Alteration of Substrate Selectivity through Mutation of Two Arginine Residues in the Binding Site of Amadoriase II from *Aspergillus* sp.†

Xinle Wu,‡ Shu G. Chen,‡ J. Mark Petrash,§ and Vincent M. Monnier*,‡

Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, and Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Received January 11, 2002; Revised Manuscript Received February 1, 2002

ABSTRACT: Amadoriases I and II are deglycation isoenzymes from *Aspergillus* sp. of potential relevance for treatment of diabetic complications resulting from excessive protein glycation. Amadoriase II has a preference for anionic substrate with a K_m of 0.23 and 2.53 mM for fructosylglycine and fructosylpropylamine, respectively. In contrast, the corresponding K_m values for amadoriase I are 9.75 and 0.023 mM, respectively. Chemical modification of amadoriase II with *p*-hydroxyphenylglyoxal, a specific arginine-modifying reagent, resulted in an inhibition of enzyme activity toward fructosylglycine, while having less effect on the enzymatic activity toward fructosylpropylamine. Peptide mapping and subsequent mass spectrometry analysis suggest that Arg^{112} is one of the sites of *p*-hydroxyphenylglyoxal modification. Sequence alignment between amadoriase I and amadoriase II revealed that two glutamic acids in amadoriase I align to Arg^{112} and Arg^{114} in amadoriase II. Site-directed mutation of amadoriase II (R112E, R114E) resulted in reversal of the enzymatic activities toward fructosylglycine and fructosylpropylamine. Our results suggested that Arg^{112} and Arg^{114} are responsible for the high affinity of amadoriase II toward anionic substrates and determine the substrate selectivity of the enzyme.

Amadoriase[1] catalyzes the oxidation of Amadori products by oxygen to yield glucosone, amine, and hydrogen peroxide (*1*). Amadori products are formed at an early stage of nonenzymatic glycation (*2*, *3*). Reducing sugars such as glucose attach to the amino groups of proteins by forming a Schiff's base that subsequently undergoes rearrangement to form the stable Amadori product (*4*). Glycation causes the browning of food during long-term storage, a problem in the food industry. In vivo, the glycation of lens crystallins has been associated with enhanced susceptibility to oxidation, high molecular weight, and covalent cross-link formation (*5*, *6*). Glycated plasma proteins are recognized by regions at the surface of macrophages and aortic, endothelial, and mesangial cells (*7*−*9*). Accelerated glycation has been implicated in the development of diabetic complications and the aging process (*10*−*13*).

Since amadoriase catalyzes the oxidase reaction in which fructosyllysine is deglycated, it has potential application in the enzymatic determination of glycated substance and disease prevention. Amadoriase activity has been reported in several strains of fungi (*14*−*19*), and a few amadoriase enzymes have been isolated and cloned from different species (*1*, *20*−*22*). Previously, we reported the isolation and cloning of two amadoriase isoenzymes from *Aspergillus* sp. from soil, which we named amadoriases I and II (*1*). Both amadoriase I and amadoriase II are monomers of molecular mass around 50 kDa. They have FAD as a cofactor and share 51% sequence identity (*1*, *21*).

When different glycated amines were tested as substrates of amadoriases I and II, we observed a striking difference in the substrate selectivity between the two isoenzymes. Amadoriase II has a high activity toward the substrate with a carboxylate group while amadoriase I shows little activity. Because amadoriase II prefers substrates with a carboxylate group, which is negatively charged, we hypothesized that positively charged amino acid residues such as lysine and arginine may possibly serve as functional groups in the binding site of the enzyme.

In this study we have probed the role of arginine residues in the binding site of amadoriase II using the arginine modification reagent *p*-hydroxyphenylglyoxal that has been widely used in experiments to identify functional arginine residues (*23*, *24*). Using a combined approach based on chemical modification and site-directed mutagenesis, we show that two arginine residues (Arg^{112} and Arg^{114}) are essential for substrate selectivity of amadoriase II.

EXPERIMENTAL PROCEDURES

Materials. 10-Acetyl-3,7-dihydroxyphenoxazine was obtained from Molecular Probes (Eugene, OR). Fructosylpropylamine and fructosylglycine were synthesized as reported by Takahashi et al. (*1*). Ni-NTA Superflow resins were purchased from Qiagen (Valencia, CA). The hydroxyapatite matrix was from Bio-Rad (Hercules, CA), and *p*-hydroxy-

† This study was supported by NEI grants EY07099 and EY930-11373.

* To whom correspondence should be addressed. Fax: (216) 368-0495. Phone: (216) 368-6613. E-mail: vmm3@po.cwru.edu.

‡ Case Western Reserve University.

§ Washington University School of Medicine.

[1] Abbreviations: amadoriase, fructosyl amino acid oxidase (EC 1.3.5); HRP, horseradish peroxidase; PAAP, propylamine Amadori product (fructosyl propylamine); GAP, glycine Amadori product (fructosylglycine); HPG, *p*-hydroxyphenylglyoxal; TFA, trifluoroacetic acid; LC-MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; DTT, dithiothreitol.

10.1021/bi025539j CCC: $22.00
Published on Web 03/09/2002

phenylglyoxal was from Pierce (Rockford, IL). Modified sequencing grade trypsin was from Roche (Indianapolis, IN). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA), and BL21 Star competent cells were from Invitrogen (Carlsbad, CA). Oligonucleotides for site-directed mutagenesis were prepared by IDT (Coralville, IA). All other chemicals were from Sigma (St. Louis, MO).

Enzyme Preparation. Recombinant amadoriase I was expressed in *Escherichia coli* and purified as described in Wu et al. (*21*) through Ni, His-tag affinity chromatography. The amadoriase II gene was ligated onto the pET23d expression vector (Novagen, Madison, WI) as an *Eco*RI−*Nco*I fragment and transformed into *E. coli* strain BL21-(DE3) pLysS. The cells were harvested 4 h after induction with 0.5 mM isopropyl β-D-thiogalactose (IPTG) at 30 °C and frozen at −70 °C. Cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and broken by three cycles of 30 s sonications. The insoluble fraction was removed by centrifugation, and the whole cell extract was precipitated by 50% $(NH_4)_2SO_4$. The pellet after centrifugation was resuspended in 10 mM NaH_2PO_4 (pH 8.0) and dialyzed against the same buffer overnight. After dialysis, the cell extract was applied to a hydroxyapatite FPLC column (12 × 88 mm, Bio-Scale MT10, Bio-Rad) equilibrated with 10 mM NaH_2PO_4 (pH 8.0). After the column was washed with 50 mL of equilibration buffer, elution was carried out with a 100 mL linear gradient of phosphate buffer (pH 8.0) from 10 to 200 mM with a flow rate of 2 mL/min. Fractions with enzymatic activity were pooled for future assay.

Enzymatic Activity Assay. Enzymatic activity was determined by Amplex Red (Molecular Probes) assay that measures the hydrogen peroxide generation. The reaction mixture contained 25 μL of enzyme solution (4−40 μg/mL) in 10 mM HEPES buffer (pH 8.0), 25 μL of substrate (fructosylpropylamine/fructosylglycine) in the same buffer, and 25 μL of a 5 μg/mL 10-acetyl-3,7-dihydroxyphenoxazine and 20 μg/mL horseradish peroxidase (HRP) mixture in a total volume of 75 μL in a 384-well microplate. The plate was kept in 37 °C, and the fluorescence (λ_{ex} = 560 nm, λ_{em} = 590 nm) was measured with a microplate reader (Spectrafluor Plus, Tecan, Research Triangle Park, NC).

Modification of Amadoriase II with p-Hydroxyphenylglyoxal. Five hundred microliters of purified amadoriase II (1 mg/mL) was incubated with 3 mg of *p*-hydroxyphenylglyoxal (HPG) at room temperature in the dark in 10 mM HEPES buffer, pH 8.0. Aliquots were withdrawn at various intervals and diluted by 10 mM HEPES buffer, pH 8.0, for activity assay.

Tryptic Peptide Mapping. After incubation with HPG, amadoriase II solution was passed through a gel filtration column (10 DG, Bio-Rad) equilibrated with 10 mM HEPES buffer, pH 8.0, to remove excess unreacted HPG. The modified protein was reduced by incubating with 3 mM DTT at 50 °C for 10 min and room temperature for 1 h. Iodoacetamide (10 mM) was added, and the mixture was incubated with reduced amadoriase II in the dark for 1 h at room temperature. The final solution was digested with trypsin using a ratio (w/w) of 1:20 at 37 °C overnight. Control unmodified amadoriase II was also treated with the same procedure.

Table 1: Kinetic Constants of Wild-Type and Mutant Amadoriases[a]

	GAP			PAAP		
amadoriase	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
I (wild type)	9.75	0.6	6.6×10^{-2}	0.023	5.8	250
II (wild type)	0.23	3.2	14	2.53	2.7	1.1
II (R112L)	0.77	3.0	3.9	1.16	3.1	2.7
II (R114L)	1.93	3.0	1.5	3.04	3.7	1.2
II (R112E)	4.48	4.9	11	1.52	4.7	3.1
II (R114E)	5.11	3.5	0.7	1.59	2.0	1.3
II (R112E, R114E)	8.16	3.0	0.36	0.58	2.1	3.6

[a] Activity was measured by hydrogen peroxide assay as described under Experimental Procedures. Apparent kinetic constants were obtained by least-squares linear regression analysis.

HPLC Separation and Mass Spectral Analysis. Peptide mapping was performed on a Magic 2002 HPLC (Michrom BioResources, Auburn, CA) using a 5 μm reverse-phase Magic C_{18} column (100 Å pore size; 1.0 × 150 mm; Michrom BioResources) which was initially equilibrated in 95% mobile phase A (2% acetonitrile, 0.05% formic acid, and 0.005% TFA)/5% mobile phase B (90% acetonitrile, 0.05% formic acid, and 0.005% TFA). The tryptic digest was injected onto the column, and the column was first washed with 95% mobile phase A for 5 min. The flow rate was always kept at 50 μL/min and the temperature at 40 °C. Elution was performed using a linear gradient from 5% to 65% mobile phase B in 30 min, with UV monitoring at 214 nm. On-line liquid chromatography−electrospray ionization mass spectrometry (LC-MS) of peptide samples was performed on a Finnigan LCQ electrospray ionization (ESI) ion-trap mass spectrometer (Finnigan, San Jose, CA). LC-MS analysis was performed by connecting the HPLC outlet line directly to the mass spectrometer. Peptide was also manually collected for subsequent analysis on a Micromass Quattro II triple quadrupole mass spectrometer (Manchester, U.K.) at the Cleveland Mass Spectrometry Facility (*21*).

Site-Directed Mutagenesis. The QuikChange site-directed mutagenesis kit and synthetic mutagenic oligonucleotides were used to introduce the desired mutations. XL1-Blue supercompetent cells were transformed with mutated plasmid. Colonies were selected from Luria broth plates containing ampicillin (100 μg/mL), and plasmid was extracted. The presence of mutation was confirmed by DNA sequencing, and the plasmid was transformed into *E. coli* strain BL21-(DE3) pLysS for protein expression.

RESULTS

Kinetic Constants of Wild-Type Amadoriases I and II. Kinetic constants were determined for purified amadoriases I and II (Table 1, wild type). GAP and PAAP (Scheme 1) were used as the substrates. Amadoriase I has very high activity toward PAAP (k_{cat}/K_m = 250 $mM^{-1}\ s^{-1}$) while its activity toward GAP was negligibly low ($k_{cat}/K_m = 6.6 \times 10^{-2}$ $mM^{-1}\ s^{-1}$). In contrast, amadoriase II showed more than 10-fold higher k_{cat}/K_m for GAP (k_{cat}/K_m = 14 $mM^{-1}\ s^{-1}$) than PAAP (k_{cat}/K_m = 1.1 $mM^{-1}\ s^{-1}$). The amadoriase II activity difference between GAP and PAAP results mainly from the difference in K_m, suggesting that amadoraise II has a higher binding affinity for GAP.

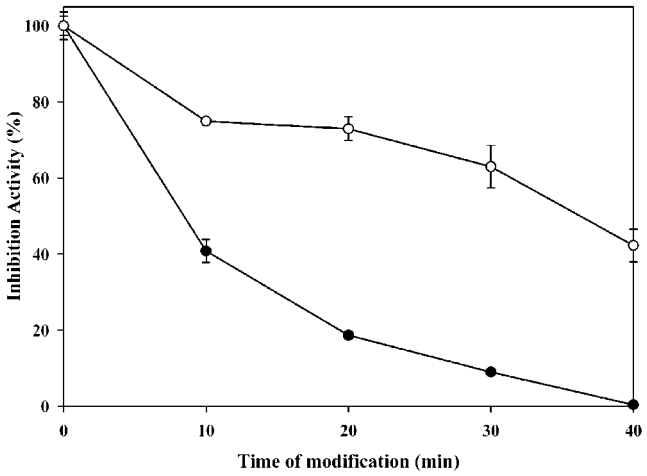
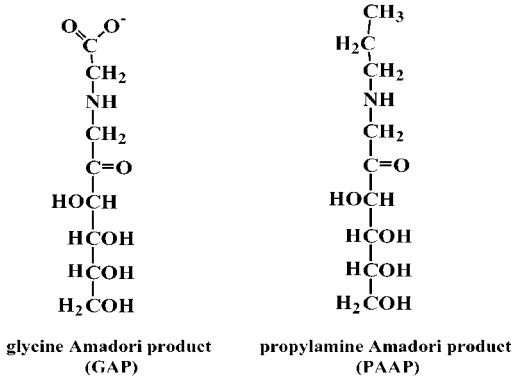


FIGURE 1: Inactivation of amadoriase II by *p*-hydroxyphenylglyoxal. The enzyme (1 mg/mL) was incubated with hydroxyphenylglyoxal (6 mg/mL) at room temperature in the dark. At the following intervals, 0, 10, 20, 30, and 40 min, aliquots were taken out and diluted 1:250 in 10 mM HEPES buffer, pH 8.0, and assayed for activity using 100 μM fructosylglycine (●) as the substrate. The enzyme solution was also diluted 1:50 by the same buffer when 100 μM fructosylpropylamine (○) was used as the substrate. The activity of the diluted enzyme was assayed by measuring H_2O_2 generation vs time. The activity of $T = 0$ min was taken as 100%, and each data point is in duplicate.

Scheme 1: Structures of the Substrates Used in the Experiments



Inactivation of Amadoriase II with p-Hydroxyphenylglyoxal. Incubation of amadoriase II with the arginine modifying reagent *p*-hydroxyphenylglyoxal resulted in a time-dependent loss of enzyme activity toward GAP (Figure 1). After amadoriase II (1 mg/mL) was incubated with hydroxyphenylglyoxal at room temperature for 40 min, 99% of the activity toward GAP was lost, while the same modified enzyme still had 42% of the original activity toward PAAP (Figure 1). The fact that modification had a larger effect on the amadoriase II activity toward GAP suggested that some arginine residue might be important for GAP binding. It is also worth noting that after the modification the activity toward both substrates decreased to a different extent, indicating other arginine residues important for the enzyme function might also be modified. On the basis of the spectrum of modified amadoriase II after excess hydroxyphenylglyoxal was removed by gel filtration and the molar extinction coefficient of hydroxyphenylglyoxal at 340 nm (1.83×10^4 M^{-1} cm^{-1}) (*23*), it was estimated that about 2−3 mol of hydroxyphenylglyoxal were incorporated per mole of enzyme.

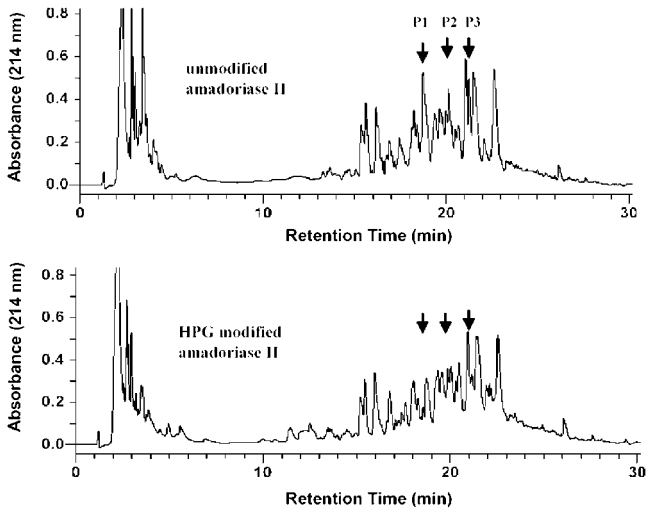


FIGURE 2: HPLC profiles of tryptic digests of modified and unmodified amadoriase II. Hydroxyphenylglyoxal-modified and unmodified amadoriases II were digested by trypsin. Digested samples were applied to HPLC for peptide mapping. Solvent A was 5% acetonitrile. Solvent B was 90% acetonitrile. The flow rate was 50 μL/min. The gradient was 5% solvent B in 5 min, 5%−65% solvent B in 30 min, and 65%−95% solvent B in 10 min. The detector was set at 214 nm. The HPLC outlet was connected directly to the mass spectrometer for on-line mass analysis. P1 was also manually collected and analyzed as described under Experimental Procedures.

Tryptic Peptide Maps of Modified and Unmodified Amadoriase II and Mass Spectral Analysis. A comparison of the tryptic peptide maps of modified and unmodified amadoriase II is shown in Figure 2. Three peptide fractions marked as P1 and P2 in the unmodified amadoriase II were reduced by 50% or more in the sample modified by HPG. The observed mass values of P3 do not match any peptide generated by trypsin digestion of amadoriase II; we suspect that peak P3 is due to contamination or nonspecific cleavage. Figure 3 shows the mass spectra of P1 and P2 analyzed on-line by LC/MS with a LCQ-ESI ion trap instrument. Assignment of P1 and P2 to their peptide sequences was made according to the observed and expected mass values of protonated ions and the known cleavage pattern of trypsin. The P1 fraction was also manually collected, and the sequence was confirmed by collision-induced dissociation (CID) tandem MS on a Micromass Quattro II triple quadrupole mass spectrometer (data not shown). Peptide sequences of P1 (residues 113−131) and P2 (residues 319−333) are listed as highlighted letters in Figure 3 below the mass spectra, and arginine residues are marked by asterisks. Modification of arginine residues at either end of the peptide will prevent the trypsin cleavage and generate a longer peptide, and a smaller amount of the original peptide will be observed from peptide mapping. On the basis of that consideration we identified Arg^{112}, Arg^{131}, and Arg^{333} as the possible sites for HPG modification. To narrow down the possibility, we prepared the sequence alignments of the corresponding region between amadoriase I and amadoriase II, which are also shown in Figure 3. Since the anionic preference only occurs in amadoriase II, and amadoriase I has the opposite substrate selectivity, the arginine of interest should thus not be conserved between those two enzymes. Only Arg^{112} is not conserved in amadoriase II, and a glutamic acid is in amadoriase I instead. A nearby arginine residue, Arg^{114}, also aligns to a

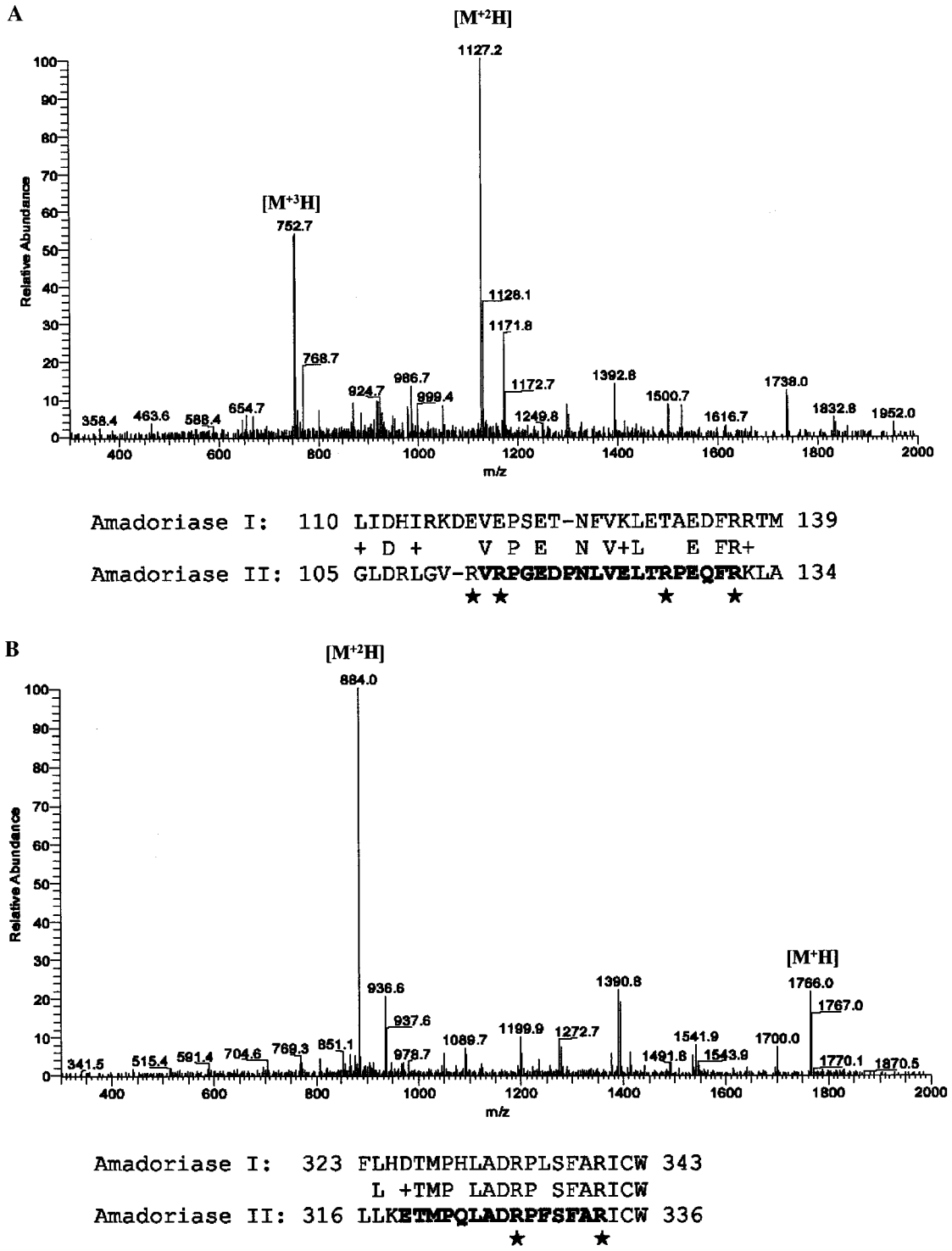


FIGURE 3: LC-MS spectrum of tryptic peptide mapping. (A) Mass spectrum of peak P1 from Figure 2 analyzed by a LCQ mass spectrometer. A major signal with a $[M^{+2}H]$ value of 1127.2 corresponded to the unmodified tryptic peptide 113−131 (expected monoisotopic mass $[M^{+2}H]$: 1126.6). The sequence of the peptide whose expected mass matches the observed mass value is listed as highlighted below the spectrum, and arginine residues were marked by asterisks. The alignment between amadoriase I and amadoriase II is also listed, and arginines are marked by asterisks. (B) Mass spectrum for P2. The signal with a $[M^{+2}H]$ value of 884.0 corresponded to the unmodified tryptic peptide 319−333 (expected monoisotopic mass $[M^{+2}H]$: 883.4).

glutamic acid in amadoriase I, based on the alignment. These data suggest that Arg^{112} and Arg^{114} are possible sites for anionic substrate binding.

Properties of the Mutants. To further identify the roles of Arg^{112} and Arg^{114} in the enzyme catalytic mechanism, site-directed mutagenesis experiments were carried out. A single mutation of each arginine to leucine and glutamic acid and a double mutation of both arginines to glutamic acid were prepared. The mutant enzymes were expressed in *E. coli* strain BL21(DE3) pLysS and purified through $(NH_4)_2SO_4$ precipitation and hydroxyapatite chromatography. The purity of the mutant enzymes was checked on sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and it was shown that the mutant enzymes were pure and had the same

migration distance on the gel as wild-type amadoriase II. The kinetic properties of the mutant enzymes are compared with the wild-type enzyme, and the results obtained are summarized in Table 1. All of the mutants have comparable k_{cat} values with the wild-type enzyme while the K_m of the mutant toward GAP increased significantly, so the overall activity (k_{cat}/K_m) decreased. The biggest increase of K_m occurs in the double mutant (R112E, R114E), almost 40-fold. On the contrary, the activity of the mutants toward PAAP was not effected, and the mutants of Arg^{112} even have a higher activity toward PAAP than the wild-type amadoriase II. These results indicate that Arg^{112} and Arg^{114} are responsible for anionic substrate binding, but not for catalysis. Interestingly, we also noticed that by changing two arginines to glutamic acid, as in amadoriase I, the K_m of the amadoriase II mutant for both substrates became similar to that of amadoriase I (Table 1), especially for GAP.

DISCUSSION

Electrostatic forces play a general role in the interactions between molecules and, as discovered in numerous cases of protein−protein interaction, enzyme−substrate binding, etc. (*25*−*27*). Side chains of charged residues, Arg, Lys, Glu, and Asp, are usually responsible for generating the electric field in the proteins and protein backbone dipoles. Those of α-helices can especially participate in the construction of a strong electric field (*28*).

Many studies showed that arginine residues can serve as positively charged recognition sites for negatively charged substrates and anionic cofactors in the enzyme active site. This suggests, as a general rule, that enzymes acting on anionic substrates or cofactors will probably contain arginine as a component of their ligand binding sites (*29*). Various α-dicarbonyls were used to modify active site arginine residues in many cases successfully (*30*). Interestingly, active -site arginines of some enzymes were indeed found to react with carbonyls much faster than free arginine itself (*30*), clearly showing that enhanced reactivity of these residues is the basis of their selective reactivity. According to Patthy et al. (*30*), the very feature that renders active site arginines able to fulfill their general anion binding role brings with it an increased reactivity toward dicarbonyls.

We started to study the anion binding of amadoriase II based on the observation that the K_m of amadoriase II toward GAP is 10 times lower than to PAAP while the major difference between those two substrates is the carboxylate group. Hydroxyphenylglyoxal modification causes much a higher decrease of the enzyme activity toward GAP compared to PAAP, suggesting reactive arginine residues in the binding site. Peptide mapping and site-directed mutagenesis confirmed that two arginine residues, Arg^{112} and Arg^{114}, are responsible for GAP affinity. The double mutant (R112E, R114E) has 10 times higher activity toward PAAP than GAP, resulting in reversal of the substrate selectivity. In other words, mutation of those arginine residues essentially converted amadoriase II to amadoriase I. We thus suspect mutation of Glu^{118} and Glu^{120} in amadoriase I to arginines would cause enhanced affinity for GAP.

The fact that Arg^{114}, followed by a proline residue, is not a digestion site of trypsin makes it difficult to conclude whether there is also modification of Arg^{114}. Although the mutagenesis data confirmed the role of Arg^{114} as the anion binding site, the different reactivity of arginine residues toward hydroxyphenylglyoxal due to their different microenviroment does not guarantee the same extent of modification of Arg^{114} as of Arg^{112}.

Aspergillus sp. has two amadoriase isoenzymes with different substrate selectivity, which allows it to be able to metabolize a broad range of glycated molecules as the nutrient source. Our results also show that change of the charge status of key residues can affect the catalysis significantly, and that might be one of nature's way to "tune" the enzyme during evolution.

ACKNOWLEDGMENT

We thank Dr. Vernon Anderson for useful discussion.

REFERENCES

1. Takahashi, M., Pischetsrieder, M., and Monnier, V. M. (1997) *J. Biol. Chem. 272*, 3437−3443.
2. Amadori, M. (1931) *Atti R. Accad. Naz. Lincei 13*, 72.
3. Kuhn, R., and Wegand, F. (1937) *Ber. 70*, 769.
4. Thorpe, S. R., and Baynes, J. W. (1982) in *The glycoconjugates* (Horowitz, M. I., Ed.) Vol. III, pp 113−132, Academic Press, New York.
5. Monnier, V. M., Stevens, V. J., and Cerami, A. (1979) *J. Exp. Med. 150*, 1098−1107.
6. Monnier, V. M., and Cerami, A. (1983) *Biochim. Biophys. Acta 760*, 97−103.
7. Salazar, R., Brandt, R., and Krantz, S. (1995) *Biochim. Biophys. Acta 1266*, 57−63.
8. Wu, V.-Y., and Cohen, M. P. (1994) *Biochem. Biophys. Res. Commun. 198*, 734−739.
9. Ziyadeh, F. N., and Cohen, M. P. (1993) *Mol. Cell. Biochem. 125*, 19−25.
10. Sell, D. R., Lapolla, A., Odetti, P., Fogarty, J., and Monnier, V. M. (1992) *Diabetes 41*, 1286−1292.
11. McCance, D. R., Dyer, D. G., Dunn, J. A., Bailie, K. E., Thorpe, S. R., Baynes, J. W., and Lyons, T. J. (1993) *J. Clin. Invest. 91*, 2470−2478.
12. Beisswenger, P. J., Moore, L. L., Brinck-Johnsen, T., and Curphey, T. J. (1993) *J. Clin. Invest. 92*, 212−217.
13. Baynes, J. W., and Monnier, V. M. (1989) *Prog. Clin. Biol. Res. 304*, 1−410.
14. Sakai, Y., Yoshida, N., Isogai, A., Tani, Y., and Kato, N. (1995) *Biosci. Biotechnol. Biochem. 59*, 487−491.
15. Horiuchi, T., Kurokawa, T., and Saito, N. (1989) *Agric. Biol. Chem. 53*, 103−110.
16. Horiuchi, T., and Kurokawa, T. (1991) *Agric. Biol. Chem. 55*, 333−338.
17. Watanabe, N., Ohtsuka, M., Takahashi, S., Sakano, Y., and Fujimoto, D. (1990) *Agric. Biol. Chem. 54*, 1063−1064.
18. Yoshida, N., Sakai, Y., Serata, M., Tani, Y., and Kato, N. (1995) *Appl. Environ. Microbiol. 61*, 4487−4489.
19. Saxena, A. K., Saxena, P., and Monnier, V. (1996) *J. Biol. Chem. 271*, 32803−32809.
20. Takahashi, M., Pischetsrieder, M., and Monnier, V. M. (1997) *J. Biol. Chem. 272*, 12505−12507.
21. Wu, X., Takahashi, M., Chen, S. G., and Monnier, V. M. (2000) *Biochemistry 39*, 1515−1521.
22. Yoshida, N., Sakai, Y., Isogai, A., Fukuya, H., Yagi, M., Tani, Y., and Kato, N. (1996) *Eur. J. Biochem. 242*, 499−505.
23. Yamasaki, R. B., Vega, A., and Feeney, R. E. (1980) *Anal. Biochem. 109*, 32−40.
24. Sun, X.-J., and Chang, J.-Y. (1990) *Biochemistry 29*, 8957−8962.

25. Xu, D., Lin, S. L., and Nussinov, R. (1997) *J. Mol. Biol. 265*, 68−84.
26. Lewis, D. F., Eddershaw, P. J., Dickins, M., Tarbit, M. H., and Goldfarb, P. S. (1998) *Chem. Biol. Interact. 115*, 175−199.
27. Nogués, M. V., Moussaoui, M., Boix, E., Vilanova, M., Ribó, M., and Cuchillo, C. M. (1998) *Cell. Mol. Life Sci. 54*, 766−774.
28. Hol, W. G. J., van Duijnen, P. T., and Berendson, H. J. C. (1978) *Nature 273*, 443−446.
29. Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977) *Science 195*, 884−886.
30. Patthy, L., and Thesz, J. (1980) *Eur. J. Biochem. 105*, 387−393.

BI025539J