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Glycation of Aspartate Aminotransferase and Conformational Flexibility

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Glycation of proteins alters biological function and changes cellular processes. Our study investigated the conformational changes that accompany glycation using the cardiac aspartate aminotransferase (cAAT). We examined the effects of brief and prolonged exposure of cAAT to glyceraldehyde (Glyc) and ribose 5-phosphate (R5P). When cAAT was briefly incubated (3.5 h) with Glyc (500 μM) or R5P (5 mM) at 37°C, cAAT activity and 1-anilinonaphthalene 8-sulfonate (ANS) binding increased relative to control. After prolonged incubation (64 h) with Glyc (500 μM) or R5P (5 mM) at 37°C, activity and ANS binding decreased relative to control. Furthermore, upon prolonged incubation of cAAT with 500 μM Glyc (14.5 h) or 2 mM R5P (64.25 h) at 37°C, the denaturation curves shifted to the right relative to control. We conclude that upon brief incubation with Glyc and R5P, cAAT exhibited a more open and flexible structure and upon prolonged incubation, a more rigid structure.

Key Words: **aspartate aminotransferase; glycation; glyceraldehyde; ribose 5-phosphate; 1-anilinonaphthalene 8-sulfonate.**

The cardiac aspartate aminotransferase (cAAT) is a useful tool in studying the effects of protein glycation. We previously used cAAT to study the anti-glycation properties of L-carnosine (1–3). cAAT resists thermal denaturation presumably due to its arrangement of α-helices (4). Despite its structural stability, cAAT is readily modified by glycation (nonenzymatic glycosylation) (5).

Several glycating agents modify AAT (6). Glyceraldehyde 3-phosphate, a glycolytic intermediate, glycates (2) the cardiac cytosolic isoform and inhibits its activity (5). Additionally, the rat liver cytosolic AAT is susceptible *in vivo* glycation (7). Glyceraldehyde and ribose are more potent as glycating agents than hexoses (8). The current study involved the use of glyceraldehyde (Glyc) and ribose 5-phosphate (R5P) as glycating agents.

Protein glycation, which occurs from lipid- and sugar-derived carbonyl compounds, alters protein structure (9). Crosslinking is the most commonly studied consequence of protein glycation (10, 11). Changes in proteolytic susceptibility (12), fluorescence (5), and transition metal binding (13) are additional features of protein glycation.

We examined the protein structural changes to cAAT as a result of exposure to the glycating agents Glyc and RP5. Interestingly, the effects of brief incubation on conformational mobility were opposite those of prolonged incubation. We assessed conformational flexibility by measuring the binding of the hydrophobic fluorescent probe 1-anilinonaphthalene 8-sulfonate (ANS), assaying enzyme activity and determining thermal denaturation curves.

MATERIALS AND METHODS

Glyceraldehyde, ribose 5-phosphate, cAAT, 1-anilinonaphthalene 8-sulfonate (ANS) and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade. cAAT (1 mg/ml) was incubated with Glyc (0–500 μM) and R5P (0–5 mM) at 24–37°C for various durations. Following incubation, samples were assayed, and comparisons were made using *t* tests (paired and unpaired) and linear regression analysis.

ANS fluorescence was used to measure changes in cAAT conformation (14). The excitation/emission wavelengths were 365 nm/460 nm with 7 nm and 15 nm slit widths, respectively. Relative fluorescence units (rfu) were obtained at 24°C using a 10 mM HEPES (pH 7.4) buffer containing ANS (15 μM) and cAAT (20 μg prot/ml). The fluorometer (Hoefer DQ 200, Amersham Pharmacia Biotech, Piscataway, NJ) was blanked with water and was calibrated with 10 mM HEPES (pH 7.4) that was set at 10. Fluorescence obtained from ANS and cAAT controls were subtracted from the samples. Fluorescence values, which were obtained from glycated cAAT samples, were normalized by setting the fluorescence values from unmodified cAAT samples to 100%.

A coupled-enzyme assay was used to measure cAAT activity (5). Samples were diluted in a 100 mM sodium phosphate buffer (pH 7.4) containing 1 mM pyridoxal 5-phosphate prior to assay 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 dehydroge-

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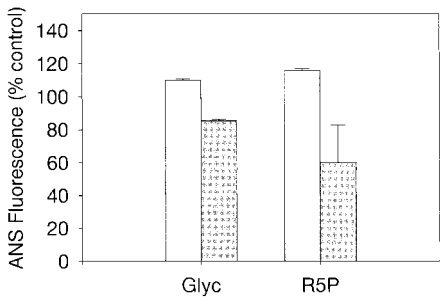

FIG. 1. ANS fluorescence as a function of brief and prolonged incubation of cAAT with Glyc and R5P. ANS fluorescence was measured as described under Materials and Methods following cAAT incubation in Glyc (500 μM) and R5P (5 mM) for 3.5 h (light gray bars) and 64 h (dark gray bars) at 37°C. Relative fluorescence units are given as a percentage of control which was set at 100%. Each group statistically differed from its respective control.

nase. Activity was presented as μmole/min per mg cAAT prot from calculations using the extinction coefficient for NADH. Glyc and R5P concentrations in the assay medium were below 10 μM.

cAAT thermal stability was determined by measuring denaturation as a function of temperature. Samples were assayed in a 10mM HEPES (pH 7.4) buffer containing 90 μg prot/ml. Denaturation was measured by light scattering (nonspecific absorbance at 450 nm) (35). A programmable Peltier-heated cell holder was used in an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech). The heating rate was set at 1.0°C/min with starting and ending temperatures set at 60°C and 94°C, respectively.

RESULTS AND DISCUSSION

We report on the differential effects of brief and prolonged incubation of glycating agents on the structure of cAAT. Following brief incubation with glycating agents, our observations suggest that the protein structure of cAAT acquired a more open conformation. Conversely, following prolonged incubations, we present evidence that the protein conformation became more rigid. The literature almost exclusively contains data showing the effects of prolonged incubation of glycating agents on target proteins. The present study presents novel evidence that brief incubation of glycating agents may exert effects that are opposite those of prolonged incubation. Furthermore, we present novel findings regarding changes in resistance to thermal denaturation.

ANS fluorescence was dependent upon the length of incubation with glycating agents (Fig. 1). When cAAT was briefly incubated with Glyc (500 μM) and R5P (5 mM) individually at 37°C for 3.5 h the fluorescence of ANS increased relative to control (Fig. 1, light gray bars). ANS is a useful probe for examining conformational changes (14) and refolding dynamics (15). ANS fluorescence increases upon protein binding (16), and ANS binding sites on proteins are hydrophobic in nature with evidence of hydrophilic contacts (17). Our observations suggest that ANS binding to modified cAAT increased relative to control. Increased ANS binding may be a consequence of a conformational change protein such that the modified cAAT exists in a more open or flexible state relative to control.

Additionally, brief incubation of cAAT (3 h at 37°C) with Glyc (125 μM) and R5P (1.25 mM) individually resulted in an increase in enzyme activity (Table 1). Due to the inter-assay variability paired *t* tests were applied to assess statistical significance between the differences that were observed. These observations are consistent with the ANS fluorescence data suggesting that brief exposure to glycating agents promote a change in protein conformation that may include increased domain mobility, which may translate into greater catalytic rates.

Prolonged incubation with glycating agents had the opposite effect. After cAAT was incubated for 64 h with Glyc (500 μM) and R5P (5 mM) individually (Fig. 1, dark gray bars) at 37°C, ANS fluorescence decreased. These observations suggest that the conformation of modified cAAT changed to a more compact structure resulting in decreased ANS binding.

Incubation of cAAT with Glyc effects the thermal denaturation curves of cAAT (Fig. 2). Upon prolonged incubation of cAAT with Glyc (500 μM Glyc for 14.5 h at 37°C) the denaturation curve shifted to the right, suggesting that a higher temperature is required to denature glycated cAAT. The Tm's (temperature at which half of the proteins are denatured) were determined for the denaturation curves of cAAT samples incubated in various concentrations of Glyc (Fig. 2, inset). The Tm increased with increasing concentrations of Glyc. These observations suggest that glycated cAAT may exist in a more compact and rigid conformation resisting the denaturing effects of temperature.

We examined the relationship of Glyc-mediated loss of function and resistance to thermal denaturation. Activity was measured following prolonged cAAT incubation with Glyc (0–500 μM) for 14.5 h at 37°C. We plotted cAAT activity as a function of Tm. The enzyme activity of Glyc-modified cAAT correlated inversely with the calculated Tm's (Fig. 3). These observations

TABLE 1

Effects of Brief Incubations of Glyceraldehyde and Ribose 5-Phosphate on cAAT Activity

Experiment	Control	+125 μM Glyc	+1.25 mM R5P
1	276.8	285.3	277.9
2	279.2	288.6	282.1
3	262.1	270.5	270.5
4	267.5	276.9	279.6
Mean	271.4	280.3	277.5
SD	±7.99	±8.20	±4.99
Significance		$P < 0.001$	$P < 0.05$

Note. Treated samples were compared with controls using paired *t* tests with matched samples with a one-tailed criteria for significance. SD, standard deviation.

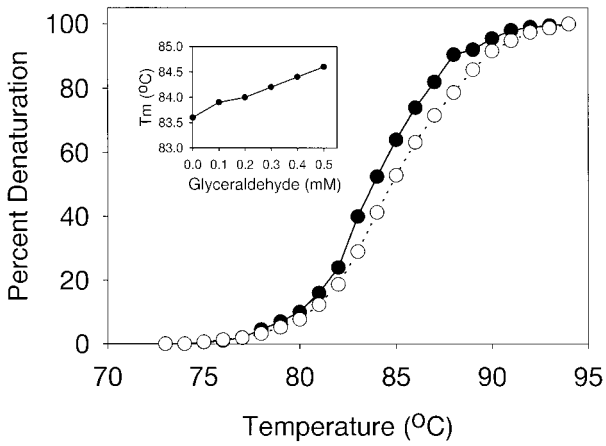

FIG. 2. Denaturation curves of unmodified and Glyc-modified cAAT. Denaturation curves were determined as described under Materials and Methods following cAAT incubation with (○) and without (●) Glyc (500 μM) for 14.5 h at 37°C. Inset: Tm is presented as a function of [Glyc] (14.5 h at 37°C). Linear regression analysis gave an $r = 0.994$, $P < 0.005$ (one-tailed), indicating a direct correlation.

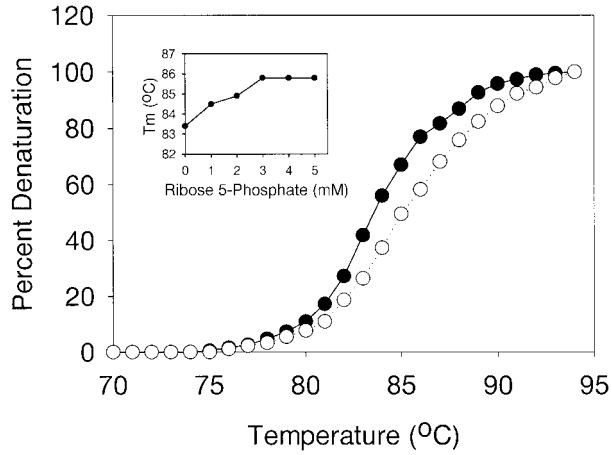

FIG. 4. Denaturation curves of unmodified and R5P-modified cAAT. Denaturation curves were determined as described under Materials and Methods following cAAT incubation with (○) and without (●) R5P (2 mM) for 64.25 h at 37°C. Inset: Tm is presented as a function of [R5P] (64.25 h at 37°C). Linear regression analysis gave an $r = 0.923$, $P < 0.005$ (one-tailed), indicating a direct correlation.

suggest that the change in conformation may contribute to the loss of functional activity.

Likewise, prolonged incubation of cAAT with R5P effects the thermal denaturation curves of cAAT (Fig. 4). The denaturation curve of R5P-modified cAAT (2 mM R5P for 64.25 h at 37–C) shifted to the right. The Tm's were determined for the denaturation curves of cAAT samples incubated in various concentrations of R5P (Fig. 4, inset). The Tm increased with increasing concentrations of R5P with an apparent maximal Tm, which was reached at 3–5 mM R5P. Additionally, the enzyme activity of R5P-modified cAAT correlated inversely with the calculated Tm's (Fig. 5). Activity measurements were made following prolonged cAAT incubation with 0–5 mM R5P (64.25 h at 37°C). These observations are consistent with those obtained with Glyc. The data suggest that protein glycation from prolonged incubation with Glyc or R5P caused a conformational change that resists denaturation and that is incompatible with catalytic activity.

The literature contains evidence regarding the cellular and molecular effects of protein glycation. Protein glycation causes crosslinking (10, 11), changes proteolytic susceptibility (12), increases transition metal binding (13), increases protein fluorescence (5), and alters signal transduction mechanisms (18). Glycation of proteins alter protein function (5), and glycation of small nitrogenous compounds yields fluorescent compounds (3), alter nutritive value (19) and produce genotoxic compounds (20). Reactivity of amino acids (21) and glycating agents (8) differ considerably. Glyc and ribose are considered fast glycating agents (8). We provide novel evidence that the duration of exposure to Glyc and R5P is crucial to determining the effects on

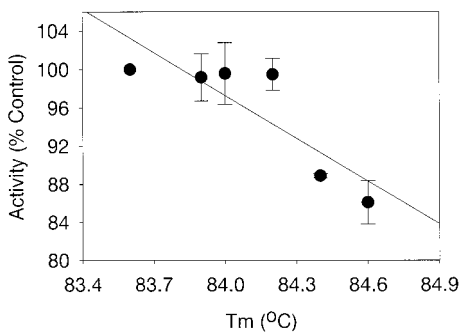

FIG. 3. Glyc-mediated loss of cAAT activity correlates with resistance to thermal denaturation. Following cAAT incubation with Glyc (0–500 μM) for 14.5 h at 37°C, activity and Tm's were determined and compared. Linear regression analysis gave an $r = 0.853$, $P < 0.025$ (one-tailed), indicating a correlation.

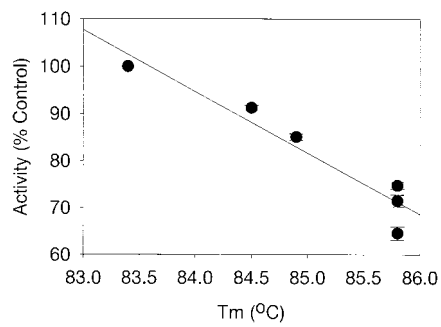

FIG. 5. R5P-mediated loss of cAAT activity correlates with resistance to thermal denaturation. Following cAAT incubation with R5P (0–5 mM) for 64.25 h at 37°C, activity and Tm's were determined and compared. Linear regression analysis gave an $r = 0.955$, $P < 0.005$ (one-tailed), indicating a correlation.

protein structure and function and that upon prolonged incubation with glycating agents protein conformation rigidifies as shown by decreased thermal denaturation and decreased ANS binding.

The biological consequence of increased protein rigidity may involve changes in protein turnover (12, 22). The inability to degrade an intracellular protein that is no longer useful may impair the balance of cellular proteases, which may impact other signal processes. Furthermore, uncatabolized protein may act an intracellular trap for other cellular waste products leading to particles such as lipofuscin.

In summary, we observed that upon brief incubation with glycating agents (Glyc and R5P), cAAT exhibited a more open and flexible structure as evidenced by increased activity and ANS binding. Upon prolonged incubation, cAAT exhibited a more rigid structure as evidenced by decreased activity, increased Tm and decreased ANS binding.

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