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The role of the thiol group in protein modification with methylglyoxal

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Abstract: Methylglyoxal is a highly reactive α-oxoaldehyde with elevated production in hyperglycemia. It reacts with nucleophilic Lys and Arg side-chains and N-terminal amino groups causing protein modification. In the present study, the importance of the reaction of the Cys thiol group with methylglyoxal in protein modification, the competitiveness of this reaction with those of amino and guanidine groups, the time course of these reactions and their role and contribution to protein cross-linking were investigated. Human and bovine serum albumins were used as model systems. It was found that despite the very low levels of thiol groups on the surface of the examined protein molecules (approx. 80 times lower than those of amino and guanidino groups), a very high percentage of it reacts (25–85 %). The amount of reacted thiol groups and the rate of the reaction, the time for the reaction to reach equilibrium, the formation of a stable product and the contribution of thiol groups to protein cross-linking depend on the methylglyoxal concentration. The product formed in the reaction of thiol and an insufficient quantity of methylglyoxal (compared to the concentrations of the groups accessible for modification) participates to a significant extent (4 %) to protein cross-linking. Metformin applied in equimolar concentration with methylglyoxal prevents its reaction with amino and guanidino groups but, however, not with thiol groups.

Keywords: methylglyoxal; protein thiol group reaction; protein modification and cross-linking; AGEs.

INTRODUCTION

Advanced glycation end products (AGEs) are elevated in diabetes and are involved in the pathogenesis of its vascular complications,[1–6] as well as in other pathological states. They are formed in the reaction of carbonyl-containing materials with N-terminal and Lys side-chain amino groups, the guanidino group of

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Arg, the sulfhydryl group of Cys in proteins, and through a series of reactions and rearrangement.[7] Some of the final products of these reactions are the well-characterized conjugates (*N*-(carboxymethyl)lysine, *N*-(carboxyethyl)lysine, *N*-(carboxymethyl)arginine, *S*-(carboxymethyl)cysteine, *S*-(carboxyethyl)cysteine and pentosidine), which are used as markers in clinical practice.

Methylglyoxal (MG) is an α-oxoaldehyde which is formed in cells primarily from triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate and glyceraldehydes-3-phosphate.[8] It is accumulated in hyperglycemia,[9,10] oxidative stress, uremia,[11] in the aging process and inflammation. As a highly reactive compound (α-dicarbonyl compounds are 20,000-fold more reactive than glucose in glycation reactions[12]), it participates in the modification of proteins[13] and DNA[14] and leads to the formation of AGEs. Molecules modified with methylglyoxal and their derivatives can affect cellular functionality *via* gene expression,[15] lead to micro- and macro-vascular complications in diabetes,[16] contribute to upregulation of inflammatory and tissue-injury-provoking molecules (interaction of AGEs and receptor RAGE), protein cross-linking and apoptosis.[17]

Amino, guanidine[16] and thiol groups[13] present on protein surfaces participate in protein modification with methylglyoxal. The products formed in the first phase of the reaction change in a series of subsequent reactions.[18] The thiol group is a strong nucleophile, at physiological pH values stronger than the amino and guanidine groups of Lys and Arg side chains. Yet, the role of thiol group in protein modification with MG has not been sufficiently investigated. The proposed mechanism of reaction of thiol with glyoxal[19] or MG[13] proved the formation of carboxymethylcysteine[19–21] and carboxyethylcysteine. These products were proposed for use as markers in pathological states[19] and attempts were made for their quantification.[22] The research on Cys side-chain modifications of thiol-dependent enzymes glyceraldehyde-3-phosphate dehydrogenase[23] and creatine kinase[19] with MG showed their inactivation during incubation. The formation of protein aggregates was observed in the treatment of albumin with MG.[18] The role of amino and thiol groups in cross-linking *via* reactive compounds, such as glutaraldehyde,[24] 4-hydroxynonenal[25] and acrolein[26] was stressed. The potential importance of SH and MG reaction in protein cross-linking has only been reported by Zeng and Davies.[27] They proposed that the product of the initial reaction between a thiol group and MG can be a target for the next reaction with an amino group or, *vice versa*, the initial product of a reaction between an amino group and MG can be a target for an SH group. Since the presence and availability of thiol groups on the protein surface is marginal compared to amino groups, the question is: what is the importance and contribution of the initial reaction between SH and MG to cross-linking? What are the target proteins for this reaction and what is the physiological role of the formed adducts?

Therefore, in this study, the time progression of the reaction of protein thiol groups (as well as of amino and guanidino groups) with methylglyoxal, detection of protein modifications, the role and contribution of the initial reaction between thiol and MG, *i.e.*, the product of that reaction, as a target for protein cross--linking were examined. Also, the effects of inhibitors on the above-mentioned reactions, especially during low-dose long-term exposition of albumin to MG, were analyzed. Human (HSA) and bovine serum (BSA) albumins were chosen as model-systems by virtue of the considerably lower thiol group content on the molecule surface compared to amino (and guanidino) group and because of their abundance and reported antioxidant properties.

EXPERIMENTAL

Materials

All chemicals were purchased from Sigma, Steinheim, Germany, unless otherwise stated. The 20 % solution of HSA was purchased from Octapharma AB, Stockholm, Sweden; thymol, 3',3",5',5"-tetrabromo-*m*-cresolsulphonephthalein (BCG), hydroquinone and bromine were from Merck, Darmstadt, Germany.

Preparation of glycated samples

HSA or BSA solutions (33 mg/mL, *i.e.*, 0.50 mM) were prepared in 0.10 M phosphate buffer (pH 7.2) under sterile conditions (sterile zone) and incubated in capped vials at 37 °C with different concentrations of MG as follows: BSA with 50 mM MG for 168 h; HSA with 100 mM MG for 24 h; HSA with 10 mM MG for 24 days. All solutions were sterile-filtered prior to incubation. For the investigations of the inhibition of the reactions, glutathione (GSH) and metformin (10 and 20 mM) were used.

Monitoring of changes of HSA and BSA molecules during incubation with MG

Aliquots of the reaction mixtures were taken at predetermined intervals during incubition and subsequently extensively dialyzed against deionized water (3 times within an hour). The albumin concentrations after dialysis were assayed by the BCG method.[28] Protein changes during incubation with different methylglyoxal concentrations were monitored using native and SDS PAGE. In brief, to 5.0 mL of 0.04 mM BCG in 0.10 M Gly buffer (pH 3.8), 0.02 mL of sample was added. The absorbance at 628 nm was measured against the reagent after 30 s.

Protein changes during the incubation with different MG concentrations were monitored using native and SDS PAG (10 % of acrylamide) electrophoresis according to protocol of Hoeffer scientific instruments.[29] 7 μL of protein samples (0.05–0.15 mg/mL) were applied. Gels were stained by Coomassie Brilliant Blue G-250 (CBB).

In each aliquot, the contents of the reactive groups were determined in triplicate.

Thiol assays

Free thiol groups were assayed spectrophotometrically according to a modified Ellman method.[30] 5,5'-Dithiobis(2-nitrobenzoic acid) reagent (100 μL of 2.0 mM solution) was mixed with 10 μL–100 μL of the sample, 100 μL of 1.0 M Tris buffer (pH 8.0) and filled up with water to 1300 μL. To avoid interferences of the browning state of modified albumin, the absorbance was measured at 412 nm against a sample blank, using a Beckman DU 50 spectrophotometer. In addition, dilution of samples (13–130 times) contributed to the minimization of the influences. Standard solutions of 2-mercaptoethanol (0.143–1.43 mM) were used for the

construction of a standard curve (y = 0.01013 + 0.7225x; r = 0.9999, p < 0.0001). The SH group concentration was also calculated using the extinction coefficient 13600 mol^{-1} dm^3 cm^{-1}.

As commercially available albumin preparations already contain oxidized Cys34,[31] the content of free thiol group was determined in untreated BSA/HSA (0.73/0.76 mole per mole of protein, respectively). The amounts of reacted thiol groups were expressed as percentages of these contents.

Guanidine assay

A spectrophotometric method[32] was used for the determination of guanidino groups. To 1.0 mL 0.020 % thymol in 1.0 M NaOH, 10 μL–100 μL sample and 100 μL 2.0 % Br_2 in 5.0 % NaOH were added and the mixture was filled up with water to 2.1 mL. The absorbance at 470 nm was measured against a reagent blank. A standard curve was prepared with standard arginine solutions in the concentration range 0.125–1.25 mM (standard curve: y = – 0.01407 + 0.30933x, r = 0.9992, p < 0.0001).

Determination of amino groups

The spectrophotometric determination of amino groups was performed as follows:[33] 100 μL sample, 100 μL 1.0 M phosphate buffer (pH 7.2), and 40 μL 0.10 M *p*-benzoquinone in dimethyl sulfoxide were mixed and water was added to a volume of 1500 μL. The absorbance at 480 nm was measured against reagent blank. A standard curve was constructed with alanine in the concentration range from 10–90 mM (y = 0.03225 + 0.00695x, r = 0.9966, p < 0.0001).

Statistical analysis

Data are expressed as the mean±standard deviation (*SD*) from a minimum of three experiments. Statistical analysis was performed by the Student's *t*-test.

RESULTS AND DISCUSSION

BSA and HSA were used as model systems to investigate the role of thiol groups in the modification and cross-linking of protein molecules *via* methylglyoxal. These proteins were chosen due to the fact that the number of amino acid side-chains is known, *i.e.*, the number of reactive groups on the protein surface that MG can react with,[18,34] and the number of thiol groups (only one) is considerably lower compared to the number of Lys (59) and Arg (24) side-chains. Considering the higher reactivity of the SH-group compared to the amino and guanidine groups at physiological pH values and the substantially lower availability for reaction, it was of interest to investigate the importance of this reaction for protein modification at different MG concentrations. On the other hand, the abundance of HSA in the serum may point to its additional role in carbonyl stress.

Modification of BSA in reaction with methylglyoxal

A reaction mixture of BSA (0.50 mM) and methylglyoxal (50 mM) in 0.10 M phosphate buffer (pH 7.2) was incubated for 7 days at 37 °C. A considerably higher concentration of MG than the physiological one (free MG in plasma is < 5 μM[35] with > 90 % MG bound to proteins[13]) was used in order to monitor and compare the reaction rates of each group (thiol, amino and guanidino) accessible

for modification. In addition, the employed MG concentration was higher (by 19 %) than the sum of reactive groups per mole of BSA surface of the protein molecule. BSA modification was monitored over time by native and SDS PAGE (Figs. 1A and 1B, respectively).

A considerable change in the mobility of BSA during native electrophoresis, *i.e.*, a decrease in the positive charge of the protein, was observed after 2 and 4 h of incubation (Fig. 1A, lanes 2 and 4, respectively). The mobility of the protein band increased with incubation time. A broadening and higher diffuseness of the protein bands during native and SDS PAGE was detected as early as after 4–6 h of incubation, which continued as the incubation proceeded further. These bands tailed to higher molecular mass values in the period from 1 to 7 days of incubation.

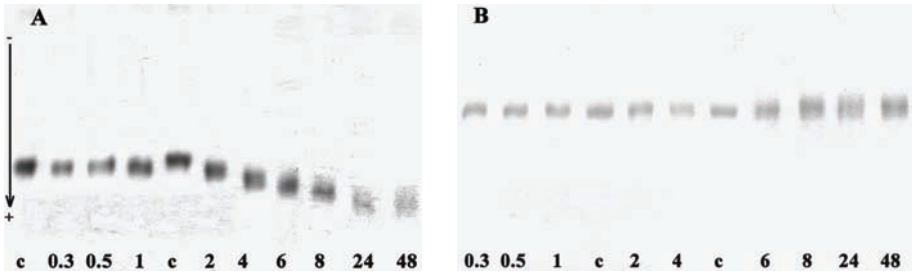

Fig. 1. Native (A) and SDS-PAGE (B) showing the changes of BSA electrophoretic mobility resulting from reaction with methylglyoxal. BSA (0.50 mM) was treated with 50 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 7 days. Lane **c** shows nontreated BSA. The marks of other lines correspond to time of incubation (h) of BSA with MG. The electrophoresis was performed on 10 % polyacrylamide gels and the bands were visualized by CBB, as detailed in the Experimental.

Comparison of the reactivity of the thiol, amino and guanidine groups of the BSA amino acid side chains with methylglyoxal

In order to determine the role and contribution of the thiol groups and to compare them with the contributions of the amino and guanidino groups to the changes in BSA as observed in the electrophoregrams, the progress of reactions of these groups with MG was monitored by their quantification in aliquots of the reaction mixture during incubation (Fig. 2).

The changes in the amounts, *i.e.*, the percentages, of reacted guanidino and thiol groups in the reaction mixture after 7 days were very similar. After 24-hour incubation, 66 % of the arginine side chains, 53 % of the thiol groups and only 21 % of the amino groups had reacted. In the following two days of incubation, the percentage of the reacted guanidino groups rose to 81 % and of thiol to 77 %, at which levels they remained until the end of incubation (7 days). The content of amino groups changed moderately from the first to the seventh day of incubation (24.8±2.4 %).

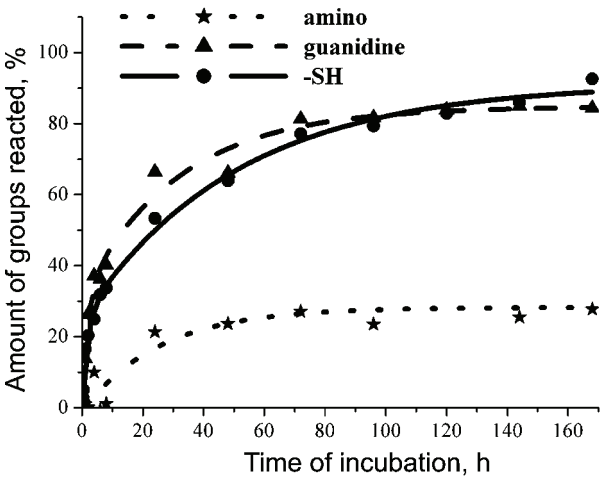

Fig. 2. Quantification of thiol, amino and guanidino groups (% of the ones reacted) during the incubation of 0.50 mM BSA with 50 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 7 days. Data are the means from three determinations. *SD* data for particular reactive group and specific time points are presented in Table I.

It is of interest to compare the changes in the contents of the reactive groups during the first 8 h of reaction, the period when modification of the protein molecules was detected by electrophoresis. The amount of guanidino groups decreased by 26 % in the first 2 h of incubation and had decreased by 37 % after 4 h. In the same time period, the amount of thiol groups decreased by 20 and 30 %, respectively. After 8 h, under the employed incubation conditions, only about 8 % of amino groups had reacted.

The contribution of the reaction of individual reactive groups to the changes of the BSA molecules observed by electrophoresis can be better realized if the percentages of the reactive groups are converted to the corresponding number of amino acid side-chains (mole) reacted per molecule (mole) of BSA (Table I). After a three-day incubation (the equilibrium state), the total content of amino acid side-chains reacted per BSA molecule was 36 (43.1 %).The contribution of MG reaction with guanidino groups to the decrease of the positive charge of BSA was higher compared to the contribution of the amino groups. The percentage of modified thiol groups to the total content of modified amino acid residues (1.55 %) was almost two times higher than that in untreated BSA (0.87 %). Modification of the SH groups can contribute to a broadening of protein bands in electrophoresis. The increased broadening of the protein bands with time reflects the production of multiple modified forms of the protein (modification with different numbers of MG molecules and different sites).

Some previous studies[36] showed that a decrease in the free thiol content in BSA could be the result of an oxidation process rather than of the glycation process with MG. For this reason, the potential oxidation of thiol group in BSA due to influence of temperature (37 °C) and the presence of oxygen in the solutions was followed by determining of the –SH content in untreated albumin (control). During the incubation, the content of –SH groups changed insignificantly. After

168 h, it had decreased by only 8 %. Since in the first hours of BSA incubation with MG, the registered decrease of free SH groups was by 20–30 % (Table I), this is mainly the result of the glycation reaction. According to the proposed mechanism,[7,13] thiohemiacetal is formed in the reaction of SH group and MG, which is converted by a Cannizzaro-type rearrangement into (1-carboxyethyl)cysteine or participates in the cross-linking of protein molecules.[27]

TABLE I. Content of reacted amino acid side-chains. BSA (0.50 mM) was treated with 50 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for various time periods

Incubation time, h	Reacted groups (mol/mol of BSA)				%[a]
	Thiol	Amino	Guanidino	Total	
2	0.149±0.010	0.66±0.12	6.29±0.15	7.10	8.50
4	0.220±0.012	3.25±0.15	8.91±0.39	12.36	14.8
8	0.247±0.001	4.72±0.32	9.64±0.15	15.77	18.9
24	0.390±0.007	12.59±1.15	15.93±0.15	28.91	34.5
48	0.468±0.011	13.96±0.92	15.86±1.08	30.29	36.2
72	0.564±0.014	15.98±1.15	19.51±0.77	36.05	43.1
168	0.677±0.009	16.36±1.01	20.25±0.77	37.28	44.5

[a]Total reacted groups as the percent of the total number of available groups

Inhibition of methylglyoxal-induced modifications of BSA by GSH and metformin

The experiment of BSA (0.50 mM) incubation with MG (50 mM) was also performed in the presence of glutathione (GSH) and metformin as inhibitors of MG reaction with amino acid side chains. The experiments were performed separately; the concentration of the inhibitors was 20 mM (2.5 times lower compared to the MG concentration). The results of monitoring BSA modification by native and SDS electrophoresis in the presence of GSH and metformin are shown in Fig. 3.

Changes in BSA mobility in native electrophoresis were observed in the presence of both inhibitors (Fig. 3A) but to a smaller extent in the presence of metformin (lanes 3, 6, and 9). Relative electrophoretic migration (REM) was determined by calculating the ratio of migration of modified BSA with nontreated albumin (Table II). Significant decreases in REM were found for BSA modified in presence of metformin compared to BSA-MG and BSA-MG + GSH after four ($p <$ < 0.01 for both) and eight ($p < 0.001$; $p < 0.05$, resp.) hours of incubation. These results are consistent with the findings which underlined metformin as potent glycation inhibitor.[37,38] On the other hand, in the presence of GSH, significant decrease of REM ($p < 0.001$ *vs*. BSA-MG) was found only after eight hours of incubation. Thus, positive charge of BSA molecules in presence of metformin is higher than in presence of GSH. A broadening of MG modified BSA (BSA-MG) bands in SDS PAGE was also found in the presence of both inhibitors after only 4 hours of incubation (Fig. 3B), but to a smaller extent in the presence of GSH (lanes 2, 5, 8) compared to the system with metformin (lanes 3, 6, 9) and without

inhibitor (lanes 1, 4, 7) for the same period of time. These findings confirm the importance of –SH reaction with MG in protein modification as well as the role of compounds that contain reactive thiol group as competitive inhibitor of glycation. The obtained results are in agreement with the established depletion of serum thiol levels and erythrocytic GSH in diabetics compared to healthy individuals.[39,40]

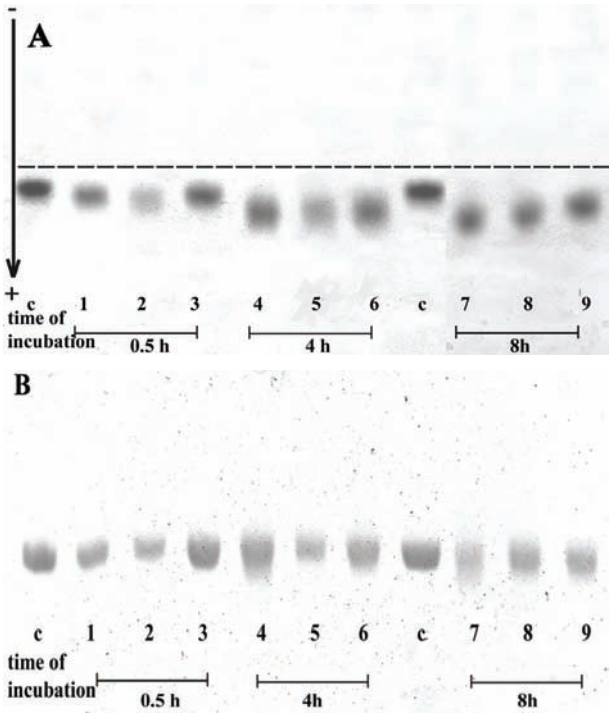

Fig. 3. Native (A) and SDS--PAGE (B) showing the changes of BSA (0.50 mM) resulting from the reaction with MG (50 mM) in the presence (20 mM) and absence of GSH and metformin. The incubation was performed in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 7 days. The marks of the lanes: lane c – – untreated BSA; lanes 1, 4 and 7 – BSA+MG; lanes 2, 5 and 8 – BSA+MG+GSH; lanes 3, 6 and 9 – BSA+MG+metformin. The electrophoresis was performed on 10 % polyacrylamide gels and the bands were visualized with CBB, as detailed in the Experimental.

TABLE II. Relative electrophoretic migrations (REM) of BSA modified with MG (BSA-MG) and in the presence of inhibitors (BSA-MG + GSH; BSA-MG + metformin). Data of REM are presented as mean±*SD* (*n* = 3) and statistical analyses were performed using the *t*-test

Time, h	REM		
	BSA-MG	BSA-MG + GSH	BSA-MG + metformin
0.5	1.044±0.007	1.055±0.027	1.043±0.008
4	1.222±0.007	1.220±0.008	1.186±0.009[a]
8	1.347±0.011	1.259±0.013[b]	1.222±0.012[c,d]

[a]$p < 0.01$ compared with BSA-MG and BSA-MG + GSH; [b,c]$p < 0.001$, compared with BSA-MG; [d]$p < 0.05$, compared with BSA-MG + GSH

HSA modification in the reaction with methylglyoxal

The investigation of BSA modification with a small excess (19 %) of MG compared to the total amount of reactive groups (84[18]) showed a significant re-

duction in the content of thiol groups and did not give cross-linking of the protein molecules. The obtained results were the basis for design of the experiments (regarding the MG concentration and duration of incubation) for the examination of HSA changes with MG. In order to determine the dependence of modification of the amino acids of the protein side chains on the MG concentration, HSA was incubated in the presence of an excess or shortage of MG compared to the total amount of reactive groups on the protein surface.

First, HSA (0.50 mM) was incubated with MG at a concentration (100 mM), considerably higher than the physiological concentration[35] and higher than the total amount of reactive groups on the protein surface[18]. The reaction mixture in 0.10 M phosphate buffer pH 7.2 was incubated at 37 °C for 24 h because after this time, intensive modification of protein molecules was detected in the experiment with BSA. The progress of MG reaction with thiol, amino and guanidino group of HSA was monitored by measuring levels of these groups in aliquots of the reaction mixture with time.

The reaction rate of the guanidine group was the highest: 48 % of available groups reacted during the first 30 min of the reaction, after which it remained unchanged (48.6±3.9 %) during the next 10 h of reaction. In the first 30 min, only 20 % of the available amino groups had reacted and after 4 h the percentage had doubled, after which time there was a gradual increase (to 49 %) after up to 10 h of incubation. The data for the progress of SH-group reaction indicates that 30 % of the groups had reacted after 30 min, 55 % after 2 h and 65 % after 10 h. At the end of the incubation period (24 h), approx. 70 % of the amino and guanidine groups and 85 % of the SH-groups had reacted.

HSA modification under these conditions was also monitored by native and SDS PAGE (Fig. 4). Changes in the charge of HSA were detected by native electrophoresis after 15 min of incubation (Fig. 4A). During 24 h of incubation, the positive charge of HSA continually declined. The changes in the charge of the HSA molecules are consistent with the change in the amounts of reacted guanidino and amino groups per HSA molecule (Table III). They were the most pronounced during the first two hours of incubation (maximum percentage of guanidino and a significant percentage of amino groups had reacted). The contribution of MG reaction with the amino groups to the change of the HSA charge increased with time.

SDS PAGE analysis (Fig. 4B) of MG modified HSA (MG-HSA) showed marked changes from unmodified HSA: there was a broadening of the peak of molecular mass 66 kDa corresponding to MG-HSA monomers. This band tailed to higher molecular mass values (extending to 90 kDa) after a very short time of incubation (about 30 min) and became more intensive with time. Also, the presence of dimers (of molecular mass *ca*. 130 kDa) and oligomers was found. These findings indicate intensive fragmentation and cross-linking of fragments (a broad band ranging between molecular masses 48–90 kDa), as well as HSA cross-

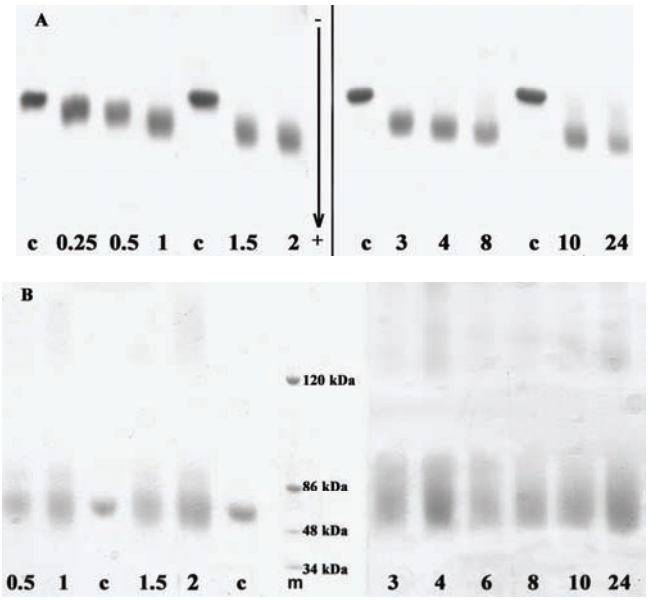

Fig. 4. Native (A) and SDS-PAGE (B) showing the changes in the mobility of HSA resulting from the reaction with MG. HSA (0.50 mM) was treated with 100 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 24 h. Lane c shows untreated HSA. The marks of other lanes correspond to the time incubation (h) of BSA with MG. Lane m – high-molecular mass standards (34, 48, 86 and 120 kDa). The electrophoresis was performed on 10 % polyacrylamide gels and the bands were visualized with CBB, as detailed in the Experimental.

-linking under the employed incubation conditions during this period of time. Taking into account that:

– the percentage of reacted guanidino groups was the highest during the first 30 min and did not change during further incubation,

– oligomers were easily detected during SDS-PAGE after two hours of incubation, during which time the amount of reacted amino and SH-groups increased (Table III) and

– the cross-linking of proteins increased with time (and the amounts of free amino and SH-groups decreased),

it can be concluded that lysine and cysteine residues play a more important role in the cross-linking of protein molecules than those of arginine.

Lysine side chains participate in HSA cross-linking *via* MG-SH (hemithioacetal) or MG-NH_2 products formed during the first step, which is consistent with an earlier proposal.[27] The obtained results are also in agreement with the proposed mechanism of MG reaction with the guanidino group,[13] in which the product of the first step participates in an intramolecular reaction with the guanidino residues of arginine, forming an imidazolone derivative, thus preventing any further linking.

TABLE III. Content of reacted amino acid side-chains. HSA (0.50 mM) was treated with 100 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for various time periods

Incubation time, h	Reacted group (mol/mol of HSA)				%[a]
	Thiol	Amino	Guanidino	Total	
0.5	0.222±0.009	11.80±0.52	11.52±0.35	23.54	28.10
2	0.413±0.009	18.90±0.38	10.15±0.17	29.46	35.17
4	0.417±0.004	24.06±0.61	11.80±0.52	36.27	43.31
8	0.459±0.013	25.28±0.52	10.96±0.43	36.70	43.81
10	0.495±0.006	28.91±0.46	11.93±0.40	41.34	49.35
24	0.647±0.004	40.71±0.48	16.8±0.33	58.16	69.43

[a]total reacted groups as the percent of the total number of available groups

Comparison of results obtained for modified BSA and HSA (Tables I and III) indicate that the total amount of reacted amino acid side-chains per mole of HSA, after 24 h of incubation, was two times higher than per mole of BSA (58.16 *vs.* 28.91). It could be said that this result conforms to the fact that the concentration of MGO in reaction mixtures with HSA was two times higher. During the entire incubation period, the amounts of reacted –SH groups were also approximately two times higher in HSA compared to BSA. The amounts of reacted Arg residues in HSA and BSA were similar after 8 and 24 h of incubation. However, it is noticeable that during the entire incubation period, the contribution of the reaction of MG with amino groups to the modification of HSA was several times higher compared to BSA. Changes in the content of amino groups brought about differences in electrophorisograms of the modified HSA compared to those obtained for BSA.

The HSA modifications and the contributions of the amino acid side chains to the changes described above result from HSA incubation with an amount of MG (200 mmol/mol HSA) that was considerably higher than the sum of the available groups (84[18]). However, it was important not only to determine the role, contributions and reactivity of each type of group (–SH, amino and guanidino) in protein modification when the amount of MG was lower than the sum of the available groups, but also to monitor changes over a longer period of time. Therefore, HSA (0.50 mM) was incubated with 10 mM MG in 0.10 mM phosphate buffer (pH 7.2) at 37 °C for 24 days. The chosen MG concentration was 10 times lower than the concentration used in the previous HSA incubation experiment and approx. 4 times lower than the sum of amino, guanidino and SH-groups on the surface of the protein molecule.

The changes in the amount (mol) of the thiol, amino and guanidino groups per mole of HSA during 24 days are shown in Table IV. In the case of MG reaction with guanidino and amino group, equilibrium was achieved after one day of incubation, with approx. 50 % (47.2±3.8 %) of the guanidino and 35 % of the amino groups reacted. Also, HSA mobility during native electrophoresis changed

considerably after the first day of incubation (Fig. 5A, lane 1), and changed slightly in the following days (lanes 2–8); differences between REM values obtained for those bands are not significant). Due to the high reaction rate of MG with the guanidino group, the percentages of Arg residues reacted during 24 h both in the case of insufficient and excess amount of MG were very high (50 and 70 %, respectively, Table V). This indicates a similar contribution of this reaction to the change in the HSA charge at different MG concentrations. The difference in the percentage of reacted amino groups at both shortage and excess of MG was more significant (35 and 69 %, respectively). Yet, the contribution of the reaction of the amino groups to the change of the HSA charge even at insufficient amount of MG was very high. After 24 h of incubation, 20.7 mol of Lys residues per mole of HSA had reacted (Table IV), which makes approx. 63 % of the total number of modified amino acid residues.

TABLE IV. Content of reacted amino acid side-chains. HSA (0.50 mM) was treated with 10 mM MG in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 24 days

Incubation time, days	Reacted group (mol/mol of HSA)				%[a]
	Thiol	Amino	Guanidino	Total	
1	0.198±0.009	20.75±0.70	11.97±0.08	32.92	39.3
3	0.430±0.006	20.39±0.83	10.40±0.23	31.22	37.3
5	0.494±0.003	23.69±0.96	11.51±0.39	35.69	42.6
7	0.528±0.012	24.08±1.11	12.22±0.23	36.83	44.0
9	0.509±0.015	28.70±0.42	13.20±0.08	42.40	50.6
15	0.514±0.010	27.45±0.14	10.85±0.23	38.81	46.3
24	0.550±0.008	29.13±0.36	10.51±0.20	40.19	48.0

[a]total reacted groups as the percent of the total number of available groups

The broadening of the HSA bands in SDS PAGE with tails to higher molecular mass values (Fig. 5B, lanes 3 and 5, obtained after 3 and 5 days of incubation) points to cross-linking of the protein molecules even with an insufficient amount of MG. The high content of reacted amino groups (compared to the MG concentration, 20 mM) as early as after 24 h of incubation points to their role in the cross-linking. Over a longer incubation period (from 9–24 days), fragmentation also emerges (the occurrence of bands with lower molecular mass values, lanes 9 and 24, Fig. 5B). Zeng and Davies[27] suggested that the initial addition of the thiol to the dicarbonyl compound and subsequent reaction with an amine can lead to cross-link formation. It could be asked what is the contribution of the SH--groups in the formation of aggregates under conditions of an insufficient amount of MG, since the number of SH-groups on the surface of the HSA molecule is negligible compared to the number of amino and guanidino groups (1:59:24).[18] After the first day of HSA incubation with 10 mM MG, 26 % of the SH-groups had reacted (0.198 mol/mol HSA), 51 % after the second day (0.388 mol/mol HSA) and equilibrium was achieved after 5 days when 65 % of the groups had

reacted (0.494 mol per mole HSA) (Table III). During this period, bands with expressed tailing to higher molecular mass values were obtained during SDS PAGE. In addition, a slight increase of reacted amino groups was found (from 35 to 40 %), which indicates the possibility of reaction with the hemithioacetal and the role of SH-group in protein modification and cross-linking. Taking into account the MG concentration (10 mM) in reaction mixture, the HSA concentration (0.50 mM), the number of reactive groups on the protein surface, the amount of reacted amino (40 %) and guanidino (50 %) groups, it can be concluded that 8.0 mM amino groups participated in cross-linking after 5 days of incubation under these conditions. Based on the amount of reacted SH-groups (65 %), *i.e.*, hemithioacetals formed during 5 days of incubation, it could be concluded that approx. 4 % of the cross-linking of the protein molecules was a consequence of hemithioacetal reaction with an amino group. This percentage is not negligible taking into account the extremely low distribution of SH-groups on the protein surface compared to amino and guanidino groups.

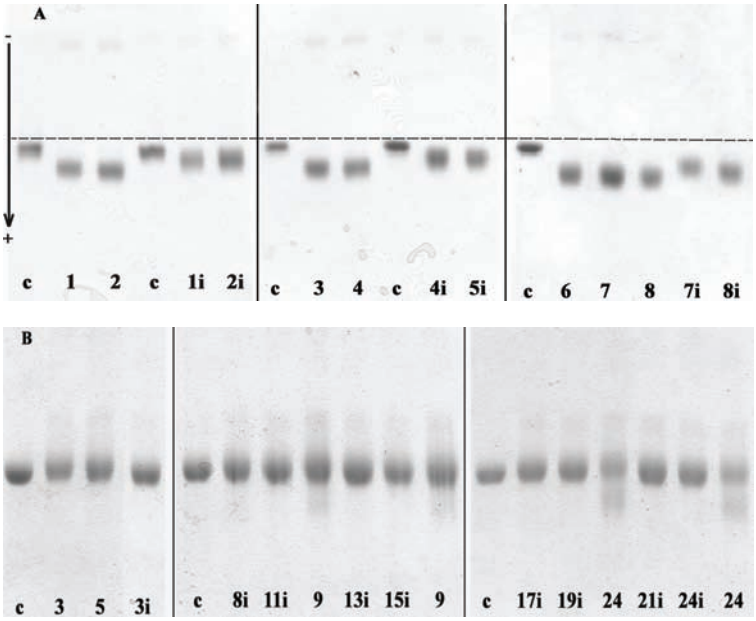

Fig. 5. Native (A) and SDS-PAGE (B) showing the changes of the mobility of HSA (0.50 mM) resulting from the reaction with methylglyoxal (10 mM). The incubation was performed in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 24 days, with (10 mM) and without metformin. Lane c shows HSA without metformin and lanes 2–24 with MG. The number of the lane corresponds to the number of incubation days. The label i beside the lane number shows that the incubation was performed in the presence of metformin. The electrophoresis was performed on 10 % polyacrylamide gels and the bands were visualized with CBB, as detailed in the Experimental.

TABLE V. Dependence of the levels of reacted thiol, amino and guanidino groups of HSA (%) on the MG concentration and incubation time. The concentration of HSA was 0.50 mM

Group	MG concentration, mM						
	100				10		
	Incubation time						
	30 min	2 h	10 h	1 day	1 day	2 days	5 days
Thiol	30	55	65	85	26	51	65
Amino	20	34	49	69	35	35	40
Guanidino	48	No change		70	50	No change	

The modification of albumin through glycation and the subsequent formation of AGEs have been shown to contribute to vascular complications in patients with diabetes. Some studies have focused on the changes of its antioxidant properties[36,41–43] and role of the thiol group (Cys 34) in these changes. Faure[43] suggested that MG can strongly impair the structure and antioxidant properties of albumin *in vitro*, leading to a modified protein similar to that isolated from diabetic patients. The results of the quantification of the reactive groups and parallel electrophoretic monitoring of HSA changes, especially at low-dose long-term exposition of albumin to MG (obtained in this paper) showed that the –SH group, in addition to the amino group, plays a role in the modification of proteins with MG and cross-linking. The significant decrease of the content of –SH groups by 26 % after 1 h incubation, *i.e.*, 72 % after 24 h of incubation, in the presence of MG may have as a consequence a decrease of the anti-oxidative capacity of albumin, which is consistent with the results of Faure *et al.*[43]

To investigate the influence of inhibitor on low-dose long-term exposition of HSA to MG, metformin was chosen. It was found that oxidative stress and glycation were significantly lower in metformin-treated patients with Type 2 diabetes mellitus.[38] In addition, experiments with BSA showed that the change of the positive charge of the BSA molecule was lower in the presence of metformin than in the presence of GSH.

The inhibitor metformin in an equimolar concentration with MG (during low-dose long-term exposition of albumin to MG), under conditions of an insufficient amount of MG, decreased the amounts of amino and guanidino groups that reacted in the first day by two to three times compared to the system without inhibitor. After a 24-h incubation, 15 % of the guanidino groups, (3.6 mol Arg residues per mole of HSA), approx. 8 % of the amino (4.7 moles of Lys side--chain per mole of HSA) and 86 % of the –SH groups (0.653 mol Cys thiol per mole of HSA) had reacted (Fig. 6). The obtained results are in agreement with the smaller changes in HSA mobility (protein charge) during native electrophoresis in the presence of metformin (Fig. 5A, lanes 1i, 2i, 4i, 5i, 7i and 8i) compared to the bands obtained without this inhibitor. Relative electrophoretic mobilities obtained for HSA bands in the presence of metformin were significantly lower

($p < 0.001$ for 1i to 7i and $p < 0.01$ for 8i) compared to the same ones without inhibitors. However, in the presence of this inhibitor, a broadening of the bands with tails to higher molecular mass values was recorded in SDS PAGE (Fig. 5B). Since all thiol groups reacted under these conditions, the product of reaction between SH-group and MG is the target for the Lys side-chain amino groups in protein cross-linking.

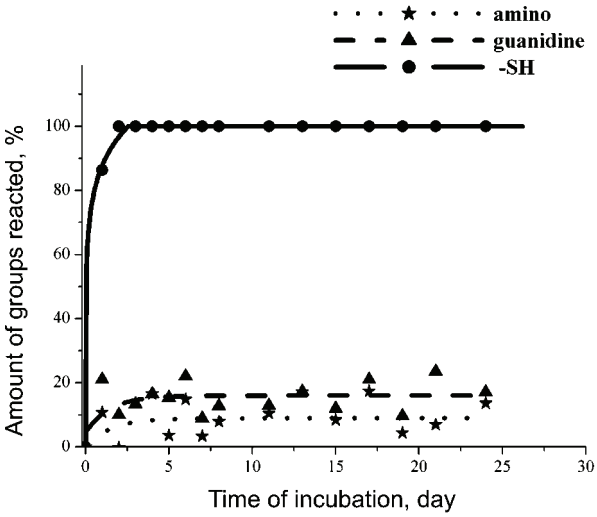

Fig. 6. Quantification of thiol, amino and guanidino groups (% of the ones reacted) during the incubation of 0.50 mM HSA with 10 mM methylglyoxal in the presence of metformin (10 mM), in 0.10 M phosphate buffer (pH 7.2) at 37 °C for 24 days. The data are the means from three determinations.

In hyperglycemia, the glycation process is accompanied by an oxidation process. By applying metformin as an antihyperglycemic agent in patients with Type 2 diabetes mellitus, the oxidation of the albumin thiol group was decreased.[38] Metformin decreases the content of toxic dicarbonyl and thereby inhibits the development of AGEs. In the present study, at the equimolar concentration of MG and metformin almost all the –SH groups reacted. This indicates that in the mixture where MG is the only reactive species, the glycation reaction of the albumin thiol group is proceeding.

An absence of fragments of lower molecular mass value (formed during 9––24 days of incubation, lanes 9 and 24, Fig. 5B) in the presence of metformin (Fig. 5B, lanes 13i, 15i, 17i, 19i, 21i, 24i), indicates the role of the reaction of guanidine with MG in the unfolding and fragmentation of the protein.

CONCLUSIONS

The results obtained indicate that thiol group reaction, despite the very small presence of thiol groups on the surface of the protein molecule, (80 times less available compared to amino and guanidino groups in HSA[18]) plays an important role in protein modification with MG and in cross-linking. Since these changes in proteins can be a cause of the development of secondary complications in dia-

betes,[16] modification of signal transduction[15,44] and the cause of various pathological states,[17] the application of MG scavenger substances is of importance in clinical practice. To date, various inhibitors of MG reactions with thiol, amino and guanidino groups have been tested, providing differences in their inhibition potency. The results presented in this paper show that metformin at equimolar concentration with MG inhibits its reaction with guanidino group but not, however, with the thiol group.

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И З В О Д

УЛОГА ТИОЛНЕ ГРУПЕ У МОДИФИКАЦИЈИ ПРОТЕИНА СА МЕТИЛГЛИОКСАЛОМ

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Метилглиоксал је веома реактивни α-оксоалдехид који се повећано ствара у хипергликемији. Реагује са нуклеофилним групама бочних остатака Lys, Arg и *N*-терминалном амино--групом, што доводи до модификације протеина. У овом раду испитивани су значај реакције SH групе са метилглиоксалом у модификацији протеина, конкурентност ове реакције у односу на реакције са амино- и гванидино-групом, ток ових реакција и њихова улога и допринос у умрежавању протеина. Као модел-системи употребљени су хумани и говеђи серум-албумин. Утврђено је да и поред веома мале заступљености SH групе на површини испитиваних молекула протеина (око 80 пута мања у односу на укупан број амино- и гванидино--група), она реагује у великом проценту (од 25–85 %). Количина изреагованих SH група и брзина реакције, време успостављања равнотеже реакције, стварања стабилног производа и допринос SH група умрежавању протеина зависе од концентрације метилглиоксала. Производ створен у реакцији SH група и недовољне количине метилглиоксала (у односу на концентрацију група доступних за модификацију) учествује у умрежавању протеина са значајним уделом (4 %). У еквимоларној концентрацији са метилглиоксалом метформин спречава његову реакцију са амино и гуанидино групама албумина, али не и са тиол групом.

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