ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 300 (2003) 75–80

BBRC

www.elsevier.com/locate/ybbrc

Carnosine promotes the heat denaturation of glycated protein

George S. Yeargans and Norbert W. Seidler*

Department of Biochemistry, University of Health Sciences, 1750 Independence Avenue, Kansas City, MO 64106-1453, USA

Received 11 November 2002

Abstract

Glycation alters protein structure and decreases biological activity. Glycated proteins, which accumulate in affected tissue, are reliable markers of disease. Carnosine, which prevents glycation, may also play a role in the disposal of glycated protein. Carnosinylation tags glycated proteins for cell removal. Since thermostability determines cell turnover of proteins, the present study examined carnosine's effect on thermal denaturation of glycated protein using cytosolic aspartate aminotransferase (cAAT). Glycated cAAT (500 μM glyceraldehyde for 72 h at 37 °C) increased the $T_{0.5}$ (temperature at which 50% denaturation occurs) and the Gibbs free energy barrier (ΔG) for denaturation. The enthalpy of denaturation (ΔH) for glycated cAAT was also higher than that for unmodified cAAT, suggesting that glycation changes the water accessible surface. Carnosine enhanced the thermal unfolding of glycated cAAT as evidenced by a decreased $T_{0.5}$ and a lowered Gibbs free energy barrier. Additionally, carnosine decreased the enthalpy of denaturation, suggesting that carnosine may promote hydration during heat denaturation of glycated protein.
© 2002 Elsevier Science (USA). All rights reserved.

Keywords: Carnosine; Glyceraldehyde; Glycation; Cytosolic aspartate aminotransferase; Thermal denaturation

Protein glycation is the product of unwanted reactions between sugars and proteins [1]. Concurrent with decreased biological activity, glycated proteins exhibit chemical adducts [2–4] and peptide crosslinking [5,6]. Glycation contributes to reduced conformational flexibility [7] and increased compaction of protein structure [8,9]. Interestingly, glycated proteins accumulate at sites of pathology [3,10,11] presumably due to impaired proteolysis of glycated substrates [12].

Carnosine (β-alanyl-L-histidine), found in brain and heart (≅ 20 mM) [13], prevents glycation [4,14] and may be useful in treating disease [16–19]. Carnosine prevents protein modification by scavenging free radicals [19], lipid peroxidative products [20], and carbohydrate oxidation products [4,6,13,21,22]. These observations demonstrate that carnosine has anti-oxidant and anti-glycation properties.

A recent study [23] suggests another beneficial property of carnosine. Glycated proteins may become carnosinylated, tagging them for cell removal [24]. While we know that glycated proteins are carnosinylated, the mechanisms of cell disposal of glycated proteins remain poorly understood. Both prevention and removal of glycated proteins may be determinants of disease because cell accumulation of glycated proteins is a proven indicator of pathology [25,26].

Since thermal instability promotes the degradation of proteins [27], we examined the effects of glycation on thermal denaturation using cytosolic aspartate aminotransferase (cAAT). cAAT was chosen because aminotransferases are susceptible to glycation [2,28,29] and glycated cAAT is found in diabetic tissues [30]. Additionally, we looked at carnosine's effect on thermal denaturation of glycated proteins. We hypothesized that glycation decreases water accessibility making proteins more heat stable and that carnosine increases the thermal instability of proteins.

Materials and methods

Materials. cAAT (pig heart, cytosolic isoform) was purchased as a lyophilized solid (Sigma Chemical, St. Louis, MO). L-Carnosine, D,L-glyceraldehyde, 2,4,6-trinitrobenzenesulfonic acid, pyridoxal 5-phosphate, and β-alanine were purchased from Sigma. All other chemicals were of reagent grade.

* Corresponding author. Fax: 1-816 283-2357.
E-mail address: nseidler@uhs.edu (N.W. Seidler).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02796-1

Preparation of glycated cAAT. Unless otherwise indicated cAAT was incubated in a 100 mM sodium phosphate buffer (pH 7.4) containing glyceraldehyde (500 μM) for 3 days at 37 °C. Following incubation, samples were dialyzed to remove unbound glyceraldehyde using Spectra/Por CE DispoDialyzers 10,000 MWCO (Spectrum Laboratories, Rancho Dominques, CA) against (1:5000) a 100 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM sodium azide. Dialyzed samples were kept at 4 °C until assayed.

Glycation assay. Amine content was determined by a spectrophotometric procedure using 2,4,6-trinitrobenzenesulfonic acid (TNBS) [2]. cAAT (0.1 mg/mL) was incubated with the amine reagent TNBS (0.2 mM) in a 50 mM sodium phosphate buffer (pH 8.3) for 20 min at 52 °C. Samples were cooled and read at 420 nm using an Ultrospec 4000 spectrophotometer (Amersham Biosciences, Piscataway, NJ) with β-alanine as a standard.

Enzyme assay. cAAT activities were determined spectrophotometrically using a coupled-enzyme assay [2]. Samples were assayed at 37 °C in an 80 mM sodium phosphate buffer (pH 7.4) containing 200 mM L-aspartate, 12 mM α-ketoglutarate, 0.2 mM NADH, and 1.1 I.U. malate dehydrogenase using an Ultrospec 4000 spectrophotometer. Activities were calculated from absorbance changes using the extinction coefficient for NADH, 6.22 absorbance of 1 μmol NADH/mL at 340 nm in a 1 cm light path.

Fluorometric detection of AGEs. Fluorescence due to advanced glycation endproducts (AGEs) was determined using an LS50B luminescence spectrometer (Perkin–Elmer, Shelton, CT). Spectra were obtained following preparation of glyceraldehyde-modified cAAT and compared with unmodified cAAT controls. The excitation wavelength was 280 nm (slit width = 15 nm) and the emission spectra were from 350 to 550 nm (slit width = 5 nm) with a scanning speed of 600 nm/min.

Thermal denaturation. Thermal denaturation profiles of unmodified and glycated cAAT (40–200 μg/mL) were measured in a 100 mM sodium phosphate buffer (pH 7.4) using a quartz cuvette placed in a programmable Peltier-heated cell holder with an Ultrospec 4000 spectrophotometer. In the experiments involving carnosine, samples were read in the presence or absence of 20 mM carnosine. Denaturation was measured as an increase in light scattering (absorbance at 450 nm) due to the unfolding and aggregation that occurs upon thermal exposure. The heating rate was set at 0.5 °C/min from 60 to 94 °C. Following each run, the $T_{0.5}$ (the temperature at which 50% of the proteins are denatured) and the average start and end absorbances were calculated using the Swift program.

Calculations. The calculations were based on a two-state mechanism, N ↔ D, with N, native structure; D, denatured structure. The apparent equilibrium constants of denaturation were obtained from data using the following equation taken from Pace [31]: $K = \alpha_N - \alpha_i/\alpha_i - \alpha_D$, where $\alpha_N = \text{absorbance}_{450\,\text{nm}}$ of native cAAT, $\alpha_i = \text{absorbance}_{450\,\text{nm}}$ of intermediate cAAT structures, and $\alpha_D = \text{absorbance}_{450\,\text{nm}}$ of denatured cAAT. Van't Hoff curves were drawn by plotting $-R \ln K$ versus $1/T$ ($R = 1.987$ cal/mol/K). Enthalpy values (ΔH) were determined from the slope obtained by linear regression analysis using SigmaPlot (SPSS). The Gibbs free energy values (ΔG) were calculated from the equation $\Delta G = -RT \ln K$. Summative ΔGs were determined by adding the individually calculated values over the temperature range used in the experiments.

Molecular modeling. The primary sequence and secondary structure data were obtained from a public database (NCBI Sequence Viewer); information submitted by Rhee et al. [32]. The target segment (Leu 20–Leu 35) was entered into CS ChemDraw and CS Chem3D Programs (CambridgeSoft, Cambridge, MA). The molecular structure was viewed as the solvent accessible surface with the ratios of water expanding the van der Waals contours. In order to compare the structure of the unmodified segment with the glycated form of the same segment, the file was first duplicated; and then the structural information was changed to include AGE adducts: Arg–pyrimidine and carboxymethyl–Lys.

Results and discussion

Glyceraldehyde-induced glycation of cAAT

Incubation of cAAT with glyceraldehyde (500 μM, 72 h, 37 °C) caused a 48% decrease in activity (Table 1). Additionally, there was a 25% loss in amine content indicating that glyceraldehyde glycated cAAT via the Maillard reaction. Given the presence of 40 primary amines (ε-amines of lysine residues and α-amines of N-termini) in the pig heart dimer, the data suggest that 10 amines per dimer were modified. This observation is consistent with previous studies [9,28]. Lysine-derived glycation products include carboxymethyl–lysine and lysine–lysine crosslinks [3]. We also observed that fluorescent AGEs were formed (Table 1). Fluorescent AGEs are diverse heterocyclic structures such as pyrroles, pyrazines, pyrimidines, and imidazolones [3]. These data are consistent with previous work [2,4,33]. In addition to distinctive absorbance and fluorescent properties, heterocyclic AGEs are also hydrophobic.

Table 1
Properties of unmodified and glycated cAAT

	cAAT	Glycated cAAT
Amine content	165 ± 2.8	123 ± 11.3
Enzyme activity	209 ± 1.6	108 ± 0.1
Fluorescence	309.3	361.3

Amine content and enzyme activity are presented as means ± SD, given in nmol per mg protein and μmol/min per mg protein, respectively, and represent duplicate readings from two experiments. Fluorescence (280 nm excitation; 440 nm emission) is presented as rfu per mg protein from a representative experiment. The difference in fluorescence represents glycation-induced AGEs [2].

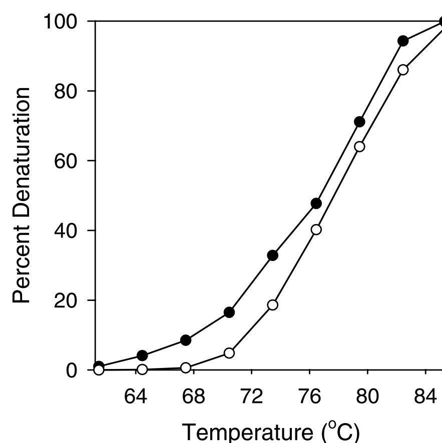

Fig. 1. Effects of protein glycation on thermal denaturation. Glycated cAAT was prepared by incubating cAAT in 100 mM sodium phosphate (pH 7.4) buffer containing 500 μM glyceraldehyde for 18 h at 24 °C prior to analysis of thermal denaturation. Closed circles, control cAAT. Open circles, glycated cAAT.

Thermal denaturation of glycated cAAT

We examined the thermostability of cAAT and glycated cAAT. Incubation of cAAT with glyceraldehyde (500 μM, 18 h, 24 °C) altered the thermal denaturation profile of the protein (Fig. 1). The denaturation curve of glycated cAAT shifted to the right of the unmodified cAAT, indicating a greater resistance to thermal unfolding. The $T_{0.5}$'s for unmodified and glycated cAAT were 74.6 °C ±1.06 (M ± SD) and 76.8 °C ±0.92, respectively (Table 2). There was a similar observation when cAAT was incubated with glyceraldehyde (500 μM) for 3 days at 37 °C increasing the $T_{0.5}$ by 2.1 °C. These findings corroborate previously published observations [7,8].

The Gibbs free energy barrier (ΔG) for thermal denaturation of cAAT was calculated for unmodified cAAT and glycated cAAT (500 μM, 72 h, 37 °C). The ΔG for glycated cAAT was over 5-fold greater than that for unmodified cAAT (Table 3). This difference suggests that a large thermodynamic barrier to thermal unfolding exists as a result of glycation. The enthalpy of denaturation (ΔH) for glycated cAAT was also higher than that for unmodified cAAT (Table 3), suggesting that glycation changes the water accessible surface. The structure of glycated cAAT is presumably in a misfolded state exhibiting significant changes in hydrophobic interactions.

Table 2
Effects of various reactive carbonyls on the thermal denaturation of cAAT

	$T_{0.5}$ (°C)	
	Unmodified cAAT	Modified cAAT
±Glyceraldehyde		
500 μM, 18 h, 24 °C	74.6	76.8
500 μM, 3 days, 37 °C	77.1	79.2
±Ribose 5-phosphate:[a]		
5 mM, 64 h, 37 °C	83.4	85.8
±Acrolein:[b]		
2 mM, 18 h, 37 °C	77.3	79.1

Data obtained as described under Materials and methods.
[a] Data from [7].
[b] Data from [8].

Proposed model of structural change to glycated cAAT

Each subunit (412 amino acids) in the cAAT dimer consists of a small and large domain. N-terminal residues (Ala 1–Glu 69) and C-terminal residues (Pro 298–Gln 412) make up the small domain, and residues Tyr 70–Asn 297 make up the large domain [32]. Residues Ala 1–Gln 11 extend from one subunit to form interactions with the neighboring subunit. Each of the two subunits in the cAAT dimer can assume two different conformations. An open-to-closed transition occurs upon substrate binding [34]. In the absence of substrate an "open" conformation exists. Upon binding of the substrate, the small domain shifts and the protein forms a "closed" conformation. The glycation experiments in this study involved incubations with cAAT in the "open" conformation.

The greatest flexibility occurs in the small domain. The following stretches of amino acids are isomorphous, that is, they do not change position upon substrate binding and hence exhibit less flexibility: Val 5–Gln 11, Leu 50–Thr 139, His 143–His 193, Thr 198–Ala 224, Glu 234–Asp 312, and Glu 318–Met 326. The isomorphous region consists of the residues that make up most of the large domain and only part of the small domain. The sequences in the small domain that exhibit enhanced flexibility include Ala 12–Val 49 and Ala 327–Gln 412, which contains an arginine–lysine (RK) pair (Arg 31–Lys 32) that represents a cluster of nucleophilic centers for glycation reactions. Given the greater intrinsic flexibility of these sequences in the small domain, there is a stronger likelihood that these amino acids would be chemically modified.

The RK sequence is found just following the first helix (Leu 16–Glu 26) and β turn (Asp 27–Pro 30) and at the beginning of the first β strand (Lys 32–Leu 35). Given the external location of this RK sequence and its position in a flexible region of the protein, we speculate that it would be a likely target for glycation. With this assumption we were interested in modeling the glycation-induced changes to this region and relate the model to the experimental observations.

Fig. 2 presents a proposed model of this RK-containing region (Leu 20–Leu 35), which includes the first helix, β turn, and β strand of the small domain. The molecular structure is depicted as the van der Waals surface plus that area inaccessible to water. The water inaccessible area of this 16-amino acid segment of cAAT

Table 3
Effects of carnosine on the thermodynamic properties of unmodified and glycated cAAT

	cAAT		Glycated cAAT	
	Minus carnosine	Plus carnosine	Minus carnosine	Plus carnosine
ΔH	103.6	111.7	112.3	97.4
ΔG	+2.0	−8.1	+11.0	+4.7

ΔH and ΔG are given in kcal/mol. Values were calculated as described in Materials and methods.

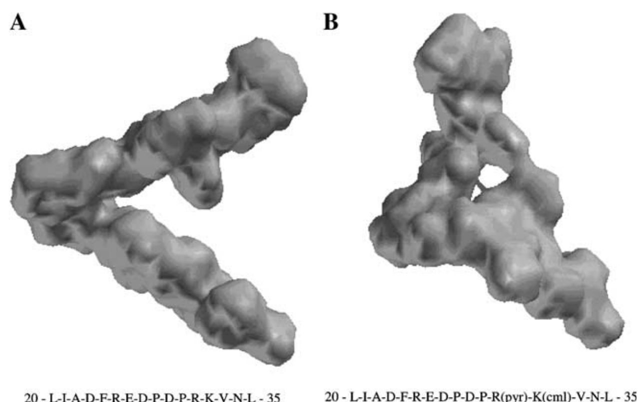

Fig. 2. Proposed model illustrating molecular surface changes due to protein glycation. (A) represents the water accessible surface of the described segment of unmodified cAAT (Leu 20–Leu 35); (B) represents the water accessible surface of the glycated form of this segment exhibiting decreased water accessibility. pyr, pyrimidine; cml, carboxymethyl.

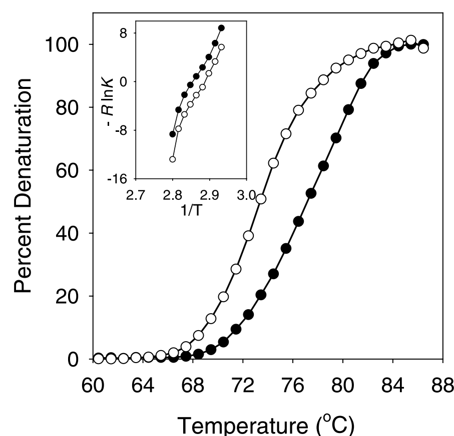

Fig. 3. Effects of carnosine on unmodified cAAT. Denaturation profiles, which were obtained as described in Materials and methods, were carried out in the presence (open circles) or absence (closed circles) of 20 mM carnosine. Inset: van't Hoff plot of unmodified cAAT with and without carnosine. Abscissa is given as K^{-1} ($\times 10^{-3}$).

is shown in its native and glycated state (Figs. 2A and B, respectively). The uppermost portion represents the helical structure (Leu 20–Glu 26) and the lower portion represents the β strand (Lys 32–Leu 35) with the bend depicting the β turn (Asp 27–Pro 30). The glycated form was made by converting the guanidine moiety to a pyrimidine at Arg 31 and by adding a carboxymethyl group to Lys 32. Arginine-derived pyrimidines and carboxymethyl-lysines are common AGEs [3].

The proposed model illustrates a localized compaction such that the protein segment now occupies a smaller space. Additionally, there is a greater molecular surface, which decreases water accessibility. This model is consistent with our data (Fig. 1; Tables 1–3) in which glycation affected thermal denaturation of cAAT. Glycated cAAT was more resistant to thermal denaturation than unmodified cAAT. Denaturation involves a dynamic change in hydration, which would be affected by glycation given the decreased accessibility of water as depicted in our model (Fig. 2).

Effects of carnosine on glycated cAAT

We examined the thermal denaturation profile of unmodified cAAT and glycated cAAT in the presence of carnosine. Carnosine enhanced the thermal denaturation of both unmodified cAAT (Fig. 3) and glycated cAAT (Fig. 4). The denaturation curves with carnosine were shifted to the left. The calculated $T_{0.5}$ with carnosine was consistently less than the $T_{0.5}$ without carnosine. Carnosine lowered the $T_{0.5}$ for unmodified cAAT by 3.8 °C (77.1 °C, without carnosine; 73.3 °C, with carnosine). Additionally, Table 3 presents the values for the Gibbs free energy barrier (ΔG) for denaturation. With unmodified cAAT carnosine lowered the ΔG from +2.0 kcal/mol (without carnosine) to −8.1 (with carnosine).

As previously mentioned glycated cAAT (500 μM glyceraldehyde for 3 days at 37 °C) increased the $T_{0.5}$ by 2.1 °C relative to unmodified cAAT. When glycated cAAT was tested in the presence of carnosine, the denaturation curve shifted to the left (Fig. 4) decreasing the $T_{0.5}$ by 2.4 °C (79.2 °C, without carnosine; 76.8 °C, with carnosine). Carnosine lowered the ΔG from +11.0 kcal/mol (without carnosine) to +4.7 (with carnosine) (Table 3), suggesting that carnosine promotes the unfolding of glycated protein.

Van't Hoff plots were drawn for unmodified cAAT (Fig. 3, inset) and glycated cAAT (Fig. 4, inset). All curves were linear as assessed by Pearson coefficients ($P < 0.01$). The slopes provided ΔH values, which are

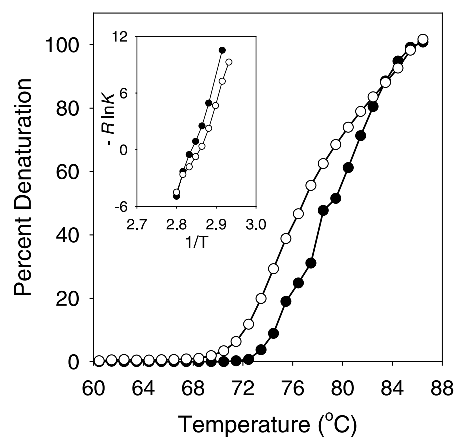

Fig. 4. Effects of carnosine on glycated cAAT. Glycated cAAT was prepared by incubating cAAT in 500 μM glyceraldehyde for 72 h at 37 °C. Denaturation profiles, which were obtained as described in Materials and methods, were carried out in the presence (open circles) or absence (closed circles) of 20 mM carnosine. Inset: van't Hoff plot of glycated cAAT with and without carnosine. Abscissa is given as K^{-1} ($\times 10^{-3}$).

presented in Table 3. The enthalpy difference (unmodified cAAT, 103.6 kcal/mol versus glycated cAAT, 112.3) is consistent with our model (Fig. 2), which suggests a change in peptide chain hydration during denaturation. The difference in enthalpies of glycated cAAT with carnosine and without carnosine (14.9 kcal/mol) further suggests that carnosine lowers the heat capacity during denaturation presumably by altering the hydration effects of glycated cAAT.

Carnosine protects against glycation of cAAT [4,6] and subsequent loss of activity [13]. Additionally, carnosine prevents neural cell toxicity [35] and protects tissues against ischemic [36] and thermal [37] injury. These effects are likely due to direct reaction with glycating agents [33].

Interestingly, carnosine reverses cataracts [38] and accelerates wound healing [39], both of which cannot be explained by carnosine's protective properties. The mechanism for removal of precipitated lens proteins in cataracts and the healing of damaged tissues may be due to the proposed role of carnosine in promoting protein turnover, particularly when proteins become chemically modified. Evidence is suggested in the observation that carnosine interferes with β-amyloid peptide nucleation and aggregation [15], a model for Alzheimer's disease.

Protein turnover in cells is a two-step process involving first a localized thermal unfolding, which is typically rapid, and then a slower proteolytic event [40]. Carnosine may initiate the early step in protein removal by assisting in the unfolding of damaged proteins. The mechanism of removal of damaged protein is crucial to understanding the potential utility of carnosine and carnosine-derivatives as therapeutic agents.

In summary, we observed that glyceraldehyde glycated cAAT, as evidenced by decreased activity, decreased amine content, and increased fluorescent AGEs. Glycated cAAT resisted thermal denaturation, increasing the $T_{0.5}$, Gibbs free energy barrier and enthalpy for denaturation. Carnosine promoted thermal denaturation of glycated cAAT by lowering the Gibbs free energy barrier and affecting peptide hydration during heat denaturation. We also propose a model in which glycated proteins are more compact with decreased accessibility to water.

Acknowledgments

The authors gratefully thank the participation of Timothy G. Morgan in the preparation of the manuscript. This study was supported in part by a grant from the Division of Research at the University of Health Sciences.

References

[1] H. Vlassara, M.R. Palace, Diabetes and advanced glycation endproducts, J. Intern. Med. 251 (2002) 87–101.

[2] C. Fitzgerald, T.A. Swearengin, G. Yeargans, D. McWhorter, B. Cucchetti, N.W. Seidler, Non-enzymatic glycosylation (or glycation) and inhibition of the pig heart cytosolic aspartate aminotransferase by glyceraldehyde 3-phosphate, J. Enzyme Inhib. 15 (1999) 79–89.

[3] J.W. Baynes, S.R. Thorpe, Glycoxidation and lipoxidation in atherogenesis, Free Radic. Biol. Med. 28 (2000) 1708–1716.

[4] N.W. Seidler, G.S. Yeargans, Effects of thermal denaturation on protein glycation, Life Sci. 70 (2002) 1789–1799.

[5] P. Chellan, R.H. Nagaraj, Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes, Arch. Biochem. Biophys. 368 (1999) 98–104.

[6] N.W. Seidler, Carnosine prevents the glycation-induced changes in electrophoretic mobility of aspartate aminotransferase, J. Biochem. Mol. Toxicol. 14 (2000) 215–220.

[7] N.W. Seidler, I. Seibel, Glycation of aspartate aminotransferase and conformational flexibility, Biochem. Biophys. Res. Commun. 277 (2000) 47–50.

[8] J.L. Southwell, G.S. Yeargans, C. Kowalewski, N.W. Seidler, Acrolein modifies and inhibits cytosolic aspartate aminotransferase, J. Enzyme Inhib. Med. Chem. 17 (2002) 19–23.

[9] N.W. Seidler, C. Kowalewski, Methylglyoxal-induced glycation affects protein topography, Arch. Biochem. Biophys. (2003), in press.

[10] A. Gugliucci, M. Bendayan, Histones from diabetic rats contain increased levels of advanced glycation endproducts, Biochem. Biophys. Res. Commun. 212 (1995) 56–62.

[11] H.P. Hammes, A. Alt, T. Niwa, J.T. Clausen, R.G. Bretzel, M. Brownlee, E.D. Schleicher, Differential accumulation of advanced glycation end products in the course of diabetic retinopathy, Diabetologia 42 (1999) 728–736.

[12] K. Sebekova, R. Schinzel, H. Ling, A. Simm, G. Xiang, M. Gekle, G. Münch, S. Vamvakas, A. Heidland, Advanced glycated albumin impairs protein degradation in the kidney proximal tubules cell line LLC-PK1, Cell. Mol. Biol. 44 (1998) 1051–1060.

[13] M.C. Jackson, J.F. Lenney, The distribution of carnosine and related dipeptides in rat and human tissues, Inflamm. Res. 45 (1996) 132–135.

[14] T.A. Swearengin, C. Fitzgerald, N.W. Seidler, Carnosine prevents glyceraldehyde 3-phosphate-mediated inhibition of aspartate aminotransferase, Arch. Toxicol. 73 (1999) 307–309.

[15] G. Münch, S. Mayer, J. Michaelis, A.R. Hipkiss, P. Riederer, R. Muller, A. Neumann, R. Schinzel, A.M. Cunningham, Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of β-amyloid peptide, Biochim. Biophys. Acta 1360 (1997) 17–29.

[16] H. Hiraishi, T. Sasai, T. Oinuma, T. Shimada, H. Sugaya, A. Terano, Polaprezinc protects gastric mucosal cells from noxious agents through antioxidant properties in vitro, Aliment. Pharmacol. Ther. 13 (1999) 261–269.

[17] J.W. Lee, H. Miyawaki, E.V. Bobst, J.D. Hester, M. Ashraf, A.M. Bobst, Improved functional recovery of ischemic rat hearts due to singlet oxygen scavengers histidine and carnosine, J. Mol. Cell. Cardiol. 31 (1999) 113–121.

[18] S. Dukic-Stefanovic, R. Schinzel, P. Riederer, G. Münch, AGEs in brain ageing: AGE-inhibitors as neuroprotective and anti-dementia drugs? Biogerontology 2 (2001) 19–34.

[19] G.I. Klebanov, Yu.O. Teselkin, I.V. Babenkova, I.N. Popov, G. Levin, O.V. Tyulina, A.A. Boldyrev, Yu.A. Vladimirov, Evidence for a direct interaction of superoxide anion radical with carnosine, Biochem. Mol. Biol. Int. 43 (1997) 99–106.

[20] A.R. Hipkiss, V.C. Worthington, D.T. Himsworth, W. Herwig, Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite, Biochim. Biophys. Acta 1380 (1998) 46–54.

[21] A.R. Hipkiss, H. Chana, Carnosine protects proteins against methylglyoxal-mediated modifications, Biochem. Biophys. Res. Commun. 248 (1998) 28–32.

[22] A.R. Hipkiss, J. Michaelis, P. Syrris, Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent, FEBS Lett. 371 (1995) 81–85.

[23] C. Brownson, A.R. Hipkiss, Carnosine reacts with a glycated protein, Free Radic. Biol. Med. 28 (2000) 1564–1570.

[24] A.R. Hipkiss, C. Brownson, Carnosine reacts with protein carbonyl groups: another possible role for the anti-ageing peptide, Biogerontology 1 (2000) 217–223.

[25] T. Miyata, S. Sugiyama, D. Suzuki, R. Inagi, K. Kurokawa, Increased carbonyl modification by lipids and carbohydrates in diabetic nephropathy, Kidney Int. Suppl. 56 (1999) S54–S56.

[26] E.B. Frye, T.P. Degenhardt, S.R. Thorpe, J.W. Baynes, Role of the Maillard reaction in aging of tissue proteins. Advanced glycation endproduct-dependent increase in imidazolium cross-link in human lens proteins, J. Biol. Chem. 273 (1998) 18714–18719.

[27] D.A. Parsell, R.T. Sauer, The structural stability of a protein is an important determinant of its proteolytic susceptibility in *Escherichia coli*, J. Biol. Chem. 264 (1989) 7590–7595.

[28] M. Okada, A. Sogo, N. Ohnishi, Glycation reaction of aspartate aminotransferase by various carbohydrates in an in vitro system, J. Nutri. Biochem. 5 (1994) 485–489.

[29] M. Beranek, J. Drsata, V. Palicka, Inhibitory effect of glycation on catalytic activity of alanine aminotransferase, Mol. Cell. Biochem. 218 (2001) 35–39.

[30] M. Okada, Y. Murakami, E. Miyamoto, Glycation and inactivation of aspartate aminotransferase in diabetic rat tissues, J. Nutr. Sci. Vitaminol. 43 (1997) 463–469.

[31] C.N. Pace, The stability of globular proteins, CRC Crit. Rev. Biochem. 3 (1975) 1–43.

[32] S. Rhee, M.M. Silva, C.C. Hyde, P.H. Rogers, C.M. Metzler, D.E. Metzler, A. Arnone, Refinement and comparisons of the crystal structures of pig cytosolic aspartate aminotransferase and its complex with 2-methylaspartate, J. Biol. Chem. 272 (1997) 17293–18302.

[33] N.W. Seidler, S.S. Shokry, J. Nauth, Properties of a glycation product derived from carnosine, J. Biochem. Molec. Biol. Biophys. 5 (2000) 153–162.

[34] C.A. McPhalen, M.G. Vincent, D. Picot, J.N. Jansonius, A.M. Lesk, C. Chothia, Domain closure in mitochondrial aspartate aminotransferase, J. Mol. Biol. 227 (1992) 197–213.

[35] A.R. Hipkiss, J.E. Preston, D.T.M. Himsworth, V.C. Worthington, N.J. Abbot, Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells, Neurosci. Lett. 283 (1997) 135–138.

[36] S.L. Stvolinsky, M.L. Kukley, D. Dobrota, M. Matejovicova, I. Tkac, A.A. Boldyrev, Carnosine: an endogenous neuroprotector in the ischemic brain, Cell. Mol. Neurobiol. 19 (1999) 45–56.

[37] L.I. Deev, E.N. Goncharenko, A.A. Baizhumanov, M.Ia. Akhalaia, S.V. Antonova, S.V. Shestakova, Protective effect of carnosine in hyperthermia, Biull. Eksp. Biol. Med. 124 (1997) 50–52.

[38] M.A. Babizhayev, A.I. Deyev, V.N. Yermakova, Y.A. Semiletov, N.G. Davydova, V.S. Doroshenko, A.V. Zhukotskii, I.M. Goldman, Efficacy of *N*-acetylcarnosine in the treatment of cataracts, Drugs R. D. 3 (2002) 87–103.

[39] P.R. Roberts, K.W. Black, J.T. Santamauro, G.P. Zaloga, Dietary peptides improve would healing following surgery, Nutrition 14 (1998) 266–269.

[40] H.A. Johnson, K.O. Devaney, Thermal instability in the endoplasmic reticulum of the rat hepatocyte, Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 40 (1982) 357–364.