Proteasome Inhibition in Glyoxal-treated Fibroblasts and Resistance of Glycated Glucose-6-phosphate Dehydrogenase to 20 S Proteasome Degradation in Vitro*

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Glycation and glycoxidation protein products are formed upon binding of sugars to NH₂ groups of lysine and arginine residues and have been shown to accumulate during aging and in pathologies such as Alzheimer’s disease and diabetes. Because the proteasome is the major intracellular proteolytic system involved in the removal of altered proteins, the effect of intracellular glycation on proteasome function has been analyzed in human dermal fibroblasts subjected to treatment with glyoxal that promotes the formation of Nε-carboxymethyl-lysine adducts on proteins. The three proteasome peptidase activities were decreased in glyoxal-treated cells as compared with control cells, and glyoxal was also found to inhibit these peptidase activities in vitro. In addition, the activity of glucose-6-phosphate dehydrogenase, a crucial enzyme for the regulation of the intracellular redox status, was dramatically reduced in glyoxal-treated cells. Further analysis was performed to determine whether glycated proteins are substrates for proteasome degradation. In contrast to the oxidized glucose-6-phosphate dehydrogenase, both Nε-carboxymethyl-lysine- and fluorescent-glycated enzymes were resistant to degradation by the 20 S proteasome in vitro, and this resistance was correlated with an increased conformational stability of the glycated proteins. These results provide one explanation for why glycated proteins build up both as a function of disease and aging. Finally, Nε-carboxymethyl-lysine-modified proteins were found to be ubiquitinatinated in glyoxal-treated cells suggesting a potential mechanism by which these modified proteins may be marked for degradation.

Glycation and glycoxidation products are formed by the binding of sugars or of aldehyde/ketone adducts to the accessible free ε-NH₂ group of the basic amino acid constituents of a protein (1, 2). The end result of this process is the formation of several forms of advanced glycation/glycoxidation end products (AGE)‡ that alter the structure and function of proteins. Glycation and glycoxidation are well known to occur in the extracellular compartment during aging and in pathologies such as diabetes and Alzheimer’s disease (3–6). In the cytosol, the glycation process is more complex due to the fact that glycation agents can be generated not only by the catabolism of sugars but also by lipid, amino acid, as well as ascorbate metabolism (2, 7). Nε-Carboxymethyl-lysine (CML) and fluorescent AGE, such as pentosidine, are two forms of AGE known to accumulate in cells during aging (6, 8). CML brings additional negative charge to the glycated protein, whereas pentosidine and cyclic AGE formation leads to intra/intermolecular cross-links. In contrast to the complex network of antioxidant defenses that limit the deleterious effects of oxidation on macromolecules (9), the cellular defenses against other post-translational damage such as glycation have not been extensively studied (10, 11).

Indeed, no specific proteolytic pathway has been shown to degrade cytosolic AGE. Because these structures are present in aggregates of cross-linked proteins called AGE-pigment-like fluorophores, mainly accumulating in lysosomes during aging (8, 12, 13), a defect in the lysosomal degradation pathway has been suggested (6, 14, 15). In addition, AGE have been shown to be resistant to ATP-dependent proteolysis (16), and proteasome activity is impaired in kidney and liver of rats during diabetes, a condition that favors glycated protein formation (17).

The proteasome is the main cytosolic proteolytic complex responsible for the degradation of damaged protein and general protein turnover (18, 19). The 20 S proteasome, which is ubiquitin and ATP-independent, is involved in 70–80% of the selective recognition and degradation of the mildly oxidized proteins in the cytosol (19, 20). The 26 S proteasome, formed upon association of the 19 S regulatory complex with the 20 S catalytic core, has also been proposed to play a role in oxidized protein degradation in ubiquitin and ATP-dependent pathways (21, 22). An age-related decline of the peptidase activities of both the 20 S and 26 S proteasomes has been reported in cells and tissues during aging in rat (23–25) or in human (26–29). Therefore, the reported decrease of the 20 S proteasomal activity may explain, at least in part, the accumulation of highly oxidized and aggregated proteins in the cytosol of cells ob...
served during aging (30). Finally, it has been demonstrated that 4-hydroxy-2-nonenal (HNE) cross-linked proteins, as well as lipofuscin/ceroid fluorescent pigments, act as inhibitors of the proteasome (31–33) which may also contribute to the accumulation of these damaged proteins in aged cells.

In this study, the fate of the proteasome has been evaluated after treatment of human dermal fibroblasts with the carbonyl compound glyoxal under conditions that promote the formation of CML-modified proteins (34, 35). The status of the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) has also been investigated because it is the first enzyme in the pentose phosphate pathway that catalyzes the formation of NADPH, important for the maintenance of intracellular redox balance (36–38).

Furthermore, it has been demonstrated previously that G6PDH activity is decreased with age (39), is affected by oxidation, and that the oxidized enzyme is susceptible to proteolysis by the 20 S proteasome in vitro (40). Therefore, the susceptibility of CML- and fluorescent AGE-modified G6PDH to degradation by the 20 S proteasome has been investigated.

In contrast to the oxidized protein, both the CML- and fluorescent AGE-modified enzymes were resistant to degradation by the 20 S proteasome. To explain the observed resistance of glycated protein to proteolysis by the 20 S proteasome, we have investigated several structural parameters of CML- and fluorescent AGE-G6PDH as compared with the oxidized and native forms by using biophysical characterization.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—G6PDH from Leuconostoc mesenteroides was purchased from Worthington. Casein fluorescent isothiocyanate (38 μg of FITC/mg of protein) was purchased from Sigma. If not specified otherwise, all other chemicals were purchased from Sigma and were of highest analytical grade available.

**Rabbit anti-CML polyclonal antibody** raised against CML-modified bovine serum albumin was provided by Dr. H. Bakala (Université Denis Diderot, Paris 7). Rabbit immune sera raised against 20 S rat proteasome was obtained from Assay Research (Silver Spring, MD) as described previously (23).

**Quantification and Isolation of CML-modified Proteins from Fibroblasts**—Many cultures of human dermal fibroblasts were obtained from M. Moreau (Inserm U505, Université Pierre et Marie Curie, Paris 6) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO₂. Two days after seeding, cells were treated with 0.25 mM glyoxal for 1 week. The medium was changed every 2 days. Cells remaining viable during incubation, 37 °C, were harvested by centrifugation for 10 minutes at 10,000 × g.

Some were obtained from Assay Research (Silver Spring, MD) as described previously (27) to measure the amount of CML-modified protein in glyoxal-treated fibroblasts. Purification of CML-modified proteins was achieved using a CNBr-activated Sepharose column coupled with polyclonal anti-CML antibodies. Crude cellular extracts were loaded on the column previously equilibrated with PBS, pH 7.4, and then washed with the same buffer. The CML-modified proteins were eluted with 0.1 M glycine, pH 2.8, and the fractions were immediately neutralized with 2 M Tris-HCl, pH 8. The eluted proteins were subjected to SDS-PAGE on a 12% gel and electrophoresed onto a nitrocellulose membrane. The blot was then processed utilizing polyclonal anti-CML antibody (1/1000) and anti-ubiquitin antibody (1/1000) (Dako, Trappes, France).

**Preparation of the Different Modified Forms of G6PDH**—CML-modified G6PDH was prepared by reductive methylation of G6PDH (41). Briefly, 6 mg of G6PDH was incubated with 45 mM glyoxylic acid and 150 mM sodium cyanoborohydride for 24 h at 37 °C in 0.1 M sodium phosphate buffer, pH 7.8. Fluorescent AGE-modified G6PDH was prepared using the procedure of Grandhée and Monnier (42) with minor modifications. G6PDH (6 mg) was incubated with 100 mM ribose and 1 mm diethylenetriaminopentaacetic acid in 0.1 M sodium phosphate buffer, pH 9.0. After 1 week of incubation at 37 °C, samples were diluted 10 times with PBS, pH 7.4, and dialyzed twice for 24 h against the same buffer. Total cyclic fluorescent AGE were characterized by fluorescence analysis (excitation, 375 nm; emission, 445 nm) in a Kontron SFM 25 spectrofluorimeter (Saint-Quentin en Yvelines, France) and pentosidine content was measured using the method from Odetti et al. (43). Protein concentration was determined by the Lowry method (Bio-Rad), and the samples were stored at −20 °C. Metal-catalyzed oxidation of the enzyme was achieved as described previously (32). Prior to use, the various forms of G6PDH were chromatographed through a Microsec (Amersham Pharmacia Biotech) equilibrated with PBS, pH 7.4. The eluted fractions were concentrated by using a Centricon-30 system (Millipore, Bedford, MA).

**Measurement of G6PDH Activity**—The enzymatic reaction was performed at 25 °C by incubating 25, 50, or 75 μg of different forms of G6PDH diluted in 1 ml of 50 mM Hepes, pH 7.8, 100 mM KCl in the presence of 10 mM NADP⁺ and 100 μM glucose 6-phosphate. G6PDH activity was determined by following the appearance of NADPH spectrophotometrically at 340 nm for 5 min.

G6PDH activity in crude cellular extracts was determined by the same method using 250 μg of protein. Assaying G6PDH activity in biological samples is complicated by the presence of endogenous 6-phosphogluconate dehydrogenase, the second enzyme of the pentose phosphate pathway, which also produces NADPH. Therefore, the G6PDH activity of G6PDH was determined after inactivation of 6-phosphogluconate dehydrogenase by maleimide at a final concentration of 5 mM (44).

20 S Proteasome: Purification, Peptidase Activities, and Western Blot Analysis—20 S proteasome was purified from human placenta according to the procedure described previously (40) with minor modifications (45). Peptidase activities of the proteasome were assayed using fluorescent peptides, succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC), for the chymotrypsin-like activity, N-t-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin (LSTR-AMC) for the trypsin-like activity, and N-benzoyloxycarbonyl-Leu-Glu-Lys-β-naphthylamide (LLE-NA) for the peptideglutamyl-peptide hydrolase activity as described previously (27) with minor modifications. The mixture, containing 25 μg of crude homogenate total protein in 25 mM Tris-HCl, pH 7.5, was incubated at 37 °C with the appropriate peptide substrate (LLVY-AMC at 25 μM or LLE-NA at 150 μM or LSTR-AMC at 40 μM) in a final volume of 200 μl. Enzymatic kinetics were conducted in a temperature-controlled microplate fluorimeter reader (Fluostar Galaxy, bMG, Stuttgart, Germany). Excitation/ emission wavelengths were 350/440 nm and 340/410 nm for aminomethylcoumarin and β-naphthylamine, respectively. Peptases activities were plotted as total fluorescence against time and the peptidase activity of the crude extract in the presence of 20 μM proteasome inhibitor N-Cbz-Leu-Leu-phenylalanine (MG132). For inactivation experiments, 2 μg of purified proteasome were incubated with various concentrations of glyoxal for 30 min at room temperature. After preincubation, proteasome peptidase activities were determined as described above. The proteasome amount was estimated by Western blot analysis of cytosolic extracts. 20 μg of total proteins were separated on a 12% SDS-PAGE. Immunoblot experiments were then performed using anti-20 S proteasome polyclonal antibody (1/2500).

**Proteolytic Degradation of the Various Forms of G6PDH and FITC-Casein**—Proteolysis of the various forms of G6PDH at different concentrations was achieved in the presence of 0.3 μM of purified 20 S proteasome at 37 °C in PBS, pH 7.4. At different times, a 20-μl aliquot was removed and processed as described above. The proteasome activity was estimated by measuring the emission intensities of FITC fluorescent peptides at 515 nm (excitation at 495 nm). Peptidase activities were determined as total fluorescence and an aliquot of 20 μM ANSA to protein was obtained after subtraction of the emission of ANSA alone and for ANSA in the presence of the different forms of G6PDH. The proteasome activity was determined by following the appearance of ANSA fluorescence in a Kontron SFM 25 spectrofluorimeter (Saint-Quentin en Yvelines, France). Fluorescence increase occurring upon binding of ANSA to protein was obtained after subtraction of the emission.
spectra of ANSA alone.

Circular Dichroism Spectra—Native and modified forms of the protein were diluted to a final concentration of 1 mg/ml in 10 mM potassium phosphate buffer, pH 7.4, and dialyzed against the same buffer overnight at 4 °C. CD spectra were recorded in a Jasco-Jyv CD6 spectropolarimeter (Longjumeau, France) at 20 °C, using 1-cm or 0.1-mm path length cells for the near (250–320 nm) and far (190–260 nm) UV analysis, respectively. Each spectrum results from averaging five successive individual scans with a 0.5-nm step and an integration time of 2 s per step. Buffer contribution was eliminated by subtraction of the corresponding spectrum acquired under the same conditions.

Urea Denaturation—Denaturation was performed by incubating 0.3 µL of the different forms of G6PDH in 20 mM potassium phosphate, pH 7.2, with increasing concentration of urea (from 0 to 8 M). The mixture was gently stirred for 3 h at 20 °C. At each urea concentration, the fluorescence emission spectrum (300–450 nm, excitation 290 nm) of the sample was determined. The native enzyme fraction was obtained from the ratio F – Fd/Fn – Fd and plotted as a function of urea concentration. F is the fluorescence at 320 nm of the sample at a given concentration of urea; Fn and Fd are the fluorescence intensities (320 nm) of the sample at 0 M and 8 M urea, respectively.

RESULTS

Protein Modification and Proteasome Activity in Glyoxal-treated Cells—The effects of intracellular glycation on proteasome peptidase activities were analyzed in primary cultures of human dermal fibroblasts treated for 1 week with 0.25 mM glyoxal. These conditions induced the formation of CML-modified proteins without affecting cell viability. Indeed, using an enzyme-linked immunosorbent assay with anti-CML polyclonal antibody, intracellular CML-protein adducts exhibited a 5-fold increase in the treated cells as compared with the control cells (data not shown). The three main proteasome peptidase activities, chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities, were monitored in cellular extracts of treated and untreated fibroblasts. As shown in Fig. 1A, all three activities decreased significantly in the glyoxal-treated cells as compared with the control cells. To determine whether the observed decline in the proteasome peptidase activities was due to a decreased proteasome content, the amount of proteasome was estimated by Western blot analysis of cellular extracts, using an anti-proteasome polyclonal antibody (Fig. 1A). No change in proteasome content was detected in treated cells indicating that the loss of proteasome activity is likely due to an alteration of proteasome function. To determine whether the observed decline of proteasome activity could be explained by direct inactivation of the 20 S proteasome by glyoxal, peptidase activities were assayed after incubation of purified proteasome with various concentrations of this compound. The results presented in Fig. 1B indicate that all three proteasome peptidase activities were inhibited with 0.1 mM glyoxal and to a higher extent with increasing concentrations.

To examine whether CML-modified proteins are substrates for ubiquitin conjugation, an immunopurification of the crude extracts from glyoxal-treated and control cells was achieved on an anti-CML polyclonal antibody affinity column. After elution, the purified proteins were submitted to Western blot analysis using anti-CML and anti-ubiquitin antibodies. As shown in Fig. 2, the major bands corresponding to CML-modified proteins are also ubiquitinated, indicating these proteins may be marked for degradation by the 26 S proteasome. Among cytosolic enzymes that may be targets for glyoxal-induced modification, the fate of G6PDH was investigated because this enzyme has an essential lysine residue in its active site. Moreover, it plays a critical role in maintenance of intracellular redox status, and this enzyme is a well characterized model for the effects of various forms of modifications on susceptibility to proteasome degradation. We observed a 60% loss in G6PDH activity in glyoxal-treated cells defining this enzyme as a target for glyoxal-induced inactivation and also as a good model for
Proteasome Inactivation and Glycation

Characterization of the Various Modified Forms of G6PDH—Because CML-modified and fluorescent AGE-modified proteins are known to accumulate with age, these glycated forms of G6PDH were prepared and used to assess their susceptibility to proteolysis by the 20 S proteasome in vitro as described under “Experimental Procedures.” Oxidatively modified G6PDH was also prepared to be used as a positive control. The formation of CML on protein was revealed by immunoblotting experiments using anti-CML antibodies. Amino acid analysis indicates a loss of 4 lysines in the CML-modified G6PDH and a loss of 9 lysines plus 4 arginines in the fluorescent AGE-modified form (data not shown). Pentosidine content (2.25 nmol/mg of enzyme) was measured by high performance liquid chromatography analysis, and the presence of other cyclic AGE structures was detected by spectrofluorometry. No intermolecular cross-links were detected after SDS-PAGE indicating that this modified G6PDH exhibits only pentosidine or other cyclic AGE cross-links within the same polypeptide (data not shown). The activities of both oxidatively modified and CML-modified G6PDH declined 50%, whereas the fluorescent AGE-modified form was more resistant to proteolysis than the native form. Kinetic parameters for degradation of the various forms of G6PDH (Table I) were calculated from direct linear plots (not shown) according to the representation of Eisenthal and Cornish-Bowden (46). The apparent $K_M$ for the CML-modified G6PDH was almost the same as for the native enzyme (41.8 and 42.7 $\mu$M, respectively), whereas fluorescent AGE-modified and oxidized G6PDH exhibited a lower $K_M$ (13 and 8 $\mu$M, respectively). The $k_{cat}$ value was about the same for the native, the CML-modified, and the oxidized G6PDH, whereas this turnover number was decreased for the fluorescent AGE-modified G6PDH. As a substrate of the proteasome, the oxidatively modified protein was competing to almost 50% for the degradation of casein, whereas the CML- and the fluorescent AGE-modified protein did not exhibit an enhanced inhibitory effect as compared with the native enzyme (Fig. 3B).

Binding of the Hydrophobic Fluorescent Probe ANSA to the Modified Forms of G6PDH—Because glycated proteins build up both as a function of disease and aging, it was important to investigate potential reasons why these proteins are not recognized and degraded by the proteasome. Hydrophobic surface exposure has been proposed to be the recognition signal for 20 S proteasome degradation. To determine whether the glycated forms of G6PDH expose hydrophobic regions at their surface, we have monitored the binding of the hydrophobic fluorescent probe ANSA which exhibits an increase and a shift in fluorescence when associated with surface-exposed hydrophobic sequences (Fig. 4). As opposed to free radical oxidation of protein which results in increased ANSA binding, no binding was observed with the CML- and fluorescent AGE-modified G6PDH. Oxidative modification of G6PDH has also been shown to promote dissociation of the dimeric enzyme that may explain hydrophobic surfaces exposure at the monomer-monomer interface (40). As expected, no dissociation of the dimer was observed for both the native and the glycated forms of the enzyme using gel filtration on a Superose 6 column (Amersham Pharmacia Biotech) (data not shown).

Structural Properties of the Modified Forms of G6PDH—Structural changes that may occur upon G6PDH modification were analyzed by far- and near-UV CD for secondary and tertiary structural alterations, respectively. The CD spectra of oxidized, fluorescent AGE- and CML-modified were nearly identical in the far-UV region, indicating no change in secondary structure (Fig. 5A). The slight change observed for the oxidized protein at 190 nm is not accompanied by a change at

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**Table I**

| Substrate          | $K_M$  | $k_{cat}$ | $k_{cat}/K_M$ |
|--------------------|--------|-----------|---------------|
| Native             | 42.7 ± 1.11 | 17.6 ± 1.5 | 0.41          |
| CML-modified       | 41.8 ± 1.07 | 17.7 ± 0.3 | 0.42          |
| AGE-modified       | 13.0 ± 0.06 | 4.5 ± 0.2  | 0.35          |
| Oxidized           | 8.0 ± 0.05  | 16.7 ± 0.2 | 2.1           |

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further investigation of glycoprotein degradation by the proteasome.

**Fig. 3.** Effect of G6PDH modifications on proteasome-mediated proteolysis. A, proteolysis of native and modified forms of G6PDH (at 18 $\mu$M) by the proteasome was performed and monitored as described under “Experimental Procedures.” The rates of proteolysis are presented as percentage of the value obtained for native G6PDH (bars represent the mean ± S.E. of three independent experiments). B, proteolysis of FITC-casein by the proteasome in the presence of various forms of G6PDH. Proteolysis of FITC-casein was determined in the presence of native and modified G6PDH. The rate of FITC-casein degradation alone was set as 100%. The bars represent the S.E. of the mean for three independent experiments.
enzymes with different conformational stabilities. G6PDH is indicative of a heterogenous population of modified curves obtained for both CML- and fluorescent AGE-modified glycated forms of G6PDH appeared more structurally stable in the presence of increasing concentrations of urea. As shown in Fig. 6A, the thermal denaturation was followed by monitoring the enzymatic activity, the fluorescent AGE-modified enzyme at wavelengths greater than 290 nm can be explained by the absorbance properties of the AGE adducts. To assess potential changes in the structural stability of the modified forms of G6PDH, thermal denaturation experiments were performed at 45 °C as depicted in Fig. 6A. Because the thermal denaturation of the native and the various modified forms of G6PDH was assayed by equilibrium denaturation experiments in the presence of increasing concentrations of urea. As shown in Fig. 6B, glycated forms of G6PDH appeared more structurally stable than native enzyme, whereas the oxidized form of the enzyme was far less stable. Moreover, the shape of the denaturation curves obtained for both CML- and fluorescent AGE-modified G6PDH is indicative of a heterogenous population of modified enzymes with different conformational stabilities.

**DISCUSSION**

Modifications of protein by glycation/glycoxidation, as evidenced by the presence of CML, pentosidine, and vespertilysine adducts on proteins, are formed during the progression of various diseases and as a result of normal aging (4–6, 47). The present study was undertaken to determine whether the glycation process and the subsequent accumulation of glycated proteins affect the proteasomal system, which is responsible for intracellular degradation of ubiquitinated and oxidized proteins (18, 19). Indeed, proteasome function has been reported to be impaired during human aging as a result of both the decreases of its intrinsic activity and protein content (27, 30). Contributing factors may include the occurrence of structural modifications (28) or the inhibitory effects of cross-linked HNE-modified protein and lipofuscin (31–33). Protein glycation was induced by treatment of human dermal fibroblasts with glyoxal, a carbonyl compound, under conditions known to promote the formation of CML-modified proteins in cultured rat sensory neurons (35) and fetal lung cells (15). In this latter study, the proteolytic activity as well as the expression of lysosomal cathepsin D were shown to decrease, indicating that AGE formation and/or subsequent oxidative stress can alter the lysosomal protein degradation pathway. In the present study, we provide evidence that the proteasomal system is impaired in glyoxal-treated cells. Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities of the proteasome are significantly decreased, although no change in proteasome content could be detected (Fig. 1A). In *in vitro* incubation of purified 20 S proteasome with glyoxal indicated that glyoxal can inactivate all three proteasome peptidase activities (Fig. 1B). This finding raises the possibility that the proteasome is a target for glyoxal-mediated inactivation in *vivo*. However, the sensitivity of proteasome peptidase activities to glyoxal appears to be slightly different in glyoxal-treated cells as compared with that of 20 S proteasome in *in vitro*; the peptidylglutamyl-peptide hydrolase activity is the most sensitive to inhibition in *vivo* and the least sensitive in *in vitro*. This apparent
discrepancy can be explained by the fact that within cells the proteasome, one of the protein targets for glyoxal, is also present as a complex with the 19 S regulatory particle to form the 26 S proteasome. Moreover, cellular AGE-modified proteins have been shown to promote oxidative stress (48–50), and the proteasome peptidylglutamyl-peptide hydrolase activity is known to be readily inactivated by oxidants (23). In addition, an inhibitory effect of glycated proteins, as reported previously for HNE cross-linked proteins and lipofuscin (32, 33), cannot be excluded.

We also found that G6PDH in glyoxal-treated cells exhibits a dramatic decrease in activity. This inactivation could be due to the formation of CML on the active site lysine residue. This would be expected to result in a decline in reduced glutathione regeneration and subsequent alterations in redox control modulation. This, in turn, would affect the activity of GSH- and NADPH-dependent enzymes involved in the detoxification of reactive carbonyls, main precursors of AGE (11). The drop of G6PDH activity may also explain, at least in part, the increase in intracellular oxidative stress associated with AGE formation (49, 50). Interestingly, G6PDH activity, shown to be sensitive to oxidative stress (36–38), decreased with age (39).

G6PDH is a target for glyoxal-mediated inactivation that has been extensively studied with respect to the effects of various post-translational modifications on susceptibility to degradation by the 20 S proteasome (40, 51). Therefore, the susceptibility of glycated G6PDH to degradation by the 20 S proteasome was investigated in vitro. Two forms of glycated G6PDH, non-fluorescent, minimally modified by CML and highly modified by fluorescent AGE with intramolecular cross-links such as pentosidine, were assayed for the degradation by the 20 S purified proteasome. As opposed to oxidatively modified G6PDH which is a good substrate of the 20 S proteasome, these two forms of glycated proteins are no more susceptible to proteolysis by the proteasome than the native enzyme (Fig. 3A). In fact, as shown in Table I, almost similar $k_{cat}$ and $K_M$ values were obtained for CML-modified and native G6PDH. Due to a lower $k_{cat}$, fluorescent AGE-modified G6PDH appeared more resistant than the native enzyme, despite a lower $K_M$. Finally, in contrast to oxidized G6PDH, both native, CML-, and fluorescent AGE-modified G6PDH exhibited about the same specificity constant $k_{cat}/K_M$. These findings provide one explanation for the observed resistance of glycated proteins to proteolysis. The formation of intramolecular cross-links generated by cyclic fluorescent adducts, such as pentosidine, may explain this resistance toward proteasome degradation, as already observed with HNE cross-linked G6PDH (31, 32, 40).

Oxidized protein degradation by the 20 S proteasome has been studied extensively by different groups (20, 52–55). Mild oxidation leads to an increased proteolytic susceptibility, whereas highly oxidized protein becomes resistant to degradation by the 20 S proteasome. It has been shown that mildly oxidized proteins exhibit decreased thermal and thermodynamic stability (56, 57) and bind more efficiently the hydrophobic fluorescent probe ANSA (40). Both the exposure of hydrophobic surface on modified proteins and the loss of secondary structure have been proposed to act as a recognition signal for binding and degradation of the substrate protein by the 20 S proteasome (19, 54, 55). Because glycated proteins are poor substrates of the 20 S proteasome, structural studies of the glycated forms of G6PDH aimed at assessing their secondary and tertiary structure as well as their conformational stability were performed. These structural properties were then compared with those of the native and oxidized forms of the enzyme. As opposed to oxidized G6PDH, hydrophobic amino acids are not exposed at the surface of glycated proteins, as shown in ANSA-binding experiments (Fig. 4). Moreover, the secondary as well as the tertiary structures of these proteins, as monitored by far- and near-UV CD, respectively (Fig. 5, A and B), were nearly identical to those of the native counterpart. CML-modified G6PDH is more thermostable than the native enzyme (Fig. 6A), and both CML- and fluorescent AGE-modified G6PDH exhibit an increased resistance toward urea denaturation (Fig. 6B). Furthermore, the shape of the transition curves indicates that both CML- and fluorescent AGE-modified enzymes represent a heterogeneous population of modified proteins. Indeed, depending on the nature and the number of intramolecular cross-links (pentosidine and versiperylamine), the fluorescent AGE-modified proteins may have different conformational stabilities. Additional negative charges brought by the carboxymethylation reaction may stabilize the protein conformation as well. Taken together, these findings show that, as opposed to oxidative modification that promotes an increased flexibility and decreased conformational stability of the protein, glycation of G6PDH induces an increased structural stability that correlates with the observed resistance of these modified proteins to degradation by the 20 S proteasome.

We have shown that CML-modified proteins in glyoxal-treated cells are also ubiquitinated (Fig. 2). Interestingly, HNE-modified proteins generated upon oxidative stress were also found to be ubiquitinated (58). These observations raise the possibility that the ubiquitin-26 S proteasome pathway is implicated in the removal of these modified proteins. However, our in vitro results suggest that even if ubiquitinated chains are recognized by the 19 S complex, glycated proteins, as well

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**Fig. 6.** Thermal and conformational stabilities of the different forms of G6PDH. A, heat inactivation of the different forms of G6PDH. Native, oxidized, or CML-modified G6PDH (0.02 μM) was incubated at 45 °C for the indicated times. Heat inactivation was determined as described under “Experimental Procedures.” C, CML-modified G6PDH; ○, native G6PDH; ●, oxidized G6PDH. The points represent the means, and the bars the S.E. of the mean for three independent experiments. B, urea-induced denaturation of the various forms of G6PDH. Denaturation was performed by incubating 0.3 μM of the different forms of G6PDH with an increased amount of urea (from 0 to 8 M) and was followed by monitoring the fluorescence emission spectrum of the samples (300–450 nm, excitation 290 nm). The folded enzyme fraction was determined at 320 nm and plotted as a function of urea concentration as described under “Experimental Procedures.” ○, native G6PDH; ●, oxidized G6PDH; □, CML-modified G6PDH; +, fluorescent AGE-modified G6PDH.
as HNE-modified proteins, are resistant to degradation by the 20 S catalytic core of the proteasome. Future studies will address whether ubiquitination of CML-modified proteins is a marking step for their degradation by the 26 S proteasome. On the other hand, ubiquitination of glycated proteins, if not a 26 S marking step for their degradation by the 26 S proteasome, may also be a signal for their targeting to other pathways such as protein degradation by the lysosomal pathway (59, 60).

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