Molecular Cloning and Expression of Amadoriase Isoenzyme (Fructosyl Amine:Oxygen Oxidoreductase, EC 1.5.3) from Aspergillus fumigatus*

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Motoko Takahashi, Monika Pischetsrieder‡, and Vincent M. Monnier§

From the Institute of Pathology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Amadoriase is an enzyme catalyzing the oxidative deglycation of Amadori products to yield corresponding amino acids, glucosone, and $H_2O_2$. We previously reported the purification and characterization of two amadoriase isozymes from Aspergillus sp. that degrade both glycated low molecular weight amines and amino acids (Takahashi, M., Pischetsrieder, M., and Monnier, V. M. (1997) J. Biol. Chem. 272, 3437–3443). To identify the primary structure of the enzymes, we have prepared a cDNA library from Aspergillus fumigatus induced with fructosyl propylamine and isolated a clone using polyclonal anti-amadoriase II antibody. The primary structure deduced from the nucleotide sequence comprises 438 amino acid residues with a predicted molecular mass of 48,798 Da. The deduced primary structure exhibits the presence of an ADP-binding motif near the NH$_2$ terminus. The identity of the amadoriase II cDNA was further confirmed by expression in Escherichia coli cells with an inducible expression system. Northern blotting analysis revealed that amadoriase II was induced by fructosyl propylamine in a dose-dependent manner.

Nonenzymatic glycation of proteins has been implicated in the development of diabetic complications and the aging process (1). This reaction leads to dysfunction of target molecules through formation of sugar adducts and cross-links. Considerable interest has focused on the Amadori product that is the most important "early" modification during nonenzymatic glycation in vitro and in vivo. Amadori products are precursors of protein cross-links, fluorescent and UV-active compounds, and glycoxidation products such as pentosidine and N$^\epsilon$-(carboxymethyl)lysine (2–4). Furthermore, Amadori products are a potent source of reactive oxygen species (5, 6) and can be taken up by specific receptors in macrophages and mesangial and aortic cells (7–9).

To elucidate the effects of nonenzymatic glycation, we (and other investigators (14–19)) have searched for novel ways to deglycate proteins enzymatically (10–13). In a previous paper, we reported the presence of amadoriase isozymes in Aspergillus sp. that could degrade both glycated low molecular weight amines and amino acids (13). Most deglycating enzymes isolated so far (13–15, 18, 19), except for a Pseudomonas enzyme (11, 12), have several features in common, such as 1) molecular mass of approximately 50 kDa, 2) FAD as a cofactor, and 3) the release of glucosone and $H_2O_2$ during the catalytic deglycation of glycated amino acids. Since these products are potentially highly damaging molecules, we hypothesized that implication of such enzymes in diabetes might contribute to the development of complications (13).

In this paper, we describe the cloning, sequencing, and expression of cDNA of amadoriase II from a cDNA library of Aspergillus fumigatus from soil (13).

EXPERIMENTAL PROCEDURES

Materials—Fructosyl propylamine was prepared as described previously (13). All other materials were analytical grade.

Strains and Growth Conditions—Escherichia coli strains XL-1 Blue MRF$^+$ and SOLR (Stratagene) were used. A. fumigatus was isolated from soil and cultivated as described previously (13). Batch cultures were grown in nutrient broth containing 1 mg/ml fructosyl propylamine, and incubation was carried out at 37 °C for 3 days aerobically.

Preparation of Antiserum—After anesthetizing, a New Zealand White rabbit was immunized by injection into popliteal lymph nodes of 50 µg of purified amadoriase II protein (13) emulsified in complete Freund’s adjuvant (Sigma). Three weeks later, booster injections were performed subcutaneously with 100 µg of enzyme emulsified in incomplete Freund’s adjuvant. Antiserum was obtained 10 days after the booster injection. Nonspecific antibody was reduced by incubating with XL1-Blue MRF$^+$ lysate before use.

Construction of the Aspergillus sp. cDNA Library—Total RNA was extracted from A. fumigatus (1 g) according to Chomczynski and Sacchi (20). Poly(A)$^+$ RNA was obtained from total RNA using an oligo(dT)-cellulose (Pharmacia Biotech Inc.) column. Five µg of poly(A)$^+$ RNA were converted to a double-stranded cDNA and ligated to the Uni-ZAP XR vector using a ZAP cDNA synthesis kit (Stratagene) according to the manufacturer’s instruction. The resulting DNA was packaged using Gigapack Gold III (Stratagene) and amplified in E. coli XL1-Blue MRF$^+$. A library containing 4.0 × 10$^6$ clones was made, of which approximately 99% were recombinants.

Cloning of Amadoriase II cDNA—Screening of the cDNA library was carried out as follows. After transferring plaques onto nitrocellulose filters (Micron Separations Inc.), plaques presenting amadoriase II epitopes by isopropyl-$bt$-thiogalactopyranoside induction were detected by anti-amadoriase II antiserum and goat anti-rabbit IgG alkaline phosphatase conjugate. Positive clones were converted to phagemids carrying cDNA inserts between EcoRI and XhoI sites of pBlueScript SK$^-$ by helper phage superinfection as described in the Uni-ZAP XR manufacturer’s manual (Stratagene). Nucleotide sequences of both strands were determined by the dyeoxy chain termination method (21) using Sequenase version 2.0 (U. S. Biochemical Corp.) and [35S]dATP$^+$ (Amersham Life Science, Inc.) and by ALF DNA Sequence with Auto Cycle$^+$ sequencing kit (Pharmacia).

Expression of Amadoriase II cDNA in E. coli Cells—The plasmid carrying amadoriase II cDNA was transformed into E. coli XL1-Blue MRF$^+$. The transformant was precultured in 10 ml of LB medium

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U82830.

‡ To whom correspondence should be addressed. Tel.: 216-368-6613; Fax: 216-368-0495; E-mail: vmm3@po.cwru.edu.

1 The abbreviations used are: dATP$^+$, deoxyadenosine 5’-O-(1-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.

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on the membrane were exposed to Kodak XAR films at 2 days with an intensifying screen.

Enzyme activity was monitored by the release of glucosone as measured by a colorimetric reaction with a galactosidase fusion protein. Cells were disrupted by sonication, and the supernatant was subjected to enzyme assays.

**Assays for Enzymatic Activity**—The enzyme activity was monitored by the release of glucosone as measured by a colorimetric reaction with o-phenylenediamine as described previously (13). Briefly, the enzyme solution (10 mM Tris-HCl, 10 mM NaCl, 50 mM NaF, 20 mM sodium phosphate, pH 7.4, 10 mM o-phenylenediamine, 10 mM fructose pyrophosphate in a final volume of 1 ml). After incubation at 37 °C for 2 h, the absorbance at 320 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produces 1 absorbance unit at 320 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produces 1 absorbance unit at 320 nm.

Protein Determination—Protein concentration was determined by the method of Bradford (22) with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Protein analysis by SDS-PAGE was carried out according to Laemmli (23). The library constructed in Uni-ZAP XR was screened with primer32P-labeled amadoriase II cDNA as a probe. The blots were transferred to nitrocellulose (Bio-Rad), and probed using anti-amadoriase II serum and goat anti-rabbit IgG alkaline phosphatase conjugate. The arrows indicate the locations of molecular mass markers. A. fumigatus amadoriase II cDNA was considered to have a new family of fructosyl amine:oxydoreductase.

**Immunoblotting**—Crude extracts were subjected to 10% SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and probed using anti-amadoriase II serum as described under "Experimental Procedures." The arrows indicate the locations of molecular mass markers. A. fumigatus amadoriase II cDNA was considered to have a new family of fructosyl amine:oxydoreductase.

**RESULTS AND DISCUSSION**

The library constructed in Uni-ZAP XR was screened with antisera against amadoriase II of A. fumigatus. By screening 5 × 10⁵ plaques, we obtained six positive clones. Sequence analysis of one of these clones is shown in Fig. 1. The amadoriase II coding sequence was considered to have a 1314-base pair coding for a novel protein of 438 amino acids (nucleotide positions 174–1487). The open reading frame is preceded by a 173-base pair 5′-noncoding region and followed by a 20-base pair 3′-noncoding region and a poly(A) tail. We assumed the ATG codon at positions 174–177 as the translational initiation codon, for the first methionine (13). The molecular mass calculated and corrected for loss of one methionine was 48,798 daltons, which is in agreement with values previously estimated by SDS-PAGE (13). Potential ribosome-binding sites (AGGA) were located 8 bases before the start codon. We assumed the 21 deduced amino acid residues were consistent with the NH₂-terminal sequence obtained from the purified protein except for the first methionine (13). The molecular mass calculated and corrected for loss of one methionine was 48,798 daltons, which is in agreement with values previously estimated by SDS-PAGE (13). Potential ribosome-binding sites (AGGA) were located 8 bases before the start codon. Close inspection revealed that amino acids present at positions 8–37 satisfy all of the 11 consensus sequence requirements for an ADP-binding domain (25). Conserved features include three glycine residues (Glu13, Glu15, and Glu18), which allow for a sharp turn between the first β-strand and the α-helix, a core of six hydrophobic amino acids (Leu2, Leu4, Ala5, Leu8, Val13, and Val18), and the presence of an acidic residue (Asp17) that forms a hydrogen bond with the 2′-OH group of the FAD adenine ribose. These are capable of forming a structural motif that interacts with the bottom of the ADP moiety of the FAD cofactor (25).

A search for protein homology using BLAST (26) revealed that amadoriase II has no significant similarities with mammalian enzymes reported so far, implying that amadoriase II is an enzyme representing a new family of fructosyl amine:oxydoreductase.
Deglycating Isoenzymes from Aspergillus fumigatus

Northern blot analysis of total RNA from A. fumigatus. Northern blot analysis was carried out with total RNA prepared from A. fumigatus cultured in the presence of various concentrations of fructosyl propylamine for 3 days. Each lane contains 10 μg of total RNA. The concentrations of fructosyl propylamine are indicated under the lanes. Ethidium bromide staining showed comparable amounts of RNA in each lane. The arrows indicate the positions of the 18 and 28 S ribosomal RNAs.

Using amadoriase II cDNA as a hybridization probe, Northern blotting analysis was performed for an A. fumigatus that was cultured in the presence of various concentrations of fructosyl propylamine. As shown in Fig. 3, amadoriase II was induced in a dose-dependent manner. This result suggests the existence of a mechanism for up-regulation of amadoriase II by an Amadori compound. To our knowledge, no other protein has been reported that is inducible by an Amadori compound. However, it is unclear yet whether the Amadori product binds to an intracellular receptor or whether it induces gene expression through indirect pathways, such as oxidative stress.

In summary, we have described the primary structure of amadoriase II from A. fumigatus, which was selected by using fructosyl adamantranine and cultured in the presence of fructosyl propylamine. Although the BLAST search detected no significant homology of amadoriase II with any human protein in the data base, the possible occurrence of amadoriase homologues in human tissue could have considerable implications for the development of diabetic complications. While growing evidence implicates Amadori products in the formation of H₂O₂, the mechanism and source of H₂O₂ formation in vivo in relationship to the Maillard reaction remains to be solved. Although metal-catalyzed oxidation of Amadori products can generate glucosone and H₂O₂ in vivo (6, 27, 28), the existence of an enzyme such as amadoriase suggests a possible role of flavin as an electron acceptor in oxidation of Amadori products.

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Note Added in Proof—While this publication was in preparation, Yoshida et al. (20) published two homologous sequences. Sequence comparison revealed that amadoriase II from A. fumigatus exhibits 82% identity and 92% similarity with fructosyl amino acid oxidase from Aspergillus terreus (accession number Y09020) and 36% identity and 65% similarity with the same enzyme from Penicillium janthinellum (Y09021), respectively, when calculated without gaps.

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