growing family of fructosyl amino acid oxidase enzymes that can deglycate amino acid residues in proteins. The first fully characterized enzyme was described by Horiuchi and colleagues (31) in Corynebacterium sp., followed by a similar enzyme from Aspergillus sp. by the same group (32). Both enzymes regenerate the free amino acid and produce H_2O and glucosone. The first was obtained from soil containing fructosyl glycine as sole carbon and nitrogen source. It is a dimeric protein with identical 44-kDa subunits, which degrade glycated amino acids with a K_m value in the submillimolar range. It also has activity against D-erythropentulosyl-glycine and D-tagatosyllysine but not against glycated alkylamines, β-amino acids, l-mino acids and D-amino acids, N'-fructosyl lysine, or ε-amino caproic acid. The Aspergillus enzyme was obtained from the soil Aspergillus sp.1005 whose spores were grown in a medium containing 1% fructosyl glycine. It is a dimeric protein of 83 kDa with identical 43-kDa subunits, i.e. similar to that of the Corynebacterium enzyme but significantly different from our strain. The fungal enzyme had activity against many glycated amino acids, including N'-fructosyl lysine but, in contrast to the enzymes described in this work, had no activity against glycated alkylamines. The K_m for fructosyl glycine was 2.2 mM, i.e. in the same range as for our enzymes. The inability of Horiuchi’s enzymes to deglycate the Amadori products of alkylamines apparently relates to the selection method.

Recently, Sakai et al. (23) isolated a fructosyl lysine oxidase from the fungus Fusarium oxysporum using N'-fructosyl N'-Z-lysine as a sole nitrogen source. The monomeric enzyme has, like our enzymes, a molecular mass of 50 kDa. The K_m for N'-fructosyl lysine was very low (0.22 mM), but little activity was observed against fructosyl valine. A small amount of activity against glycated poly-L-lysine but not against glycated protein was observed. In contrast to the other enzymes of Horiuchi, fructosyl lysine oxidase had covalently bound FAD.

Finally, Genzyme scientists described in a European patent (33) the presence of deglycating enzymes in the bacterial groups Klebsiella and Corynebacterium, the fungi Acremonium and Fusarium, and the yeast genus Debaryomyces grown in media containing glycated butylamine as sole nitrogen source. All enzymes obtained generated glucosone and H_2O. One enzyme isolated from Debaryomyces vanrijiae had a K_m of 80 µM for glycated butylamine, a pH optimum from 7.0 to 8.5, and highest stability between pH 5.0 and 7.5. Molecular weights of these enzymes are undetermined.

In spite of the failure to induce soil organisms to produce enzymes with activity against glycated proteins, the discovery of inducible amadoriase isoenzymes in our fungus, together with the reports above describing the presence of amadoriase enzymes in several genetically unrelated organisms such as prokaryotes, fungi, and yeast, raises the important question of the evolutionary significance of deglycating enzymes. Except for the Pseudomonas enzyme described earlier (17), whose isolation was recently described (34), all amadoriase enzymes that
regenerate the intact amine also generate the highly reactive molecules glucosone and H₂O₂. In view of the growing number of data which directly implicate H₂O₂, oxidative stress, and Maillard reaction intermediates in diabetes (35, 36), the question thus arises as to whether amadoriase enzymes occur in human tissues. The sequence homology between amadoriase isoenzymes and mammalian D-amino acid oxidase suggests that deglycating or related enzymes could occur in the human. To our knowledge there has been no systematic investigation of that question, except for a previous short report, the conclusions of which were questioned (37, 38). Cloning of amadoriase cDNA, which is in progress in our laboratory, will provide important information for future research in the possible occurrence of fructosyl amino acid oxidase in mammalian tissues.

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