Purification and Characterization of a Membrane-bound Deglycating Enzyme (1-Deoxyfructosyl Alkyl Amino Acid Oxidase, EC 1.5.3) from a Pseudomonas sp. Soil Strain*

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Searching for novel approaches for uncoupling glycation from hyperglycemia as a cause of diabetic complications, a Pseudomonas sp. soil strain containing a membrane-bound enzyme that deglycates amino acids under release of free fructosamine was isolated (Gerhardinger, C., Marion, S. M., Rovner, A., Glomb, M., and Monnier, V. M. (1995) J. Biol. Chem. 270, 218–224). This enzymatic activity was found to be very sensitive to inactivation by most detergents. From the plasma membrane (~3 mg/ml protein concentration), the enzyme could be solubilized in active form using 10 mM 3-[3-chloromidopropyl]-dimethylammonio]-2-hydroxy-1-propanesulfonate aided by 2 M NaCl and 10% glycerol (27% optimal solubilization yield). The supernatant from a 55% saturation (NH4)2SO4 cut was fractionated onto a phenyl-Superose column and enzymatic activity was eluted with a sharp peak of enzyme activity. Analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a major band at 106 kDa and, on isoelectrofocusing gel, a pI of 5.1. The protein was confirmed by affinity labeling with 14CN and N3, suggestive of copper as a likely cofactor. Identification of the protein was confirmed by affiliating with 14CN and isoelectrofocusing. The “amadoriase” activity was also inhibited by Hg2+, Ag+, Cu2+, and Zn2+ and had Km and Vmax values of 0.14 mM and 0.48 unit/ml (16 units/mg of protein), respectively, for (1-deoxyfructosyl) aminocaproate. Significant activity was noted to exceed with the spontaneous binding of sugar aldehydes with the free amino groups on protein or peptide to form a Schiff’s base, which rearranges to more stable Amadori product (4). The Amadori product can undergo a complex series of chemical rearrangements to form irreversible advanced glycation end products (AGEs) (4–6). A growing number of studies in recent years suggest that glycation and the formation of AGEs are involved in the pathology of diabetic complications. AGEs are found in several human tissues (7) and are increased in diabetic human serum (8). Studies by several groups provide more direct evidence demonstrating a relationship between levels of AGEs and development of diabetic complications (9). Formation of AGE moieties in tissues and on matrix components such as vessel wall and kidney is thought to contribute to diabetic pathologies such as vascularopathy and atheroma formation (10). More direct evidence is the demonstration that transgenic mice that overexpress the human equivalents of human AGEs develop a disease state similar to diabetes (11). Further, in a separate study, a salutary influence on development of diabetic nephropathy could be achieved by treatment with monoclonal antibodies against glycated albumin to db/db mice (12). Current investigations suggest interaction of specific receptors to AGEs and involvement of AGE-receptor dependent-sensitive pathways to promote cellular responses leading to diabetic complications (13). Therefore, based on these observations, it can be concluded that the formation of Amadori products is likely to be a central event in AGE formation and the subsequent pathogenesis of diabetic complications.

Thus, it is conceivable that inhibition of the glycation cascade at the primary level of Amadori product formation may prevent the subsequent deleterious events and thus prove to be an effective way to control the glycation-dependent complications and to fully understand the role of nonenzymatic glycation in diabetes and aging. In addition, this might also help to assess mechanistic links and biologic consequences arising from excess nonenzymatic glycation of proteins in diabetes from that of other mechanisms such as the aldose reductase pathway (15). In a novel approach, we searched for enzymes with Amadori product splitting activity, and in doing so, we earlier isolated a Pseudomonas sp. bacterial soil strain by selective culture technique that is able to utilize Amadori product as the sole carbon source (1). Since this strain was forced to grow on synthetic Amadori product, fructosyl aminocaproate, Amadori product-splitting enzymatic activity was induced, which surprisingly cleaved the Amadori product at the N-alkyl

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1 The abbreviations used are: AGE, advanced glycation end products; PAEG, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]- dimethylammonio]-2-hydroxy-1-propanesulfonate; FPLC, fast protein liquid chromatography; fructosyl, 1-deoxyfructosyl; ribosyl, 1-deoxyribosyl.
bond under consumption of O₂, thereby forming fructosamine and adipic acid. In addition, an Amadori-binding protein was isolated and purified using glycated Sepharose lysine as an affinity support (16).

Here, we describe the successful solubilization, purification, and characterization of this “amadoriase” enzyme that is able to decompose the Amadori product into metabolizable free fructosamine and adipic acid under consumption of O₂ from the plasma membrane of a Pseudomonas aeruginosa sp. from soil.

EXPERIMENTAL PROCEDURES

Materials

Most of the detergents were obtained from a kit provided by Boehringer Mannheim or Sigma. CHAPSO, lysolecithin, bovine serum albumin, poly-l-lysine, and isoelectrofocusing markers were from Sigma. K⁴CN was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Nutrient broth was obtained from Difco. Immobilon transfer membrane was from Millipore Corp. (Bedford, MA). Dowex AG50W-X4–400 was from Aldrich. SDS-PAGE reagents, molecular weight markers, and Bradford protein assay kit were from Bio-Rad. Isoelectrofocusing gels, chromatographic FPLC column (prepacked), and PD-10 columns were obtained from Pharmacia Biotech Inc. Silica gel-coated aluminum thin layer chromatography plates (0.2 mm thick) were obtained from Alttech (Deerfield, IL). Other chemicals were of analytical grade.

Isolation and Growth of Microorganism

The previously isolated Pseudomonas sp. soil strain (1) was cultured in complete medium (nutrient broth, 8 g/liter) and supplemented with 5 g/liter fructosyl aminocaproate to induce the enzyme activity in the culture medium (1). Typically, one batch consisting of 7–8 liters of culture medium was used, and several batches were grown simultaneously in bottom-notched plastic 4-liter flasks (Nalgene) at 37 °C aerobically for 24 h.

Solubilization and Purification of Amadoriase

Extraction and Solubilization of Amadoriase—Cells were harvested by centrifugation at 5000 × g for 15 min. The cell pellet was washed once with minimal salt (KH₂PO₄, 3 g/liter; K₂HPO₄, 7 g/liter; (NH₄)₂SO₄, 0.1 g/liter) and centrifuged at 5000 × g for 15 min. The cell pellet was kept frozen at −20 °C until used. Cells were thawed and suspended (1:2, w/v) in freshly prepared 50 mM sodium phosphate buffer (pH 7.4) combining 1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride. The resulting thick cell suspension was lysed in the cold using a French press at 6000–7000 p.s.i. two or three times. Cell lysis was efficient when the cell concentration of 7000 × g for 20 min was used to remove unbroken cells and the viscous material formed during the lysis process. This crude extract was subjected to 165,000 × g force for 1.5 h using a 70 Ti fixed angle rotor and an Ultracentrifuge (Beckman, model L-4060M). The pellet containing the plasma membrane (designated P₂M fraction) was immediately processed for further solubilization. A temperature of 4 °C was maintained throughout purification to prevent the loss of enzyme activity.

The P₂M fraction was washed with 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM NaCl for 30 min with occasional stirring and centrifuged at 150,000 × g for 1 h. A firm brown membrane pellet was obtained. This pellet was solubilized with freshly prepared solubilization buffer using an autopette (5-ml variable volume) with back and forth motion of the solution. Solubilization buffer contained 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 2 mM NaCl, and 10 mM CHAPSO. Care was taken to avoid frothing. Solubilization was also achieved using the same solubilization buffer except lysolecithin instead of CHAPSO was used at 0.5 mg/ml of protein. In both cases, solubilization was started by adding 25 μl of 20% Amadori product solution. Oxygen consumption was recorded and converted to μmol of O₂ consumed per min (18). One unit of enzyme activity was defined as the amount of enzyme that caused the consumption of 1 μmol of O₂/min under the above assay condition. To confirm that the activity measured by O₂ electrode relates to product formation, we incubated the enzyme extract for 24 h with two inhibitors (NaCN and HgCl₂) that almost resulted in complete inhibition of the product fructosamine formation (data not shown).

Ammonium Sulfate Precipitation—To the solubilized enzyme, (NH₄)₂SO₄ was slowly added with constant slow stirring to give 50% saturation. The solution became cloudy and was centrifuged at 10,000 × g for 10 min to remove the precipitated proteins. The supernatant that still contained enzyme activity was brought to 45% (NH₄)₂SO₄ saturation, and the solution was again cleared from the precipitated proteins. To the 45% (NH₄)₂SO₄ saturated solution, (NH₄)₂SO₄ was further added to reach 55% saturation. The solution was kept for another 15 min to allow complete precipitation of proteins followed by centrifugation at 10,000 × g for 15 min to obtain a clear supernatant.

Phenyl-Superose Chromatography—The clear supernatant from the last step (~8 ml) was loaded onto a phenyl-Superose HR 5/5 column using a superloop, and chromatography was performed on FPLC (Pharmacia). The column was pre-equilibrated with 6–7 ml of 50 mM sodium phosphate buffer, pH 7.4, 10% glycerol, 2 mM (NH₄)₂SO₄, and 3 mM of CHAPSO (Buffer A). After loading the sample, the column was washed with 3 ml of Buffer A, and elution was performed with a decreasing gradient from 2 to 0 mM (NH₄)₂SO₄ in the same buffer conditions. Fractions of 1 ml were collected. Protein concentration and enzyme activity were measured in all fractions, and fractions containing the enzyme activity were pooled. Enzyme activity-containing fractions (~6 ml from two phenyl-Superose HR 5/5 columns (used immediately one after the other), were collected and used for next step.

Removal of (NH₄)₂SO₄ Using PD-10 Column—PD-10 columns (Sephadex G-25 pre-packed columns) were pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.2, 10% glycerol, 0.15 mM NaCl, and 3 mM CHAPSO (buffer composition for the next step). 2–3 ml of the pooled enzyme-containing fractions was passed to a PD-10 column, and the eluate was collected in 3.5 ml of the same buffer.

Mono Q Chromatography—About 3–4 ml of the last enzyme-containing fraction was loaded using a superloop onto a Mono Q HR 5/5 column (prepacked from Pharmacia) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.2, 10% glycerol, 3 mM CHAPSO (buffer composition of the previous step). 2–3 ml of the pooled enzyme-containing fractions was passed to a PD-10 column, and the eluate was collected in 3.5 ml of the same buffer.

Synthesis of Fructosyl Aminocaproate and Other Glycated Substrates

Fructosyl aminocaproate was prepared by the modified method of Finot and Mauron (17) and as described previously (16), which yielded a pure Amadori product as confirmed by thin layer chromatography and proton NMR (16).

Other glycated substrates were prepared as described earlier (1, 15, 16). Fructosyl glycine was a generous gift from Dr. Tatsuo Horiuchi (Kikkoman Corp., Noda, Japan). Glycated bovine serum albumin, ribated bovine serum albumin, and glycated poly-l-lysine (30–70,000 daltons) were prepared by incubating at 37 °C for 30 days as described previously (1, 16).

Assay of the Amadoriase Activity

Amadoriase enzyme activity was assayed polarographically with a Clark type O₂ electrode by determination of O₂ consumption during the decomposition of its substrate. The standard assay mixture, which comprised a total volume of 1.5 ml with 0.1 mM sodium phosphate buffer, pH 7.4, and 50–200 μl of the enzyme solution, was continuously stirred within the electrode chamber at 25 °C. After equilibration, the reaction was started by adding 25 μl of 20% Amadori product solution. Oxygen consumption was recorded and converted to μmol of O₂ consumed per min (18). One unit of enzyme activity was defined as the amount of enzyme that caused the consumption of 1 μmol of O₂/min under the above assay condition. To confirm that the activity measured by O₂ electrode relates to product formation, we incubated the enzyme extract for 24 h with two inhibitors (NaCN and HgCl₂) that almost resulted in complete inhibition of the product fructosamine formation (data not shown).

Determination of Optimum Reaction Conditions and Substrate Specificity

Amadoriase activity was determined at different pH and temperatures. For optimal pH, enzyme activity was determined in a variety of buffers (sodium phosphate, sodium citrate, glycine-NaOH) that gave a pH range of 5–10.5. The effect of temperature was evaluated by keeping
the reaction mixture at different temperatures for 15 min followed by immediate chilling on ice to stop the reaction.

### Binding Study with K\(^{14}\)CN and Determination of Molecular Weight

To the purified enzyme (250 \(\mu\)l), 20 \(\mu\)l of K\(^{14}\)CN was added (5 \(\mu\)Ci). The mixture was incubated for 30 min at 25°C with constant shaking, and the radioactivity was analyzed in 5 \(\mu\)l of the mixture. After the incubation, the unbound K\(^{14}\)CN was separated by passing the mixture to Sephadex G-25 (preequilibrated with 1 mM CHAPSO) in a syringe under centrifugal force. The resultant solution collected from the bottom of the syringe was concentrated on Savant Speedvac model AS160.

This sample containing enzyme bound with \(^{14}\)C-labeled cyanide was subjected to isoelectrofocusing under standard conditions as described below. The isoelectrofocusing gel was then cut in 2-mm thin slices and disrupted in a minimal amount of SDS-PAGE sample buffer and the whole content was transferred in a scintillation vial for measurement of the radioactivity with a Beckman liquid scintillation counter model 8000. In a separate experiment, the pooled protein bands corresponding to the radioactivity containing band were electroeluted using a Bio-Rad electroleuter model 422 in 25 mM Tris and 0.1% SDS at a fixed constant current of 10 mA for 5–6 h. The electroeluted protein in the solution (500–600 \(\mu\)l) was dialyzed against 5 mM Tris and 0.01% SDS for 24 h and concentrated using a Savant Speedvac. The sample was then analyzed on SDS-PAGE.

### SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on a 7.5% precast gel from Bio-Rad following the method of Laemmli (19). The polyacrylamide gels were dried on a Bio-Rad gel drier model 583 for 1.5 h at 80 °C under vacuum. The molecular weight was determined by extrapolating from the semilogarithmic plot of the relative mobility of standard proteins run simultaneously.

### Isoelectrofocusing Experiment

Isoelectrofocusing of the purified enzyme was performed on the precast isoelectric focusing gels (pH 3–9). Sample preparation and isoelectrofocusing were carried out following the instructions provided by Pharmacia Biotech Inc. and using the Phast System (Pharmacia).

### Protein Measurement

The amount of protein was assayed with a kit provided by Bio-Rad. The assay was based on the method of Bradford (20), and bovine serum albumin was used as a standard.

### Amino-terminal Sequence Analysis

The purified protein was subjected to SDS-PAGE and electroblotted on Immobilon membrane (Millipore) using 0.025 M Tris, 0.192 M glycine, and 10% methanol. The membrane was washed thoroughly with water and stained with Coomassie Blue, and the blot was air-dried. The 106-kDa protein band of the enzyme was cut out. Sequence analysis was performed at the Molecular Biology Core Laboratory (Department of Biochemistry, Case Western Reserve University) with an Applied Biosystems 477A gas phase sequencer equipped with an on-line analyzer for phenylthiohydantoin-derivatized amino acids. The NH\(_2\) terminal segment was analyzed with the Swiss-Prot and DNAStar programs to search for its homology with known protein sequences.

### RESULTS

The amadoriase enzyme was bound to the plasma membrane of the bacterium and found to be extremely susceptible to inactivation by many detergents. Generally, detergent concentrations below the critical micellar concentration could not cause sufficient protein extraction. In contrast, higher concentrations caused solubilization but loss of enzyme activity.

**Solubilization of Amadoriase**—Two detergents, lyssolecithin and CHAPSO, were found useful. A systematic approach was utilized to define the optimal conditions for solubilization with minimal loss of activity, and Table I describes conditions required for optimal solubilization. In essence, optimal solubilization could only be achieved in the presence of 2 mM NaCl and 10% glycerol. High salt promoted more protein extraction, and glycerol had stabilizing activity. The effect of NaCl on the efficiency of amadoriase enzyme solubilization is concentration-dependent, and increased solubilized membrane proteins as a function of NaCl concentration also yielded more enzyme activity, indicating an active solubilized form of the enzyme (data not shown). Maximal solubilization was reached at a concentration of 2.0 mM NaCl, an effect that was independent of detergent type i.e. lyssolecithin or CHAPSO. The detergent:protein ratio significantly affected the solubilization of amadoriase, and the highest yield of active amadoriase, 48 and 27%, was obtained at a ratio of 0.5 and 2.0 mg lyssolecithin and CHAPSO, respectively, per mg of protein (Table I). Although lyssolecithin caused 48% yield (at a relatively lower detergent:protein ratio), it was abandoned because solubilized proteins were found heavily aggregated when analyzed on gel filtration (molecular mass between 5 and 5000 kDa). Attempts to use guanidine hydrochloride, urea, organic solvents, or sonication to disaggregate the proteins were all in vain (data not shown). In contrast, optimal solubilization with 10 mM CHAPSO was satisfactory, and all subsequent procedures for solubilization of amadoriase enzyme involved 10 mM CHAPSO in the above mentioned solubilization buffer.

**Purification of Amadoriase**—The results of the amadoriase purification procedure are summarized in Table II. The data in Table II and Figs. 1 and 2 were taken from one complete amadoriase purification sequence. This purification procedure was found to be quite reproducible.

The first major step in the purification scheme is the (NH\(_4\))\(_2\)SO\(_4\) saturation of the solubilized enzyme. The addition of (NH\(_4\))\(_2\)SO\(_4\) up to 55% saturation promoted stepwise precipitation of ~71% of membrane protein. However, at 55% (NH\(_4\))\(_2\)SO\(_4\) saturation (2 m final concentration), amadoriase activity did not precipitate but remained in solution. This procedure resulted in an increase of 8-fold purification from the crude extract due to increase in the specific activity (Table II).

Hydrophobic interaction FPLC was performed in 3 mM CHAPSO and 10% glycerol. Under these conditions, the majority of amadoriase activity emerges from the column as a sharp peak with the last protein-containing fractions, suggesting its extreme hydrophobic nature (Fig. 1). This step caused a further rise in the specific activity to 2.11 units/mg of protein (16.2-fold purification from crude extract). Anion exchange FPLC on a Mono Q column was performed after removal of (NH\(_4\))\(_2\)SO\(_4\) by Sephadex G-25 packed PD-10 columns and replacement with 150 mM of NaCl. A 3 mM CHAPSO and 10% glycerol concen-
tration was kept to prevent loss of activity or membrane protein precipitation. The amadoriase activity appeared as a sharp peak between 450 and 550 mM of NaCl (Fig. 2A). The chromatography was performed at a low pH (6.2), which caused most protein to elute through the column and to separate the peak from small adjoining peaks. This resulted in a purified amadoriase with a molecular mass of 106 kDa on SDS-PAGE (Fig. 2B). Purified amadoriase had an isoelectric point at pH 5.1 when analyzed by isoelectrofocusing (Fig. 3A). The purified enzyme was checked for its reaction products on thin layer chromatography by incubating with fructosylaminocaproate as previously performed with crude enzyme extract (1), and a spot corresponding to free fructosamine was detected by ninhydrin and triphenyltetrazolium chloride (data not shown).

Labeling with [14C]KCN—Because small contaminants were noticed in the SDS-PAGE gel, an affinity labeling experiment was performed with KCN, which is a potent enzyme inhibitor. The enzyme was reacted with radiolabeled [14C]-cyanide, and protein-bound radioactivity was separated from free K[14CN] by Sephadex G-25. In similar experiments, radiolabeled cyanide has been ligated to Cu-containing amine oxidase (21), and cyanide has been shown to bind directly to copper (22–24). Nonspecific binding was checked with bovine serum albumin and found to be only ~9% of the specific binding to amadoriase (data not shown). These data are suggestive for the presence of copper in the enzyme. When analyzed by isoelectrofocusing gel, the concentrated [14CN]-ligated amadoriase radioactivity was found at the same place of the thick band at pH 5.1 (Fig. 3A). Furthermore, the electroeluted protein from bands corresponding to pH 5.1 showed a single band at 106 kDa on SDS-PAGE as can be seen in Fig. 3B. These results thus confirm a monomeric molecular mass of amadoriase of 106 kDa.

Optimal Reaction Conditions and Effect of Metals and Other Compounds—As can be seen in Fig. 4A, the amadoriase showed maximum activity at pH 7.0 in sodium phosphate buffer and pH 6.0 in sodium citrate buffer. Activity below or above the pH range of 5.5–8.0 caused significant lowering in the enzyme activity (Fig. 4B). Some residual activity remained at pH 9, and above that it could represent enzyme partially resistant to denaturation or possibly base-catalyzed degradation of Amadori product in glucosone. The amadoriase activity was found unstable when kept at room temperature or above (Fig. 4B). At lower temperatures, i.e., 4°C or ~20°C, the enzyme activity was stable only for a few days. Several compounds and metal ions were added to the standard enzyme assay mixture prior to the addition of fructosylaminocaproate to investigate their effect on amadoriase activity. While not significantly altered in

### Table II

| Purification of the Amadoriase enzyme from the plasma membrane of the Pseudomonas soil strain |
|---------------|---------------|---------------|---------------|
|               | Activity (units/ml) | Protein (mg/ml) | Specific Activity (units/mg) | Purification (fold) |
| Crude extract | 1.70           | 13.0           | 0.13          |                  |
| Membrane fraction | 1.54          | 3.0            | 0.51          | 3.9              |
| Solubilized fraction | 0.46          | 1.2            | 0.38          | 3.0              |
| Phenyl-Superose HR 5/5 | 0.42          | 0.20           | 2.11          | 16.2             |
| Mono Q HR 5/5 | 0.40           | 0.03           | 15.38         | 118.3            |

**Fig. 1.** Elution profile of amadoriase enzyme from phenyl-Superose FPLC. Amadoriase purified as described under “Experimental Procedures” was loaded onto a FPLC phenyl-Superose HR 5/5 column equilibrated in 50 mM Na/PO₄ buffer (pH 7.4), 10% glycerol, 3 mM CHAPSO, and 2 mM (NH₄)₂SO₄. The column was first washed with equilibration buffer, and elution was performed with a decreasing linear gradient of (NH₄)₂SO₄ from 2 to 0 mM in the same buffer. Fractions (1 ml) were collected and analyzed for protein concentration (●) and enzyme activity (E).

**Fig. 2.** A, elution profile of amadoriase enzyme from Mono Q FPLC. Purified amadoriase as described under “Experimental Procedures” was loaded onto a prepacked FPLC Mono Q HR 5/5 column equilibrated in 50 mM Na/PO₄ buffer (pH 6.2), 10% glycerol, 150 mM NaCl, and 3 mM CHAPSO. An increasing linear gradient of NaCl from 0.15 to 1 M was applied in the same buffer. Fractions (0.6 ml) were collected and analyzed for protein concentration (●) and enzyme activity (E). B, SDS-PAGE analysis of the purified amadoriase from Pseudomonas sp. plasma membrane. Lane 1, molecular mass standards: myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa). Lane 2, purified amadoriase enzyme from Pseudomonas sp. plasma membrane (see “Experimental Procedures”).
A number of potent enzyme inhibitors were found to be cyanide, azide, and phenylhydrazine (inhibitors of the carbonyl group in quinone cofactor present in copper-amine oxidases (22)) did not cause any effect on the amadoriase activity (Table III).

**Table III**

| Addition          | Activity % |
|-------------------|------------|
| None              | 100        |
| MnCl₂             | 118        |
| AgNO₃             | 60         |
| HgCl₂             | 0          |
| MgCl₂             | 107        |
| CaCl₂             | 83         |
| ZnSO₄             | 67         |
| CuSO₄             | 60         |
| Na₂N₃            | 0          |
| NaCN             | 100        |
| EDTA             | 93         |
| Dithiothreitol    | 87         |
| Phenylhydrazine   | 107        |
| Semicarbazide     | 100        |
| Aminoguanidine    | 93         |

Diethylenetriamine pentaacetic acid was used at 8 mM final concentration in the assay mixture.

reducing agent. Compounds such as aminoguanidine as well as phenylhydrazine and semicarbazide (inhibitors of the carbonyl group in quinone cofactor present in copper-amine oxidases (22)) did not cause any effect on the amadoriase activity (Table III).

**DISCUSSION**

The amadoriase enzyme described above is unusual in several aspects. First, it cleaves the Amadori product at a very unexpected site, i.e. the alkylamine instead of the ketoamine bond (1) (Scheme 1; brackets signify “not isolated”). Second, it can cleave the borohydride-reduced Amadori product (Table IV), suggesting that the pyranose or furanose configuration of the sugar is not necessary for binding of the Amadori product. Third, its activity is not limited to fructosyl amino acids as evidenced by its ability to degrade ribosylated substrates (Table IV). In the case of aminocaproic acid as a substrate, the oxidized product was adipic acid (1). Thus, this enzyme differs considerably from those isolated from soil using a similar approach, whereby the Amadori product is cleaved oxidatively under release of the intact amine and formation of glucosone.
and H₂O₂ (30–32).

In further contrast to the amadoriases described so far, the current enzyme was found to be an integral membrane protein that could be solubilized and purified in active form only with CHAPSO, whereby solubilization was greatly aided by 2 M NaCl. Purification using chromatographic techniques such as metal-chelating resins, chromatofocusing, and gel filtration were not useful, but satisfactory results were obtained with a combination of hydrophobic and ion exchange chromatography.

A likely cofactor appears to be copper. Similar to most copper-dependent enzymes (such as cytochrome oxidase, amine oxidase/diamine oxidase, ascorbate oxidase, urate oxidase, galactose oxidase, phenolase, laccase, and dopamine B hydroxylase (21–24, 28)), this amadoriase is involved in an oxidoreduction type of reaction and is inhibited instantly by CN⁻ and N₃⁻. Cyanide binds directly to the copper (21–24), and this enzyme also shows binding to ¹⁴C-labeled cyanide (Fig. 3), suggesting the presence of a copper atom at its active site. Previous studies indicate copper-dependent enzymes to be less well characterized, and the role of copper in relation to the mechanism of catalysis remains unclear for many enzymes (23, 25, 26). However, some characteristics of copper-dependent amine oxidases (i.e. molecular mass of 90 kDa and isoelectric point between 4.5 and 6.5 (23)) showed similarity to amadoriase. We estimated, based on cyanide labeling, a stoichiometric ratio of 16 cyanide molecules binding per enzyme molecule, which may reflect an equal or smaller number of copper atoms/enzyme molecule, depending upon the symmetry or free coordination sites of copper in the enzyme. However, the molar ratio of cyanide molecules to the enzyme may be less, since the separation of bound ¹⁴CN using Sephadex G-25 may not be quantitative. The inability of chelating agents to inactivate the enzyme (Table III) suggests that copper is present in a tightly bound (27).

The precise mechanism of action of amadoriase is unknown. It could be suggested based on the previous studies that Cu²⁺ may be reduced by the substrate (28) and reoxidized by air in the catalytic cycle, where copper is important in binding O₂ (21, 24). However, there is still a lack of direct evidence that copper is the O₂ binding site or is directly involved in the electron transfer. On the other hand, the lack of inhibition of amadoriase activity by either semicarbazide or phenylhydrazine suggests the absence of a quinone type of organic cofactor, such as 2,4,5-trihydroxyphenylalanine quinone, which was recently discovered in copper-amine oxidases (29). It cannot be ruled out that the lack of inhibition of these compounds could be linked to their short exposure to the enzyme or to a protective effect of detergent.

Curiously, in contrast to many oxidases, our enzyme was not found to generate H₂O₂. In that regard it resembles the group of copper proteins (i.e. ascorbate oxidase, laccase, or catechol oxidase). Based on its properties, the enzyme is currently best classified as 1-deoxyfructosyl alkyl amino acid oxidase (EC 1.4.3.5), but this classification may have to be revised when more details on the mechanism of action of the enzyme become available.

The purified amadoriase has a molecular mass of 106 kDa and a pI of 5.1. However, it is possible that the enzyme may be in more than monomeric form in the native condition. SDS-PAGE under nonreducing conditions failed to reveal any distinct band (data not shown), possibly because of protein aggregation and formation of disulfide bridges when heated at 90°C in the absence of dithiothreitol during sample preparation.

The low Kₘ and high Vₘₐₓ is suggestive of a relatively high affinity of this amadoriase enzyme toward fructosyl aminocaproate (Fig. 5). The Kₘ of purified amadoriase is found to be lower than when it was embedded in the membrane (i.e. 0.21 mM (1)), which is to be expected, since the purified enzyme is free from steric hindrances. As discussed above, the enzyme showed broad substrate specificity with many glycated amino acids but was unable to oxidize alkylamine Amadori products or simple amine (Table IV). The data so far presented here strongly point out the necessity of a carboxyl group for proper enzyme activity. In addition, it had no activity against glycated proteins, suggesting steric hindrance or the necessity of the enzyme to “fold” over the substrate.

### Substrate specificity of the purified Amadoriase enzyme

| Substrate               | Activity (%) |
|-------------------------|--------------|
| -Fructosyl aminocaproate| 100          |
| -Fructosyl lysine       | 158          |
| -Fructosyl glycine      | 83           |
| Ribosyl &-aminoacaproic acid | 67  |
| Ribosyl bovine serum albumin | 0  |
| Fructosyl propylamine   | 0            |
| Fructosyl N-α-acetyl lysine | 67  |
| Glycated poly-L-lysine  | 17           |
| Sodium borohydride-reduced -fructosyl aminocaproate | 67 |
| e-Ribose α-t-Boc lysine | 83           |
| e-fructosyl α-t-Boc lysine | 108  |
| e-Aminocaproic acid     | 0            |
| α-Glucose               | 0            |
| α-Ribose                | 0            |
| L-Lysine                | 0            |
| Glycine                 | 0            |
The inability of this and other amadoriase enzymes (30–32) to deglycate proteins raises the question of whether it is altogether possible to find enzymes with the desired activity for the potential utilization in mammalian models of diabetic complications. At the same time, however, it raises the question of whether deglycating enzymes with activity against glycated low molecular weight substrates have been conserved throughout evolution. Such enzyme would regenerate the free amino acid, which would then be available for protein synthesis. In hyperglycemia, however, such enzymes might contribute toward the development of diabetic complications, especially if they produce glucose and H$_2$O$_2$ in presence of elevated levels of glycated amino acids. Although it is not known whether free fructosamine would also degrade into NH$_3$ and form H$_2$O$_2$ and glucose, the absence of such a pathway could make this enzyme useful for in vivo testing of the contribution of glycated amino acids toward cellular dysfunction in transgenic models of diabetes.

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