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Original article

Proteasomal degradation of glycated proteins depends on substrate unfolding: Preferred degradation of moderately modified myoglobin

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ABSTRACT

The Maillard reaction generates protein modifications which can accumulate during hyperglycemia or aging and may have inflammatory consequences. The proteasome is one of the major intracellular systems involved in the proteolytic degradation of modified proteins but its role in the degradation of glycated proteins is scarcely studied. In this study, chemical and structural changes of glycated myoglobin were analyzed and its degradation by 20S proteasome was studied. Myoglobin was incubated with physiological (5–10 mM), moderate (50–100 mM) and severe levels (300 mM) of glucose or methylglyoxal (MGO, 50 mM). Glycation increased myoglobin's fluorescence and surface hydrophobicity. Severe glycation generated crosslinked proteins as shown by gel electrophoresis. The concentration of advanced glycation endproducts (AGEs) N-ε-carboxymethyl lysine (CML), N-ε-carboxyethyl lysine (CEL), methylglyoxal-derived hydroimidazolone-1 (MG-H1), pentosidine and pyrraline was analyzed after enzymatic hydrolysis followed by UPLC-MS/MS. Higher concentrations of glucose increased all analyzed AGEs and incubation with MGO led to a pronounced increase of CEL and MG-H1. The binding of the heme group to apo-myoglobin was decreased with increasing glycation indicating the loss of tertiary protein structure. Proteasomal degradation of modified myoglobin compared to native myoglobin depends on the degree of glycation: physiological conditions decreased proteasomal degradation whereas moderate glycation increased degradation. Severe glycation again decreased proteolytic cleavage which might be due to crosslinking of protein monomers. The activity of the proteasomal subunit β5 is influenced by the presence of glycated myoglobin. In conclusion, the role of the proteasome in the degradation of glycated proteins is highly dependent on the level of glycation and consequent protein unfolding.

1. Introduction

The Maillard reaction describes the non-enzymatic reaction between reducing carbohydrates and amino acids, peptides or proteins and leads to the formation of a group of heterogeneous chemical structures. These so-called Amadori products are 1-amino-1deoxyketose structures, which are formed during early stages of the reaction. In further course stable reaction products called advanced glycation endproducts (AGEs) are formed [1–3]. The presence of AGEs in human tissues originating from the endogenous reaction of glucose and proteins, was linked to the development of age-related diseases or metabolic disorders, such as Alzheimer's or diabetes [4,5]. In the state of hyperglycemia when excessive levels of circulating glucose are present, the risk of protein modifications caused by the Maillard reaction increases [6–8]. It was shown previously that the occurrence of AGEs in vivo can lead to the production of promoters for oxidative stress [9] and inflammatory processes [10,11]. This may impact the development of multiple diseases and, therefore, the presence of high amounts of circulating AGEs is generally seen as potentially harmful. To prevent an excessive accumulation of modified proteins, cells are equipped with intracellular degradation systems and one of them is the proteasome [12,13]. The proteasome is a protein complex consisting of the 20S "core" proteasome and a set of regulator proteins that can change its activities and substrate specificities. The 20S proteasome is composed of two outer α-rings and two inner β-rings and each ring consists of seven subunits whereby only β-subunits show catalytic activity. In mammalian proteasome, three β-subunits show proteolytic activity of the following types: caspase-like (β1), trypsin-like (β2) and

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chymotrypsin-like (β5) [14]. In relation to advanced glycation end-products, the β2-subunit might be of special importance since it cleaves at lysine, arginine and cysteine residues [14] and those amino acids are the major targets of glycation reactions [15] and potential protein modifications might impact the degradation efficiency of this specific subunit. For continuous protein turnover, most proteins are tagged with ubiquitin to be recognized and degraded by the 26S proteasome. Structurally abnormal, misfolded proteins, such as unfolded or oxidized proteins are degraded ubiquitin-independently by the 20S proteasome whereby surface hydrophobicity is the driving factor for proteasomal recognition [16]. It was shown in a previous study of our group that glycated proteins impact proteasomal degradation. BSA modifications with high levels of glucose, fructose, ribose, glyoxal or MGO led to crosslinked proteins which were subsequently not degraded by the 20S proteasome [17]. Similar results were reported for glucose-6-phosphate dehydrogenase which was modified with glyoxylic acid or ribose [18]. The authors concluded that an increased conformational stability of the glycated proteins is responsible for the proteolytic resistance. In contrast, it was shown that the concentration of protein oxidation markers in diabetic compared to nondiabetic subjects do not differ and it was assumed that increased proteolysis might explain the unchanged levels [19,20].

A common drawback of studies dealing with proteasomal degradation of AGEs is the use of very high levels of glycation agents, which do not resemble physiological conditions (e.g. 5–10 mM glucose). Moreover, chemical characterization of glycated proteins is often performed superficially (e.g. confirmation of glycation only by fluorescence spectroscopy) and the conclusions drawn from those studies should be treated with caution.

The aim of our study was to modify the model protein myoglobin followed by extensive chemical characterization and relate those changes to proteasomal degradation. The heme protein myoglobin is the major oxygen-carrying protein in muscle cells and was chosen for the study due to its high abundance of potential glycation-sites in form of lysine residues and its monomeric structure [21]. Moreover, for the structurally similar protein hemoglobin it was shown that glycation leads to the formation of glycated hemoglobin HbA1c and that the abundance of this modification correlates with increased blood glucose levels [22,23]. In general, it was found that glycation of physiological proteins alters proteolysis by the ubiquitin-proteasome system (UPS) and lysosome/autophagy pathway (LPS) [24]. Whether glycation alters the degradability of myoglobin and the involvement of the ubiquitin-independent 20S proteasome was not studied until now. Our results show that glycation of myoglobin leads to an increase as well as to an impairment of proteasomal degradation, depending on the severity of structural changes due to glycation. The results contribute to a better understanding of the structural requirements for the proteolysis of glycated proteins.

2. Material and methods

2.1. Chemicals

Myoglobin (≥90%) was purchased from Worthington. Glucose, sodium dihydrogen phosphate, disodium hydrogen phosphate, HEPES, potassium chloride, sodium borohydride, sodium hydroxide, hydrochloric acid, trichloroacetic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium carbonate and potassium hydroxide were obtained from Carl Roth (Karlsruhe, Germany). Methylglyoxal, aminopeptidase solution, pepsin and prolidase were obtained from Sigma-Aldrich (Steinheim, Germany). Pronase E was from Merck (Darmstadt, Germany). Dansyl chloride, 8-Anilino-1-naphthalenesulfonic Acid (ANSA), formic acid and perfluoropentanoic acid (NFPA) were purchased from Alfa Aesar (Karlsruhe, Germany). Borate buffer (0.4 N, pH 10.2) was obtained from Agilent (Böblingen, Germany) and artificial proteasome substrates Suc-Leu-Leu-Val-Tyr-AMC and Ac-Arg-Leu-Arg-AMC were obtained from Enzo Life Sciences (Lörrach, Germany). All AGE standards (N-ε-carboxymethyllysine (CML), N-ε-carboxyethyllysine (CEL), pentosidine, pyrraline and methylglyoxal-hydroimidazolone 1 (MG-H1)) were purchased from Iris Biotech (Marktredwitz, Germany). LC-MS grade water was from VWR (Darmstadt, Germany) and LC-MS grade acetonitrile was from Merck (Darmstadt, Germany). For all experiments except LC-MS/MS, ultrapure water was used prepared by a Milli-Q Advantage A10 water system from Merck (Darmstadt, Germany).

2.2. Glycation of myoglobin

Myoglobin (10 mg/ml) was incubated in phosphate buffer (100 mM, pH 7.4) with varying concentrations of glucose (5, 10, 50, 100 and 300 mM) and MGO (50 mM) for 4 weeks at 37 °C. After incubation, the samples were dialyzed against distilled water for three days followed by lyophilisation of the retentate. A control of native myoglobin incubated in phosphate buffer without added glucose or MGO was used as a control. All lyophilized protein samples were stored at −20 °C until analyses.

2.3. Gel electrophoresis

SDS-PAGE was performed with a 15% polyacryamid gel (molecular weight of myoglobin is approximately 17 kDa). Samples containing 8 µg of protein were diluted with SDS sample buffer (0.25 M Tris pH 6.8 with 8% SDS and 40% glycerol) and heated to 100 °C for 7 min before application on the gel. Detection of proteins was performed by Coomassie staining.

2.4. Fluorescence measurements

To analyze fluorescence characteristics, 1 mg of protein retentate was dissolved in 1 ml HEPES buffer (50 mM, pH 7.8). The solutions were diluted 1:10 with buffer and 200 µL were transferred to a black microtiter plate. Fluorescence intensities were measured at λ_{ex} = 350 and λ_{em} = 450 nm (AGE fluorescence) and λ_{ex} = 280 and λ_{em} = 350 nm (tryptophan fluorescence) using a multimode reader Tecan Infinite M200 (Mainz, Germany).

2.5. 8-Anilino-1-naphthalenesulfonic acid (ANSA) binding

ANSA binding to the protein samples was analyzed as published previously [18] with slight modifications. Briefly, 25 µl of protein solution (1 mg/ml in 50 mM HEPES, pH 7.8; sample solutions) or 25 µl HEPES (blank solution) was incubated with 20 µl KCl (1 M in HEPES), 135 µl HEPES (50 mM, pH 7.8) and 20 µl ANSA (1 mM) in a black microtiter plate. An ANSA stock solution of 100 mM was prepared in DMSO and was further diluted in HEPES buffer. Samples were incubated at 37 °C for 30 min followed by measurement of fluorescence emission spectrum from 430 to 600 nm (λ_{ex} = 370 nm). Apo-myoglobin was prepared by Teale's method [25].

2.6. Acid hydrolysis

To release (modified) amino acids from their protein structure acid hydrolysis was performed according to Ref. [8]. Myoglobin was solubilized in ultrapure water (20 mg/ml) and an aliquot of 20 µl was incubated with 250 µl borate buffer (0.4 N, pH 10.2) and 250 µl sodium borohydride (1 M in 0.1 M NaOH) for 2h at room temperature to reduce early glycation products such as fructoselysine to avoid neo-formation of CML during heating in HCl. Protein precipitation was achieved by adding 1 ml of trichloroacetic acid (20%, w/v) and centrifugation (4 °C, 10000 rpm, 10 min). The supernatant was discarded, and the precipitate was washed with trichloroacetic acid (5%, w/v) and centrifugation was repeated. After removal of the supernatant, 1 ml of HCl

(6 M) was added to the protein pellet and the sample was heated at 110 °C for 23 h. The hydrolyzed samples were evaporated to dryness with a vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Karlsruhe). The residue was dissolved in 200 µl ultrapure water and after centrifugation (4 °C, 10000 rpm, 10 min) an aliquot was used for amino acid analysis.

2.7. Enzymatic hydrolysis

Since some glycation modifications are unstable towards acid treatment, proteins were additionally hydrolyzed by proteolytic cleavage [1]. Therefore, 25 µl of myoglobin solution (20 mg/ml in ultrapure water) were incubated with 33 µl HCl (40 mM) and 7 µl pepsin (2 mg/ml in 20 mM HCl; 29 U) at 37 °C for 24 h. The sample was then buffered at pH 7.4 by addition of 32 µl potassium phosphate buffer (500 mM, pH 7.4) and 7 µl of KOH (260 mM). After addition of 7 µl pronase E solution (20 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 400 U), the samples were further incubated at 37 °C for 24 h. An aliquot of 7 µl of a commercial aminopeptidase solution (0.5 U) and 7 µl prolidase solutions (1.7 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 1.3 U) were added and the sample was once again incubated at 37 °C for 24 h. The enzymatic hydrolysate (125 µl) was used for further analysis. The percentage release due to enzymatic hydrolysis was calculated by taking the release of leucine during acid hydrolysis as 100%.

2.8. Analysis of lysine and leucine

For derivatization, hydrolyzed protein samples (acid and enzymatic samples) were diluted 1:20 with ultrapure water and an aliquot of 25 µl was incubated with 40 µl sodium carbonate (0.1 M) and 50 µl dansyl chloride (5 mg/ml in acetone) at 40 °C for 1 h. After the addition of 10 µl HCl (3 M) and centrifugation (10,000 rpm, 10 min) 90 µl were transferred to an HPLC vial and subjected to HPLC-FLD analysis. Chromatographic analysis was performed using a Shimadzu Prominence HPLC system equipped with a pump (LC-20AC), an online degasser (DGU-20A3), an auto-sampler (SIL-20AC HT), a column oven (CTO-20AC) and a fluorescence detector (RF-20Axs). Analyte separation was achieved with a Nucleosil 100 C18 column (250 mm x 4.6 mm, 5 µm particle size, Dr. Maisch, Germany). Solvent A was 0.1% formic acid in ultrapure water and solvent B was 0.1% formic acid in ACN/ultrapure water (90/10). The gradient started with 20% solvent B and was elevated linearly to 90% B over a period of 25 min, was held at 90% B for 3 min and was changed back to 20% B in 1 min and was held there for 4 min. The flow rate was 1 mL/min, the separation was performed at 30 °C and 20 µL sample solution was injected. Peaks were detected by measuring fluorescence intensity at λ_{ex} = 340 and λ_{em} = 550 nm. Quantitation was achieved by external calibration with lysine and leucine standard solutions. Peak evaluation was managed using the software Lab Solutions Version 5.87 SP1.

2.9. Analysis of advanced glycation endproducts

AGE analysis was performed in enzymatically hydrolyzed samples. Standard addition method was used for quantification. In the sample without addition, 20 µl of enzymatic hydrolysate was mixed with 30 µl of LC-MS grade water. In the second run, 20 µl of hydrolysate was mixed with 20 µl of water and 10 µl of a standard solution 1. In the last run, 20 µl of hydrolysate was mixed with 20 µl of water and 10 µl of a standard solution 2. Standard solution 2 contained CML (4.8 µM), CEL (5 µM), MG-H1 (3 µM), pyrraline (0.6 µM) and pentosidine (2 µM) and standard 1 was a 1:1 dilution of standard 2 in ultrapure water. UPLC analysis was performed with an Acquity Ultra Performance LC system coupled to a Waters Quattro Premier XE mass spectrometer (both Waters Corporation, Milford, MA, USA). For chromatographic separation, a Zorbax RRHD Eclipse C18 column (2.1x50 mm, 1.8 µm) at a column temperature of 30 °C was used. Solvent A was 10 mM NFPA in LC-MS grade water and solvent B was 10 mM NFPA in ACN. The solvents were pumped at a flow rate of 0.4 ml/min in gradient mode (0 min, 1% B; 1 min, 1% B; 6 min, 50% B; 6.1 min, 1% B; 8 min, 1% B). The injection volume was 10 µl. The ESI source was operated in positive mode and nitrogen was utilized as the nebulizing gas with a gas flow of 650 l/h and gas temperature of 350 °C. The capillary voltage was set to 2.4 kV and the source temperature was 150 °C. Analytes were measured in MRM mode with the following transitions and optimized collision energies (CE) and cone voltages (CV). CML: 204.9 → 84.2 (q, CV 24, CE 18 V), 204.9→130.2 (Q, CV 24, CE 12 V), CEL: 219.1→84.1 (q, CV 24, CE 18 V), 219.1→130.1 (Q, CV 24, CE 12 V), pyrraline: 255.2→175.2 (q, CV 14, CE 12 V), 255.2→148.1 (Q, CV 14, CE 18 V), MG-H1: 229.2→70.1 (q, CV 26, CE 22 V), 229.2→113.6 (Q, CV 26, CE 14 V), pentosidine: 379.2→135.1 (q, CV 42, CE 44 V), 379.2→187.2 (Q, CV 42, CE 36 V). Transitions used for quantification are labeled with q and transitions used for the confirmation of the presence of the analyte are labeled with Q. Data were acquired and evaluated with the MassLynx Software (Waters, version 4.1).

2.10. Proteasomal degradation

The degradation of native and glycated myoglobin was assessed in phosphate buffered saline following addition of isolated proteasome (8 µg) to protein samples (40 µg) according to Ref. [17]. Proteasome was isolated from human blood as previously described [26]. Incubation and analysis of free amino groups was performed according to Ref. [27]. Samples were incubated at 37 °C for 2 h followed by the addition of ice-cold trichloroacetic acid (20%). The samples were left on ice for 5 min and subjected to centrifugation (10.000 rpm, 10 min, 4 °C) afterwards. An aliquot of the supernatant was used to assess the liberation of amino groups. Therefore, 25 µl of supernatant were incubated in the dark with 125 µl HEPES buffer (1 M, pH 7.8) and 50 µl fluorescamin (0.3 mg/ml in acetone) at room temperature in a black microtiter plate. After exactly 5 min incubation time, fluorescence intensity of the samples (λ_{ex} = 360 and λ_{em} = 460 nm) was analyzed using a multimode reader Tecan Infinite M200 (Mainz, Germany). Leucine was used as calibration standard and liberation of amino groups was calculated as mM leucine equivalents. Blank samples with native or modified myoglobin incubated with phosphate buffer instead of proteasome were included. Oxidized myoglobin (positive control for increased 20S proteasomal degradation) was prepared by myoglobin incubation with hydrogen peroxide for 2 h at 37 °C according to Ref. [26].

To investigate the competing influence of glycated myoglobin on the degradation of the artificial proteasome substrates Suc-Leu-Leu-Val-Tyr-AMC (β5-subunit) and Ac-Arg-Leu-Arg-AMC (β2-subunit), glycated myoglobin (42 µg) was incubated with proteasome (8 µg) and each of the artificial substrates in PBS (β5-subunit substrate: 167 µM and β2-subunit substrate: 833 µM) and the proteolytic cleavage of the substrates was continuously monitored with a multimode reader Tecan Infinite M200 (Mainz, Germany) for 2 h. The activity of the proteasome is calculated based on the slopes resulting from plotting fluorescence signal of the cleaved substrates against incubation time.

3. Results

3.1. Characterization of glycated myoglobin

Myoglobin was incubated with varying concentrations of glucose (5–300 mM) and MGO (50 mM) for 4 weeks followed by dialysis and lyophilisation. In course of the Maillard reaction the amino group of proteins reacts non-enzymatically with keto or aldehyde functions of sugar molecules. Depending on reaction time and temperature a heterogonous group of reaction products is formed. Especially during the advanced stage, cyclic structures which may exhibit fluorescence properties and crosslinking products can result from the Maillard

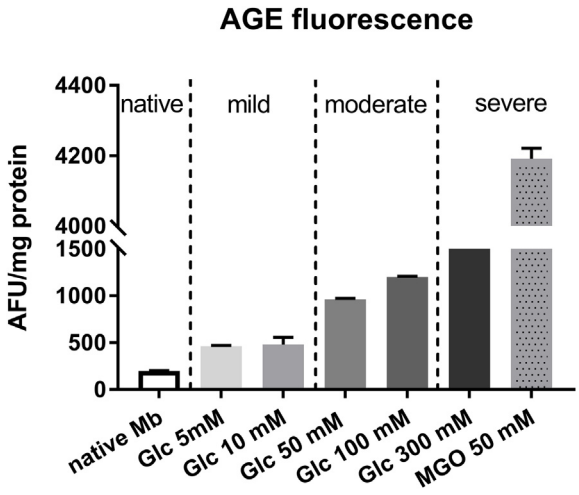

Fig. 1. AGE-Fluorescence of glycated myoglobin. Fluorescence intensity was analyzed at λ_{ex} = 350 nm and λ_{em} = 450 nm. Samples were arranged according to their glycation levels (native = unmodified protein, mild glycation = 5 and 10 mM glucose, moderate glycation = 50 and 100 mM glucose and severe glycation = 300 mM glucose and 50 mM MGO). Data are mean ± S.D., n = 3.

reaction. To assess the formation of total cyclic fluorescent AGEs, fluorescence analysis of the solubilized proteins after 4 weeks of glycation was performed. Increasing concentrations of glucose during incubation led to an increase of AGE fluorescence intensities (Fig. 1).

Myoglobin incubated with MGO showed the highest fluorescence intensity. It was shown previously, that MGO is highly reactive towards proteins under physiological conditions resulting in increased AGE fluorescence [17,28,29]. To assess whether protein crosslinking is responsible for AGE fluorescence, glycated proteins were applied to gel electrophoresis. Glycation with physiological (5–10 mM) and with moderate (50–100 mM) concentrations of glucose did not lead to a detectable increase of high molecular weight protein aggregates (Fig. 2).

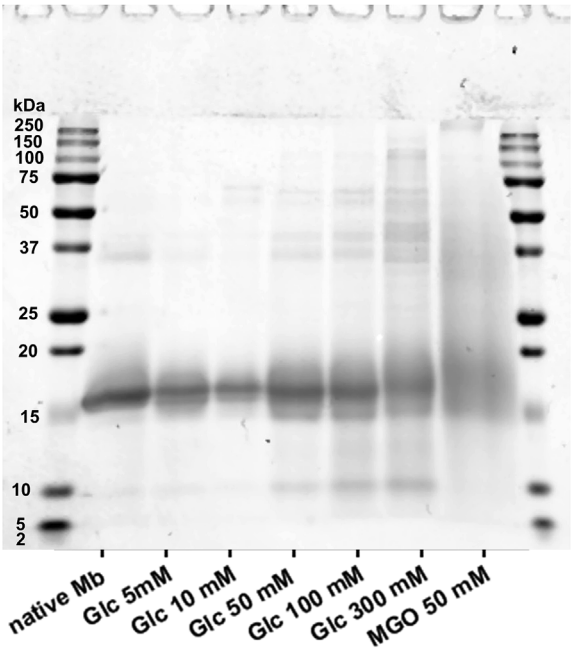

Fig. 2. Gel electrophoresis of native and glycated myoglobin. SDS-PAGE using a 15% gel was performed and detected by Coomassie staining. Molecular weight of native myoglobin is 17 kDa. One representative gel (n = 2) is shown.

In contrast, myoglobin incubation with high concentrations of glucose (300 mM) and MGO (50 mM) led to a significant increase of crosslinked products shown by a decrease of the native myoglobin lane and an increase of lanes between 40 and 150 kDa. To evaluate AGE formation in more detail, lysine modification and specific AGE structures were analyzed after enzymatic hydrolysis of glycated myoglobin. For incubation with glucose, compared to native myoglobin a maximum lysine modification of 20% was achieved. The reactive dicarbonyl compound MGO modified up to 56% of lysine residues in myoglobin (Fig. 3). To assign the lysine modification to specific AGE structures, N-ε-carboxymethyllysine (CML), N-ε-carboxyethyllysine (CEL), pentosidine and pyrraline were analyzed by LC-MS/MS after enzymatic hydrolysis of glycated proteins. Moreover, the arginine modification methylglyoxal-hydroimidazolone 1 (MG-H1) was included in the set of AGEs due its high abundance. The concentrations were expressed as µmol AGE/mmol leucine. The amino acid leucine, which is not modified in the Maillard reaction, was used as an internal reference for AGE content, thus preventing an underestimation of AGEs resulting from incomplete enzymatic hydrolysis. Myoglobin incubation with increasing concentrations of glucose led to an increase of all analyzed AGEs (Fig. 3). For the studied AGEs, CML gave the highest concentrations (2.3–59.8 µmol/mmol leucine), followed by MG-H1 (2.4–6.1 µmol/mmol leucine) and CEL (0.3–0.8 µmol/mmol leucine). Concentrations of pentosidine (7.3–105.5 nmol/mmol leucine) and pyrraline (5.3–37.5 nmol/mmol leucine) were significantly lower.

3.2. Interaction of glycated myoglobin with heme group

The heme group of myoglobin is coordinated by two histidine residues (H65 and H95) which are located in the inner protein structure. Since glycation changes the secondary structure, glycation-induced changes of the heme binding properties of myoglobin were studied by measuring the Soret band at 410 nm. Apo-myoglobin, which was prepared by acid-butanone extraction of native myoglobin, lacks the heme group and was used as a positive control and shows no Soret band (Fig. 4). When myoglobin was incubated with increasing concentrations of glucose or MGO, the Soret band decreases (Fig. 4), indicating the removal of the heme group from the heme binding pocket. This phenomenon was described previously and was explained by glucose-protein interactions which slightly alter the conformation of heme active site [21,30].

3.3. Surface characteristics of glycated myoglobin

To analyze the effect of glycation on the hydrophobicity of the protein surface, the binding of the fluorescent dye ANSA was tested. ANSA exhibits an increase and a shift in fluorescence when associated with surface-exposed hydrophobic sequences [18]. Protein glycation with concentrations higher than 5 mM glucose increased the binding of ANSA to the protein surface which is shown by an increased fluorescence signal at λ_{em} = 500 nm (Fig. 5). This result confirms previous studies [31] showing that glycation of myoglobin reduces the α-helix content of the protein. This was also confirmed by measuring tryptophan fluorescence as an indicator for the exposure of tryptophan residues to the surface of the proteins (Fig. 5). In contrast to the binding of ANSA, myoglobin incubated with MGO did not show increased tryptophan fluorescence but showed even less tryptophan fluorescence than native myoglobin.

3.4. Proteasomal degradation of native and glycated myoglobin

To analyze how glycation changes the proteolytic degradability of myoglobin, native and glycated proteins were incubated with 20S proteasome for 2 h at 37 °C. Efficiency of proteasomal degradation was analyzed by trapping liberated amino groups with fluorescamine and subsequent fluorescence measurement. The aim was to compare the

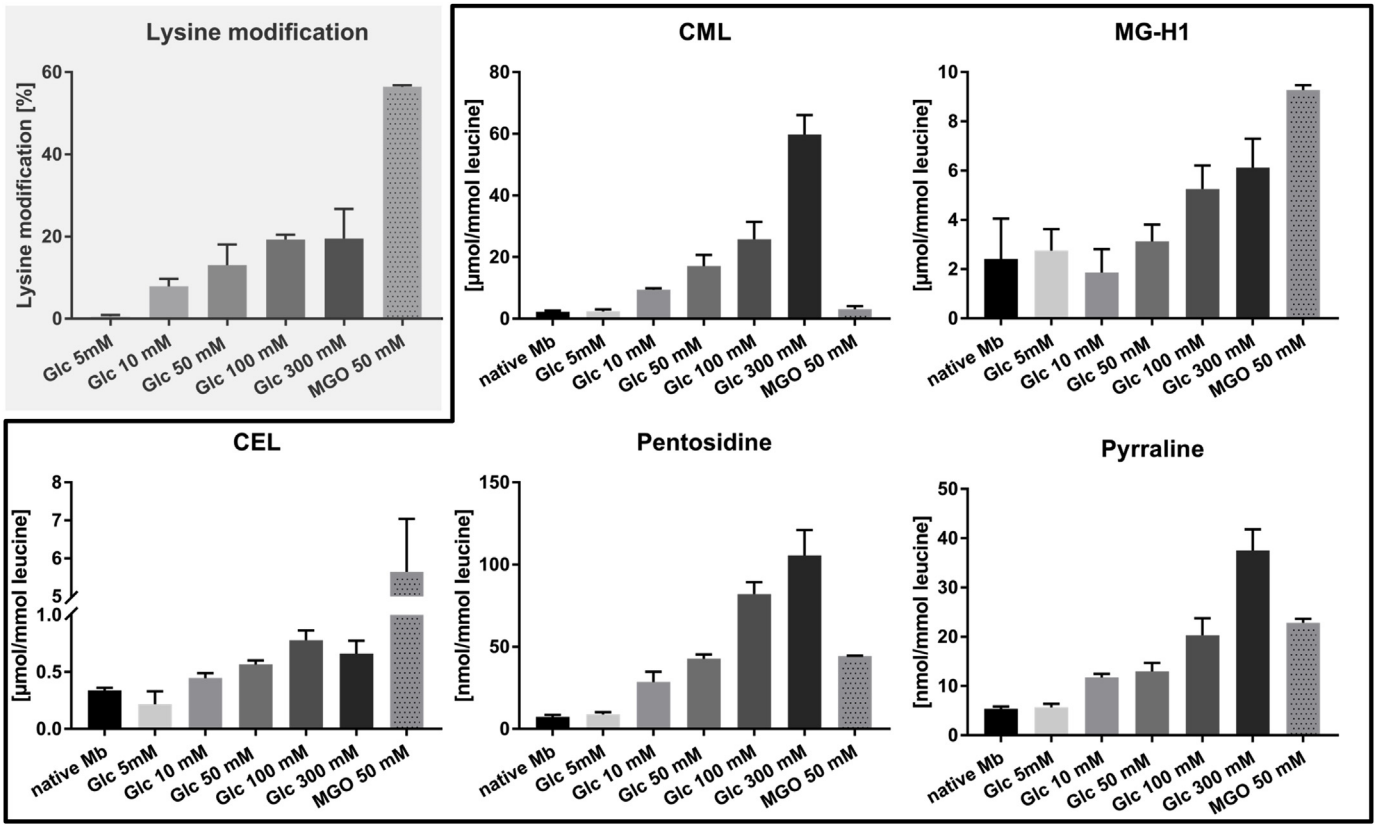

Fig. 3. Lysine modification and protein-bound AGE analysis of native and glycated myoglobin. Protein samples were enzymatically hydrolyzed and analyzed for their lysine and leucine concentration by HPLC-FLD. AGE analysis was performed with UPLC-MS/MS. Data are mean ± S.D., n = 3.

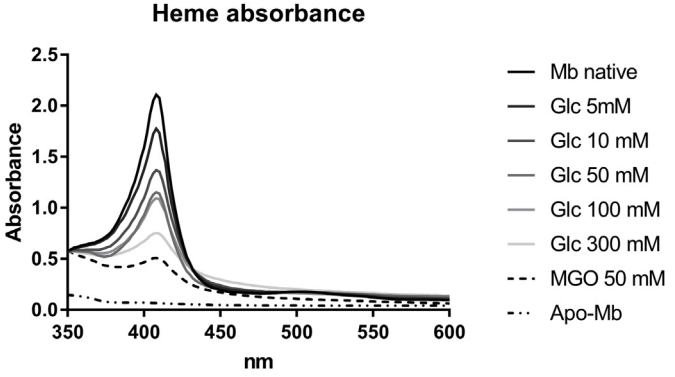

Fig. 4. UV–Vis absorbance of native and glycated myoglobin. Absorbance spectra of protein samples in HEPES buffer were recorded from 350 to 600 nm. A distinct Soret peak was observed at 410 nm. One representative measurement (n = 3) is shown.

impact of protein modifications on proteasomal degradation. Therefore, native, unmodified myoglobin was used as a control and proteasomal degradation thereof was set as 100%. Moreover, oxidized myoglobin was used as a positive control for increased 20S proteasomal degradation. Compared to native myoglobin, mildly glycated myoglobin (5 and 10 mM glucose) was degraded to 65% and therefore appears to be a poorer substrate for the proteasome than native myoglobin (Fig. 6). In contrast, proteasomal degradation of moderately glycated myoglobin was 115% for glycation with 50 mM glucose and significantly increased to 165% for glycation with 100 mM glucose ($P < 0.05$). In turn, severe glycation with 300 mM glucose leads to degradation of 97% of protein and was significantly decreased to 58% after glycation with 50 mM MGO ($P < 0.05$). To study the influence of the absence of the heme group without glycation on proteasomal degradation, apo-myoglobin was subjected to proteasomal hydrolysis. The removal of the heme group from the otherwise native myoglobin resulted in a significant increase of proteasomal degradation to 164% ($P < 0.01$).

To determine whether the modified proteins impact proteasomal function towards substrates which are efficiently cleaved by the proteasome, competition experiments were performed. Therefore, the artificial substrates Suc-Leu-Leu-Val-Tyr-AMC (specific for β5-subunit) and Ac-Arg-Leu-Arg-AMC (specific for β2-subunit) were incubated with the 20S proteasome in the presence of 20 µM of a subset of glycated myoglobins. The activity of the proteasomal subunits was calculated by plotting the arbitrary fluorescence units (AFU) of the substrate cleavage against the incubation time. Incubation of proteasome and artificial substrate alone represents unhindered cleavage and the results for the co-incubation of proteasome, artificial substrate and glycated myoglobins are shown in Fig. 7. The presence of native myoglobin decreases the degradation of the artificial substrates to approximately 65% and thus native myoglobin appears to be a competitive substrate of the proteasome. The activity of the β5-subunit towards Suc-Leu-Leu-Val-Tyr-AMC was not changed further in the presence of myoglobin incubated with 50 mM glucose, but was reduced to 53%, 48% and 36% for myoglobin incubated with 100 or 300 mM glucose and 50 mM MGO, respectively (not statistically significant). Although the activity of the β2-subunit of the proteasome was impaired to the same extent as the β5-subunit in the presence of native myoglobin (reduction to 69% activity), the additional presence of glycated myoglobin did not markedly change cleavage of Ac-Arg-Leu-Arg-AMC.

4. Discussion

Glycation is an ubiquitously present process that leads to the formation of protein modifications mainly at lysine or arginine residues. During aging, glycated proteins can accumulate and lose their function

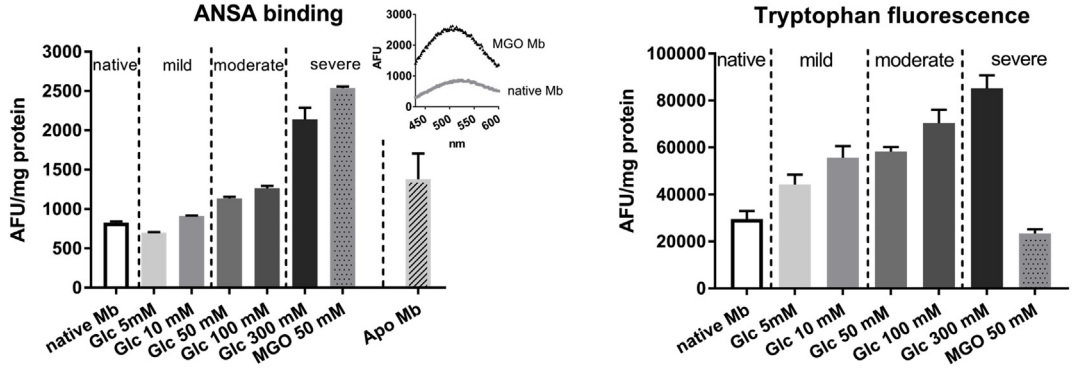

Fig. 5. Spectrofluorimetric analysis of ANSA binding and tryptophan in native and glycated myoglobin. For ANSA binding, emission spectra (λ_{ex} at 370 nm) were recorded and the peak at λ_{em} = 500 nm was used to calculated the increase of ANSA binding. Tryptophan fluorescence of protein samples were recorded in HEPES buffer at λ_{ex} = 280 nm and λ_{em} = 350 nm. Data are mean ± S.D., n = 3.

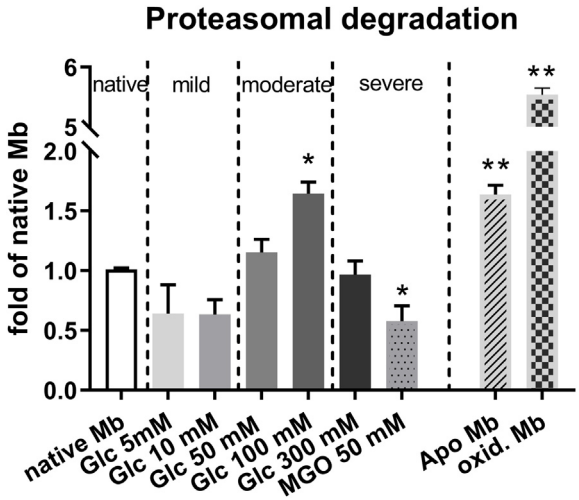

Fig. 6. Proteasomal degradation of native and glycated myoglobin. Protein samples were incubated with isolated 20S proteasome for 2 h at 37 °C. Liberation of amino groups was analyzed with fluorescamine. Leucine was used for calibration purposes. The liberated NH_2-concentration of glycated samples was referenced to native myoglobin. Data are mean ± S.D., n = 3. Hypothesis testing was performed via a two sample *t*-test between native myoglobin and the respective samples with a significance level of 0.05 (*) or 0.01 (**).

[2,32]. Under optimal conditions, accumulation of modified proteins is prevented by the proteasome which degrades and eliminates damaged proteins [14]. It was shown previously that the proteasome is unable to degrade extensively glycated proteins leading to an increased accumulation of such modified proteins in cells [17,18]. A drawback of previous studies was a missing characterization of the so-called "AGE-modified proteins" and therefore no direct correlation between AGE formation and proteasomal inhibition could be made. In the present study, myoglobin was modified with glucose and MGO and extensively characterized. To compare the results of this study with previous findings which characterized AGEs by fluorescence measurement, the spectroscopic properties of glycated myoglobins were analyzed. Glycation induced fluorescence might be either due to specific fluorescent structures such as argpyrimidine or pentosidine [33,34], or can be caused by protein crosslinking, reactions at the peptide backbone or changes in the tertiary structure [35]. Since fluorescence spectra in our study were obtained by measuring at $\lambda_{ex/em}$ = 350/450 and the fluorescence maxima of the known fluorescent AGEs argpyrimidine and pentosidine are situated at shorter wavelengths ($\lambda_{ex/em}$ = 320/380 for argpyrimidine and $\lambda_{ex/em}$ = 335/385 for pentosidine), we assume that AGE fluorescence is mainly caused by formation of crosslinks and changes of tertiary structures. This was also confirmed by studying crosslink formation by gel electrophoresis, whereby especially the highly fluorescent MGO incubated myoglobin showed a significant shift to high molecular weight adducts. Glycation was further characterized by analysing the specific AGEs CML, CEL, MG-H1, pentosidine and pyrraline. All studied AGEs increased with rising glucose concentration; however the extent of enrichment differed between the AGE species. CML showed the highest increase by factor 26 and pentosidine and pyrraline the lowest increase by factor 2.5 in myoglobin incubated with 300 mM glucose compared to native myoglobin. The increased formation of CML can be explained by the variety of its formation pathways e.g. from the Amadori compound fructoselysine but also from the reaction of glyoxal with lysine. Modification of myoglobin with MGO (50 mM) did not lead to an increase of CML but to a pronounced increase of the MGO-derived AGEs CEL and MG-H1. Surprisingly, the

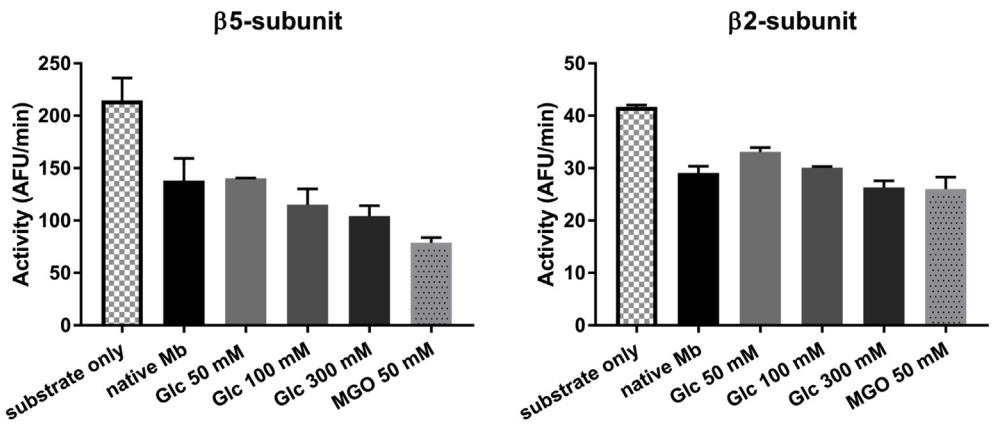

Fig. 7. Proteolysis of artificial proteasome substrates in the presence of glycated myoglobin. Artificial substrates Suc-Leu-Leu-Val-Tyr-AMC (β5-subunit) and Ac-Arg-Leu-Arg-AMC (β2-subunit) were incubated in the absence or presence of native and glycated myoglobins and the proteolytic cleavage of the substrates was continuously monitored. Activity is expressed by the slopes resulting from plotting fluorescence signal of the cleaved substrates against incubation time. Data are mean ± S.D., n = 2.

concentrations of pyrraline and pentosidine also increased during myoglobin incubation with MGO. Pyrraline is mainly formed by the reaction of 3-deoxyglucosone and lysine residues and pentosidine results from the reaction of pentose and lysine residues. The unexpected increase might be due to impurities of the commercial MGO solution [36,37] or potentially new formation pathways of these compounds. In conclusion, up to 40% of lysine modification of myoglobin incubated with 5 mM glucose could be explained by the lysine-derived AGEs CML, CEL, pentosidine and pyrraline, whereby those specific modifications explained only 20% of lysine modification of the sample incubated with 300 mM glucose. For the MGO modified myoglobin, the analyzed lysine-derived AGEs account for only 2% of modified lysine. To check whether the reduced accountability of specific AGE structures for total lysine modification was due to less efficient hydrolytic cleavage of modified amino acids from the protein structure, we compared the hydrolytic efficiency of the enzymatic versus a total (acid) hydrolysis. Therefore, the release of the unmodified amino acid leucine was used as a marker of hydrolytic efficiency. Compared to acid hydrolysis, the enzymatic hydrolysis of mildly and moderately glycated myoglobin yielded 90–94% of leucine residues. For MGO-modified myoglobin the enzymatic efficiency decreased to 74%. Therefore, the decreased accountability of specific AGE structures for total lysine modification might at least partially be due to a reduced release of certain modified amino acids. Moreover, it is known that glycation with MGO can result in the formation of the crosslinking structures methylglyoxyl-derived lysine dimer (MOLD) and methylglyoxal dimer imidazolone crosslink (MODIC). Since these compounds were not analyzed in the present study, it might be possible that those structures explain the gap between studied AGEs and total lysine modification.

Analysis of protein surface characteristics by binding of the fluorescent dye ANSA revealed that moderate glycation leads to an increase of hydrophobicity. Although glycation introduces hydrophilic groups to the protein, changes in the secondary structure thus leading to structural modifications and denaturation seem to be the predominant effects. Moreover the heme protein myoglobin exhibits a hydrophobic heme binding pocket in the inner protein structure. In native myoglobin this pocket is occupied by heme and does not present a binding site for the hydrophobic dye ANSA. However, glycation induces structural changes and thereby reduces heme binding capacity of the cavity. Therefore, the empty heme binding pocket presents an additional binding site for ANSA. Apo-myoglobin lacks the heme group and showed higher ANSA binding compared to native myoglobin. Since myoglobin incubated with 300 mM glucose and 50 mM MGO exhibit higher ANSA binding than apo-myoglobin, it is assumed that additionally to ANSA binding to the heme pocket, glycation induces structural changes of the residual protein structure, e.g. denaturation, which explain the increased hydrophobic surface. Besides ANSA binding, hydrophobicity of the proteins was studied by analyzing tryptophan fluorescence which is known to increase with denaturation. Based on tryptophan fluorescence, myoglobin incubated with MGO did not show increased denaturation which contradicts the results obtained by ANSA binding. MGO-induced glycation leads to strong crosslinking of the protein, as shown by gel electrophoresis. Myoglobin contains two tryptophan residues at position W8 and W15 in the N-terminal α-helix. Due to its exposed position, this helix might be especially prone to cross-linking reactions thus burying the tryptophan residues in cross-linked structures and thereby limiting its availability for fluorescence measurement. Moreover, it was shown that tryptophan can scavenge methylglyoxal and form β-carbolines [38] and indole can form adducts with up to three MGO molecules [39]. Those scavenging reactions possibly influence the spectroscopic properties of tryptophan thus making it less suitable for studying glycation-induced denaturation.

The major aim of this study was to evaluate the influence of glycation on protein degradation by the proteasome. It was shown that glycation-modified substrates were degraded significantly slower than unmodified substrates by the proteasomal system [24]. This is somewhat surprising since the function of the proteasome is the protection of cells against modified proteins. The present study shows that glycation per se does not negatively influence proteasomal degradation. Alterations in the proteolytic activity are critically dependent on the degree of glycation. Analysis of mildly (5 and 10 mM glucose), moderately (50 and 100 mM glucose) and severely (300 mM glucose and 50 mM MGO) glycated myoglobin revealed that proteasomal degradation was initially decreased compared to native myoglogin, increased for moderately glycated proteins and decreased again in the face of severe glycation. The reduced proteasomal degradation of mildly glycated myoglobin might be due to the glycation-induced decrease of hydrophobic surface as shown by slightly decreased ANSA binding. Hydrophobic surface exposure has been proposed to be an important recognition signal for degradation by the 20S proteasome [40]. Glycation, especially the formation of Amadori products during early stage, binds hydrophilic residues of carbohydrates to the protein structure and thereby mild glycation of proteins might reduce the recognition and cleavage of proteins by the proteasome. As the Maillard reaction progresses, changes in the structural conformation dominate the binding of hydrophilic groups and thereby lead to an increase in hydrophobicity. As previously assumed, the increased surface hydrophobicity also in this study appears to be a recognition signal for the proteasome followed by an increased proteasomal degradation of moderately glycated proteins. Although hydrophobic surface further increases for severely glycated myoglobin, those proteins appear to be poorer substrates for the proteasome. We assume that the transition from intramolecular glycation to intermolecular glycation, characterized by crosslinking, is a key attribute that decides the fate of glycated proteins towards the proteasome. Thus, our results point to the fact that initial glycation may decrease proteasomal degradation of proteins, which can be recovered with more pronounced modification but will be detrimentally affected by glycation which includes crosslinking reactions (Fig. 8B). For native, mildly and moderately glycated myoglobin, proteasomal degradation correlates with protein unfolding measured by ANSA binding (Fig. 8A).

In conclusion, glycated proteins apparently do not exhibit optimal proteasome substrates. Besides glycated proteins, other modification such as oxidation can lead to damaged proteins which ought to be degraded by the proteasome. However, when the proteasome is occupied with the degradation of glycated proteins this might limit the degradation of other substrates. Therefore, a competing experiment with artificial proteasome substrates specific for the subunits β5 and β2 in the presence of glycated myoglobin was performed. It was shown that the presence of native but to a higher extent glycated myoglobin decreases the activity of the proteolytic activity of the catalytic subunit β5 towards the artificial substrate Suc-Leu-Leu-Val-Tyr-AMC. The proteolytic activity of the β5-subunit was described to exhibit a chymotrypsin-like activity which prefers cleavage after aromatic amino acid residues and leucine. These amino acids are not modified in the Maillard reaction and thus still present potential cleavage sites for the proteasome. However, structural changes which occur in course of glycation, such as protein unfolding or crosslinking might influence the accessibility of these cleavage sites. The results suggest that glycated myoglobin interacts with the β5-subunit and depending on the degree of glycation, is degraded or serves as an inhibitor for the proteasome. In contrast, the β2-subunit, which exhibits trypsin-like activity, cleaving mainly after lysine, arginine and cysteine residues is not significantly influenced by the presence of glycated myoglobin compared to native myoglobin. Since β2-subunit cleavage sites are main targets of the Maillard reaction, this subunit might be incapable of cleaving even mildly glycated proteins. The modification of cleavage sites might reduce the interaction of the proteasome with glycated myoglobin to such a low level that glycated myoglobin does not markedly impair the proteasomal function of the β2-subunit. Our results suggest that proteasomal subunits can unequally handle protein glycation but further research is needed to study the role of different proteasomal subunits in the proteolysis of glycated proteins.

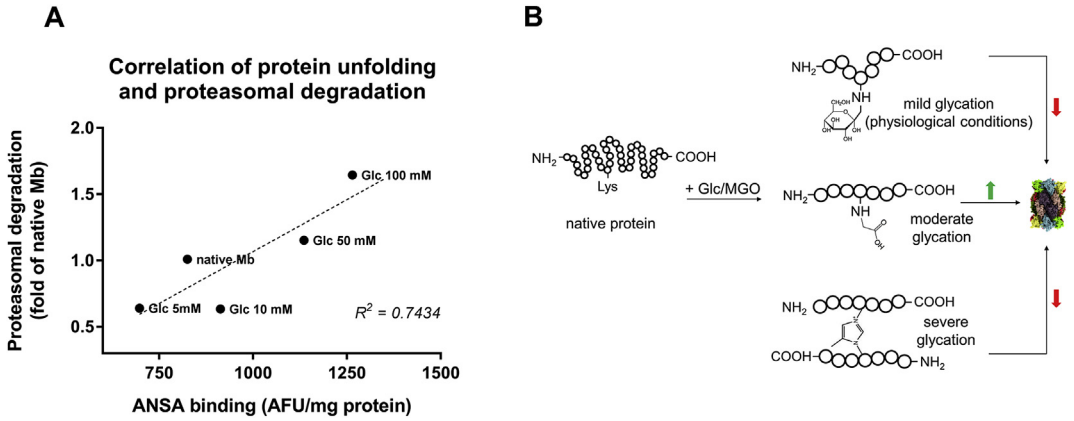

Fig. 8. Protein unfolding during glycation and proteasomal degradation. A) Correlation of ANSA binding to native, mildly and moderately glycated myoglobin and its proteasomal degradation. B) Schematic overview of proteasomal degradation of differently modified proteins: Glucose or MGO covalently modify amino acid side chains in proteins leading to mild, moderate or severe glycation. Only moderately glycated proteins increase proteasomal degradation whereas the other forms are less efficiently cleaved.

5. Conclusion

Taken together, the model protein myoglobin was glycated with various concentrations of glucose including physiological glucose concentrations. Moreover, protein crosslinking with MGO was performed. Glycated myoglobin was chemically characterized for the AGE-content and the modifications and their structural consequences were related to proteasomal degradation. We were able to show glycation of proteins per se does not hinder their proteasomal degradation, but that the extent of glycation and, hence the degree of unfolding, is of utmost importance in order to draw conclusions concerning proteolytic cleavage. We hypothesize that in cells mildly glycated proteins are poor substrates for the proteasome but proceeding glycation increases proteasomal degradation. However, chronic glycation under certain circumstances, e.g. hyperglycemia or aging, leads to extensive AGE formation and intermolecular crosslinks which cannot be degraded by the proteasome. Severe glycation might lead to AGE accumulation and potential detrimental effects. Further research is needed to examine the role of severely glycated, crosslinked proteins in cells.

Author contributions

JR designed and performed the experiments. CO and JK supported the proteasomal degradation studies. TG isolated the proteasome and gave advice and suggestions on this work. JR prepared the manuscript and CO, JK and TG critically read the manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Abbreviations

Mb: myoglobin
AGEs: advanced glycation endproducts
CML: N-ε-carboxymethyllysine;
CEL: N-ε-carboxyethyllysine;
MG-H1: methylglyoxal-hydroimidazolone 1
MGO: methylglyoxal
ANSA: 8-anilino-1-naphthalenesulfonic acid