Isolation, Purification, and Characterization of an Amadori Product Binding Protein from a Pseudomonas sp. Soil Strain*

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Sugar react nonenzymatically with protein amino groups to form a ketoamine adduct (Amadori product), which leads to the formation of advanced glycation end-products. These compounds are involved in the development of tissue modifications such as cross-linking and fluorescence in diabetes and aging. Searching for an enzyme to reverse protein glycation, we isolated a Pseudomonas sp. soil strain growing selectively on the Amadori product $\varepsilon$-fructosyl-aminocaproate. An Amadori product binding protein (ABP) was purified from the bacterial extract by single-step affinity chromatography on glycated lysine-Sepharose. The protein, a monomer of 45 kDa, did not bind to unglycated or NaBH$_4$-reduced glycated lysine-Sepharose suggesting specificity for the Amadori compound. The concentration-dependent binding of glycated aminocaproate showed saturation with $K_d = 1.49 \mu M$ and $B_{max} = 17.63 \text{nmol/mg}$ of protein corresponding to 0.8 mol/mol of protein. The binding of $\varepsilon$-l-[1-14C]fructosyl-aminocaproate to the protein was inhibited by other glucose-derived Amadori products, but not by free sugars, unglycated amines, or ribated lysine. The sequence of the first 16 NH$_2$-terminal amino acids and a GenBank search revealed that ABP is a novel protein. Its synthesis was inducible by the organism in Amadori product. Immunoblotting studies showed that ABP is not found in cell extracts from other prokaryotes, yeast, or liver homogenate and does not bind Amadori products in glycated proteins. ABP has no enzymatic activity toward glycated substrates and may thus have transport or permease function for glycated amino acids.

Nonenzymatic glycation and advanced glycation of proteins are thought to be one of the links between hyperglycemia and long term complications of diabetes and aging (1, 2). In the early step of the Maillard reaction, the aldehyde group of glucose reacts nonenzymatically with free amino groups to form a labile Schiff base, which is then converted to a more stable ketoamine by the Amadori rearrangement (3). With time the Amadori product undergoes a series of reactions leading to the nonenzymatic browning or advanced Maillard products (4), also referred to as advanced glycation end products (AGEs).

Several lines of evidence suggest that AGE accumulation in long lived proteins participates in some of the tissue alterations common to diabetes and aging such as arterial stiffening, cataract formation, and atherosclerosis (5, 6). A correlation was found between the levels of advanced Maillard reaction products and the cumulative severity of diabetic complications (7–9). In addition, AGE-modified proteins have been found to be recognized by specific cellular binding sites (10, 11), and the AGE-receptor interaction could contribute to the pathogenesis of diabetic angiopathy.

In recent years, pharmacological approaches for blocking the advanced Maillard reaction have been developed. Aminoguanidine, an inhibitor of AGE formation (12, 13), prevented the increase in regional blood flow (14) and basement membrane thickness (15), and ameliorated retinopathy (16) and neuropathy (17) in diabetic rats.

The possibility of breaking off the chain of events resulting from nonenzymatic glycation by enzymatically reversing the Amadori product could be a powerful tool to fully understand the role of nonenzymatic glycation in diabetes and aging. Searching for such approaches as a potential gene therapy of complications resulting from glycation, we have grown soil organisms on glycated substrate as sole carbon source hoping to isolate enzyme(s) that degrade Amadori products. We describe here the isolation and characterization of a novel protein from a Pseudomonas sp. soil sp. that binds selectively glycated amino acids.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**

Lysine-Sepharose, D-glucose, $\varepsilon$-aminocaproic acid, NaBH$_4$, EDTA, phenylmethylsulfonyl fluoride, N-methyl-$\varepsilon$-glucamine, N-acetyl-lysine, p-nitrophenyl phosphate, bovine serum albumin (BSA), and RNase were from Sigma, Dowex AG50XW–400 was from Aldrich. N-tert-Boc-$\varepsilon$-lysine was from Bachem (Torrance, CA). SDS-PAGE reagents, molecular weight markers, silver staining kit, and Bradford protein assay were from Bio-Rad. [U-14C]Glucose was purchased from ICN Biochemicals (Costa Mesa, CA). Alkaline phosphatase was from Boehringer Mannheim. Immobilon transfer membrane was from Millipore Corp. (Bedford, MA). Diafragma membrane MY10 and Centricron 10 were from Amicon, Inc. (Beverly, MA). All other chemicals used were analytical grade reagents.

**Synthesis of Amadori Products**

$\varepsilon$-D-fructoseyl Amino Caproic Acid—Glycated $\varepsilon$-aminocaproic acid was prepared by a modified method of Finot and Mauron (18). Briefly, a suspension of 1 M D-glucose and 1 M $\varepsilon$-aminocaproic acid was acidified to pH 3.25 with acetic acid in water and refluxed at 100 °C for 3 h. After rotary evaporation, the residue was dissolved with 0.2 M pyridine formate (pH 3.25) and applied to a Dowex AG50W-X4–400 column (10 x 20 cm). The column was eluted with 0.2 M pyridine formate saline; fructosyl-aminocaproate, $\varepsilon$-D-fructoseyl-aminocaproic acid; fructosyl glycine, $\varepsilon$-D-fructoseyl glycine; fructosyl lysine, N-tert-Boc-$\varepsilon$-(1-deoxyfructosyl)-lysine; Ribosyl lysine, $\varepsilon$-(1-deoxyribofructosyl)-lysine; fructosyl propylamine, $\varepsilon$-D-fructoseyl propylamine; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
(pH 3.25), and the effluent was monitored by thin layer chromatography on silica plates with 1-butanol/acetone/water (40:60:10). Plates were developed both with ninhydrin and triphenyltetrazolium chloride. The fractions containing the Amadori product were pooled and flash chromatographed on a silica gel column (5 × 30 cm) with methanol/ethyl acetate (2:3) as the mobile phase. The Amadori product was then concentrated by evaporation, redisolved with 150 ml of methanol and precipitated by dropwise addition into 2 liters of cold ethyl acetate. The purity was checked by thin layer chromatography and confirmed by proton NMR.

N′-4-Boc-N′-1-deoxyfructosyl-lysine and N′-acetyl-N′-1-deoxyfructosyl-lysine were synthesized as described by Finot and Maurois (18) and as below. e-1-deoxyribosyl lysine was synthesized as described previously (19). 1-deoxyfructosylglycine and 1-deoxyfructosylprolylamine were a generous gift from Dr. T. Horisuchi and from Dr. Marcus Glomb, respectively. Borohydroxide-reduced 1-deoxyfructosylaminocaproic acid was prepared through reduction in presence of 50 M excess NaBH₄. For preparative purposes, washed cells from 400 ml of culture were disrupted by three passes through a French press at 6,000–7,000 p.s.i., followed by sonication. Following centrifugation, the cell-free extract (10–15 mg of total proteins) was applied to a column of glycated lysine-Sepharose (6 ml of gel). After washing as above, the binding protein was eluted with 1 M NaCl in 0.05 M sodium phosphate buffer (pH 7.4) and concentrated to approximately 2 ml by ultrafiltration (Diaflo membrane YM10) under nitrogen. The purity was verified by SDS-PAGE both with Coomassie Blue and silver staining detection.

Induction Experiment
To investigate the effect of the presence of Amadori product in the culture medium on the expression of protein(s) binding to glycated lysine-Sepharose, cells were harvested from 400 ml of a nutrient broth culture and suspended in 30 ml of 0.05 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA. The cell suspension were withdrawn, washed with minimal salt, and resuspended with 150 ml of minimal medium supplemented with 0.5 g/100 ml fructoseaminocaproate, and incubated at 37°C. At various time points, 20 ml of the cell suspension were disrupted as above and centrifuged. An amount of each extract corresponding to 0.7 mg of total protein was chromatographed over a 1-ml column of glycated lysine-Sepharose as above, and the eluates were dialyzed, lyophilized, and analyzed by SDS-PAGE.

Binding Studies
The binding activity of the purified protein toward various substrates was characterized using three types of assays.

Binding to Glycated Lysine-Sepharose—In order to evaluate the relationship between extent of glycation of lysine-Sepharose and binding of the purified protein, a mixture of lysine-Sepharose (4 μmol of NH₂/mg of gel) and glucose (1 mM) was prepared as above and incubated at 50°C. 1-ml aliquots were withdrawn, washed, and 25 μl of packed gel were counted in duplicate using a Beckman LS 6000Sc β-counter. 8 μg of purified protein was applied to each column preequilibrated with 0.05 M sodium phosphate buffer (pH 7.4). After washing with the same buffer, columns were eluted with 5 ml of 0.2 M fructoseaminocaproate. The eluates were concentrated by ultrafiltration (Centricon 10, Amicon, Inc.), and comparable volumes of each samples were subjected to SDS-PAGE. Following Coomassie Blue staining, the gel was analyzed by scanning densitometry (UBS SciScan 5000). The optical density of the bands and the corresponding value of incorporated radioactivity were plotted versus time.

To test the binding specificity of the purified protein for the Amadori product, a series of 0.5-mI gel columns were prepared with lysine-Sepharose, Sepharose-fructosyllysine, Sepharose-fructosylaminocaproate-EDTA and Sepharose-deoxyglucosyllysine. Purified protein (8 μg) was applied to each column and chromatographed as above. Both the washes and the eluates were concentrated by ultrafiltration and analyzed by SDS-PAGE.

Equilibrium Dialysis—An equilibrium dialysis radioligand binding assay was used to further characterize the binding specificity of AAB (inhibition studies) and to evaluate the binding parameters with the model Amadori product e-fructoseaminocaproate (saturation studies). The assay was carried out in polypropylene microdialysis cells (Bel-Art Products, Pequannock, NJ) consisting of two wells (0.1 ml each) separated by a dialysis membrane. One well contained 10 μg of AAB in 0.05 M sodium phosphate buffer (pH 7.4), the other contained 60 nM of a [3H]fructoseaminocaproate solution in the same buffer. The amount of nonspecific binding was evaluated by performing the assay in the presence of an excess of unlabeled ligand. Background binding was evaluated by adding 10 μg of the competitor to the labeled ligand.

The data were analyzed by the method of Scatchard (51). The dissociation constant (Kd) and the maximal binding capacity (Bmax) were calculated from the intercept and slope of the Scatchard plot. The data were fitted to a two-site model (52), assuming that the free concentration of the ligand was in equilibrium with the radioligand.

The kinetic parameters were determined by nonlinear least squares analysis using a computer program based on the Marquardt-Levenberg algorithm (53). The goodness of fit was assessed by visual inspection and by analysis of residuals. The estimates of Kd and Bmax were obtained from the parameter estimates and their standard errors were obtained from the inverse of the Hessian matrix. The standard errors were used to calculate the 95% confidence intervals for the parameter estimates.

The binding parameters were compared using a one-way ANOVA followed by a Dunnett's multiple comparison test. The significance level was set at p < 0.05.

Enzyme-Linked Immunosorbent Assay (ELISA)
The enzyme-linked immunosorbent assay (ELISA) was used to determine the specificity of the binding of the purified protein for the Amadori product. The assay was carried out in 96-well microtiter plates (Corning) coated with 10 μg of AAB in 0.05 M sodium phosphate buffer (pH 7.4). The plates were incubated overnight at 4°C. After washing with PBS-T, the plates were incubated with 1% skim milk for 1 h to block any remaining binding sites. Following washing, the plates were incubated with the purified protein at various concentrations for 1 h at room temperature. After washing, the plates were incubated with an anti-AAB (diluted 1:1000 in 1% skim milk) for 1 h at room temperature. After washing, the plates were incubated with an anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:1000 in 1% skim milk) for 1 h at room temperature. After washing, the plates were developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate. The absorbance at 405 nm was measured using a plate reader (Bio-Tek Instruments, Winooski, VT). The data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). The IC₅₀ values were calculated using nonlinear regression analysis and the goodness of fit was assessed by visual inspection and by analysis of residuals. The estimates of IC₅₀ were obtained from the parameter estimates and their standard errors were obtained from the inverse of the Hessian matrix. The standard errors were used to calculate the 95% confidence intervals for the parameter estimates. The data were fitted to a two-site model (52), assuming that the free concentration of the ligand was in equilibrium with the radioligand.
the presence of an excess of unlabeled fructosyl-epsilon-aminocaproate (200 nm). After incubation at 4°C for 20–22 h in a rotary shaker, two aliquots of 20 μl were withdrawn from each well, and the radioactivity was measured. For the inhibition studies, the [14C]-labeled Amadori product was used at 6 μM constant concentration with and without a 10-fold molar concentration of the compound to be tested. The ability of ABP to bind different compounds was expressed as percent of the binding of [14C]-fructosyl-aminocaproate. For the saturation studies, the [14C]-labeled Amadori product was used at concentration ranging from 0.5 to 18 μM. A plot of bound Amadori versus concentration was obtained, and the binding parameters were calculated according to the Michaelis-Menten equation using nonlinear fitting analysis.

Enzyme-linked Immunoassay (ELISA) for Binding to Glycated Proteins—For investigation of the ability of ABP to bind to glycated proteins, an ELISA sandwich assay was performed. Briefly, glycated proteins (BSA and RNase) were immobilized on microtiter plates, and after blocking with unglycated BSA, ABP (10–100 ng) was added to each well. Following 16 h of incubation at 4°C, bound ABP was detected by ELISA using a monoclonal antibody to ABP (see below) and goat anti-mouse IgG conjugated with alkaline phosphatase.

Production of a Monoclonal Antibody to ABP—To investigate the ABP binding to glycated proteins and for detection in other organisms, a monoclonal antibody was produced using the methods of Schulman et al. (21) and modified by Kaetzel et al. (22).

Ascites or culture supernantant was pretreated with Seraclor Reagent (Calbiochem) for delipidation, and IgG rich ascites were purified by FPLC on a protein G Superose column (Pharmacia Biotech Inc.). The specific IgG was eluted with 0.1 M HEPES-HCl (pH 2.7). The antibody was typed by immunodiffusion in agarose gels (ICN Biocyticals) and found to belong to the class IgG.

An ELISA consisting of 100 ng/well ABP as coating agent was developed as described previously (38). Following blocking of the 96-well plate with 200 μl of 1 g/l 100 ml ovalbumin, the plate was washed 3 times with phosphate-buffered saline-Tween 20 (0.05 g/100 ml) and used for detection of purified antibodies, mouse sera, or culture supernantant using goat anti-mouse IgG conjugated with alkaline phosphatase and p-nitrophenol phosphate as substrate.

Immunoblotting Studies

Presence of ABP was sought by Western blotting in extract of Pseudomonas sp. soil strain, P. aeruginosa (ATCC 27853), Proteus mirabilis, α-Streptococcus mitis, Staphylococcus aureus, Corynebacterium striatum, Candida albicans (obtained from Microbiology Laboratory, University Hospitals of Cleveland), and rat liver (Dr. H. A. Elgawish). The proteins (16 μg each) were separated by SDS-PAGE and electrophoretically transferred to Immobilon. Following blocking with 3% BSA solutions, the membrane washed in Tris-buffered saline-Tween 20 (0.05 g/100 ml) and incubated with ABP antibody (0.4 mg/ml IgG) in 1% BSA-Tris-buffered saline-Tween 20 for 2 h. The membrane was washed twice in Tris-buffered saline-Tween 20, followed by incubation with 1:1,000 diluted goat anti-mouse IgG coupled with alkaline phosphatase in 1% BSA. Reactive bands were visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml) and nitro blue tetrazolium (0.30 mg/ml) solution.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on a 12% polyacrylamide resolving gel and 4% stacking gel according to the method of Laemmli (23). Protein samples were solubilized both with reducing and nonreducing SDS sample buffer. The gels were stained for proteins with Coomassie Blue R-250 and silver stain.

Molecular Mass Measurement

The protein molecular mass was estimated on SDS-PAGE by extrapolation from the semilogarithmic plot of the relative mobility of standard proteins run simultaneously.

Protein Measurement

Absorbance at 280 nm was used to monitor the protein elution from column chromatography. Protein concentration was measured by the method of Bradford (24) using BSA as standard.

Amino-terminal Sequence Analysis

A partial sequence of the protein was obtained according to Matsu- daira (25). Briefly, the purified protein was subjected to SDS-PAGE and electroblotted on Immobilon membrane using 1 m CAPS, 10% methanol buffer (pH 11). After staining with Coomassie Blue, the blot was air dried, and the protein band 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 NH2-terminal sequence was entered in the FASTA program to search the GenBank Sequence Data base (26).

RESULTS

Strain Selection—A mucoid and mobile P. aeruginosa soil organism was found to utilize the Amadori product as sole carbon source. The organism was isolated from soil samples by direct selection and combination of growth in broth and agar. Typing was performed at the Microbiology Laboratory of the Institute of Pathology. In order to investigate whether the growth of the isolated microorganism was due to the utilization of the Amadori product itself or to spontaneous conversion of the product to free glucose and epsilon-aminocaproate, the growth rates of the soil strain with various substrates were compared with those of a control strain of P. aeruginosa (ATCC 27853). While both strains could grow on minimal medium supplemented with glucose and epsilon-aminocaproic acid, only the soil strain grew on medium containing glycated-epsilon-aminocaproate, and none of the strains was capable of growth on glucose or aminocaproic acid only (not shown).

Isolation of the ABP—To search for protein(s) involved in recognition of the Amadori product, affinity chromatography of the bacterial extract was carried out on glycated and unglycated lysine-Sepharose as described, and the eluate was analyzed by SDS-PAGE. A single protein was found in the soil strain extract upon elution from glycated lysine-Sepharose. However, no protein band was obtained from the extract passed over unmodified affinity substrate (Fig. 1A, lanes 3 and 4).

![Fig. 1. A, SDS-PAGE of Pseudomonas sp. before and after affinity chromatography on glycated lysine-Sepharose. Lanes 1 and 7, molecular mass markers; lane 2, soil strain crude extract; lane 3, soil strain extract eluate from glycated lysine-Sepharose; lane 4, soil strain extract eluate from nonglycated lysine-Sepharose; lanes 5 and 6, extract from P. aeruginosa control strain and eluate (lane 6) from glycated lysine-Sepharose. B, ABP expression of Pseudomonas sp. soil strain at various incubation times in minimal medium containing glycated epsilon-aminocaproate. SDS-PAGE of bacterial extract eluates from glycated lysine-Sepharose. Lane 1, molecular mass markers; lanes 2–7: 0, 1, 2, 4, 14, and 24 h of incubation, respectively.](#)
suggesting that the identified protein binds to the fructosyllysine adduct of the affinity support rather than nonspecifically to the affinity support.

The protein baptized ABP was not found in the extracts from *Pseudomonas* (Fig. 1A, lane 6) and *E. coli* control strains (not shown), both unable to utilize the Amadori product. Moreover, no protein was eluted from the glycated lysine-Sepharose column when the same affinity experiment was performed with cell extract from soil strain cultivated in minimal medium supplemented with glucose and ε-aminocaproic acid or in nutrient broth (data not shown), suggesting that the synthesis of ABP is related to the presence of the Amadori product in culture medium. To confirm this hypothesis, an induction experiment was performed in which the soil strain-washed cells grown in nutrient broth culture were incubated in minimal medium containing ε-fructosyl-aminocaproate. As shown in Fig. 1B ABP became expressed in the bacterial cells incubated with the Amadori product with a lag phase of about 4 h (faint band), reaching a steady state in approximately 14 h.

**Molecular Mass and NH₂-terminal Sequence**—ABP was found to migrate on SDS-PAGE with a mobility in agreement with a molecular mass of 45 kDa. A single band was found both under reduced and nonreduced conditions, suggesting that the protein is a monomer. The sequence for the first 16 NH₂-terminal amino acid was obtained and consists of KDAVVAEPDA-

**Binding Studies**—To evaluate the relationship between the extent of glycation of lysine-Sepharose and the binding of the purified protein, the binding was studied by varying the extent of glycation of the affinity substrate. A strong correlation was found between the amount of protein bound and the extent of glycation of lysine-Sepharose (not shown).

The binding specificity of ABP for the Amadori product was investigated by affinity chromatography on various substrates. The protein was not retained by unmodified lysine-Sepharose and NaBH₄-reduced glycated lysine-Sepharose (Fig. 2, lanes 3 and 4). Moreover, the binding to the glycated substrate was not affected by carrying out the glycation reaction with EDTA (Fig. 2, lane 5), excluding, thereby, the binding to carboxymethyllysine, a degradation product of the Amadori compound that forms in presence of oxygen and metal ions (27).

Binding specificity was also studied by measuring the ability of unlabeled compounds to compete with the binding of [U-14C]fructosyl-ε-aminocaproate in the equilibrium dialysis radioligand binding assay (Table 1). Of the compounds tested, only the Amadori products of glucose were strong competitive inhibitors but not the one of ribose (ε-ribosyl-lysine). Both ε- and α-fructosyllysine were inhibitors; however, the affinity of ABP for the α-amine derivative was half of that for ε-fructosyllysine. Complete inhibition of the ABP binding to [U-14C]fructosyl-ε-aminocaproate was observed also with proteinase-digested glycated proteins. Free amines and sugars, as well as carboxymethyllysine showed no or very low inhibitory effect. Surprisingly, when the binding assay was performed with NaBH₄-reduced fructosyl-ε-aminocaproate, 75% inhibition was observed. This is in contrast with the data from binding to borohydride-reduced glycated lysine-Sepharose and with the absence of inhibition with methylglucamine, a model compound of NaBH₄-reduced Amadori product.

When the binding of glycated-ε-aminocaproate to ABP was investigated by increasing concentration of the ligand, saturation was observed (Fig. 3). A dissociation constant of 1.49 μM and a maximal binding of 17.63 nmol/mg ABP (corresponding to approximately 0.8 mol of ligand/mol of protein) were calcu-

![Fig. 2. Binding of purified ABP to different affinity columns](image-url)
Amadori Binding Protein

A monoclonal antibody to ABP was produced for determination of ABP binding to glycated proteins and for evaluation of its presence in other microorganisms, including yeast and rat liver homogenate. The antibody was able to bind ABP as demonstrated by ELISA (Fig. 4). Western blotting revealed that ABP was present in the Pseudomonas soil organism grown in Amadori product, but absent in control strain as well as in several Gram-positive and -negative bacteria, yeast, and rat liver extract.

The ability of ABP to bind to glycated proteins immobilized in microtiter plates was investigated using an ELISA sandwich assay with the monoclonal anti-ABP antibody. Proteins (BSA, RNase) were reacted with d-glucose up to 30 days, and a time course experiment was performed. However, no ABP binding was detected as a function of glycation (not shown).

**DISCUSSION**

A novel protein that specifically binds Amadori products originating from glucose and low molecular weight amines was isolated from a soil strain of Pseudomonas sp., which was selected on the basis of its ability to utilize a synthetic Amadori product as the only carbon source to grow. ABP was isolated by a one-step chromatography on glycated lysine-Sepharose and was found to be highly specific in its substrate requirements, to the extent that only Amadori products originating from glucose could compete with glycate e-aminocaproate for its binding to ABP. This finding suggests that the ABP-ligand interaction is predominantly mediated by the sugar-derived portion of the Amadori product. The fact that the protein was not retained by glycated affinity substrate after NaBH₄ reduction further suggested that the Amadori product should be in the hemiketal configuration in order to be recognized by the binding protein.

In contrast to the findings above, investigation of ABP binding properties by equilibrium dialysis revealed that the NaBH₄-reduced fructosyl-ε-aminocaproate was able to compete with the binding of the unreduced Amadori product to ABP by 75% (Table I). However, methylglucamine, a compound identical with borohydride-reduced glycated methylamine did not inhibit the binding of fructosyl-ε-aminocaproate to ABP thereby raising the question of how to reconcile these observations. Carboxymethyl-lysine did not appear to be responsible for ABP binding specificity since it was not retained by the affinity substrate when glycation of lysine-Sepharose was carried out under deaerated conditions in presence of EDTA, thereby excluding the possibility that carboxymethyllysine instead of the Amadori compound was the actual ligand (27). This result was confirmed by the fact that carboxymethyllysine inhibited the binding of fructosyl-ε-aminocaproate by only 30%. These data, suggest that ABP recognizes both the carbohydrate and the alkyl side chain of the Amadori product in the region around the ketoamine bond, with the sugar portion, however, being predominant.

The ability of ABP to bind to glycated lysine-Sepharose and enzymatically digested glycated proteins but not to intact glycated proteins suggests presence of steric inhibition. The latter was apparently absent in lysine-Sepharose, possibly due to the spacer arm linking the ε-amino group of l-lysine to the beads. The high affinity of ABP for free ligands and the decreased affinity for protein-bound Amadori product is reminiscent of similar behavior of antihapten antibodies (38, 39).

While this study was in progress, we became aware of the work of Horiuchi (31), which described the purification and properties of a fructosyl-amino acid oxidase from a soil strain of Corynebacterium sp. This enzyme, of molecular mass 44 kDa,
decomposes Amadori products of α-L-amino acid to α-ketoaldehydes (glucosone) and α-L-amino acids under oxidative conditions. In a subsequent publication, Horiiuchi et al. (32) described a similar enzyme of 43 kDa isolated from a soil strain of *Aspergillus* sp. Evidence for deglycating activity of an *Aspergillus* crude extract was also found by Watanabe et al. (33), although the latter enzyme was not isolated. In both cases, the selection of the microorganism from the soil samples was performed using an Amadori product as sole carbon source in the culture medium. The similarity of the molecular mass of α-amino acid oxidase and ABP could have been identical. This possibility was, however, excluded by the negative results obtained from experiments to investigate the potential enzymatic activity of ABP. Moreover, immunoblotting experiments performed with fructosyl-amino acid oxidase generously donated by Dr. Horiiuchi showed absence of cross-reactivity between ABP and the enzyme.

The discovery of an Amadori binding protein in the soluble extract of a *Pseudomonas* soil strain grown on Amadori product as sole carbon source, raises the question of what physiological function it could have. Since we found membrane-bound periplasmic permease protein with transport function activity will have direct relevance for cellular processing of glycated proteins. It is currently unknown whether mammalian cells secrete proteins that can bind glycated low molecular weight amines. However, studies by Erbersdobler et al. (37) have shown that free glycated lysine is absorbed, taken up by the liver, as well as excreted, suggesting that some proteins may be involved in transporting glycated amino acids across membranes.

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