

Inhibition of human muscle-specific enolase by methylglyoxal and irreversible formation of advanced glycation end products

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Abstract

Methylglyoxal (MG) was studied as an inhibitor and effective glycating factor of human muscle-specific enolase. The inhibition was carried out by the use of a preincubation procedure in the absence of substrate. Experiments were performed in anionic and cationic buffers and showed that inhibition of enolase by methylglyoxal and formation of enolase-derived glycation products arose more effectively in slight alkaline conditions and in the presence of inorganic phosphate. Incubation of 15 micromolar solutions of the enzyme with 2 mM, 3.1 mM and 4.34 mM MG in 100 mM phosphate buffer pH 7.4 for 3 h caused the loss a 32%, 55% and 82% of initial specific activity, respectively. The effect of MG on catalytic properties of enolase was investigated. The enzyme changed the K_M value for glycolytic substrate 2-phospho-D-glycerate (2-PGA) from 0.2 mM for native enzyme to 0.66 mM in the presence of MG. The affinity of enolase for gluconeogenic substrate phosphoenolpyruvate altered after preincubation with MG in the same manner, but less intensively. MG has no effect on V_{max} and optimal pH values. Incubation of enolase with MG for 0–48 h generated high molecular weight protein derivatives. Advanced glycation end products (AGEs) were resistant to proteolytic degradation by trypsin. Magnesium ions enhanced the enzyme inactivation by MG and facilitated AGEs formation. However, the protection for this inhibition in the presence of 2-PGA as glycolytic substrate was observed and AGEs were less effectively formed under these conditions.

Keywords: Enolase, inhibition, methylglyoxal, glycation, end products

Introduction

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) is a metalloenzyme that catalyses the dehydration of 2-phospho-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP) in the forward or catabolic direction in the second half of the Embden-Mayerhoff-Parnas glycolytic pathway [1]. During gluconeogenesis (anabolic pathway) the same enzyme as a phosphopyruvate hydratase catalyses the reverse reaction hydration of PEP to 2-PGA. Magnesium ions are a natural cofactor of enolase, essential in maintaining active conformation of the catalytic domain and important in the enzymatic reaction mechanism [2]. Enolase is one of most abundantly

expressed cytosolic proteins in many organisms. The native enolase molecule is a hetero- or homodimer formed by two subunits from of α , β , or γ type of monomer which are encoded by three different loci in the majority of human and mammals tissues. In skeletal muscles, the $\beta\beta$ isoenzyme predominates (over 90% of total enolase activity) [1,3].

One of the several posttranslational modifications of proteins is their glycation with reactive α -carbonyl compounds. The carbonyl groups of reducing sugars and low-molecular-mass α -oxoaldehydes formed covalent bonds with free amino groups of amino-acids in proteins. Inter- and extra-cellular proteins undergo of such modification, which affects their

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functional properties. Moreover, as a result, a heterogeneous mixture is formed of highly cross-linked protein derivatives, advanced glycation end-products (AGEs) [4–6]. Glycated proteins are less soluble and become resistant to proteolysis [6,7]. They are immunogenic and cytotoxic [8,9]. Glycation develops under physiological conditions in the course of aging of an organism. The effects of this process can be pathogenic, as increased production of AGEs in blood and soft tissues occurs during acute and chronic hyperglycemia. AGEs are a significant factor in the pathogenesis of diabetes and diabetic complications, such as retinopathy, kidney dysfunction, cataract, and atherosclerosis [9–12]. Accumulation of protein AGEs was also observed in neurodegenerative diseases [13,14].

One of the most effective glycation compounds is methylglyoxal (MG), which is more reactive with proteins than glucose [15]. It is a widely occurring compound formed by most glucose-metabolizing cells. MG is formed mainly during the transformation of phosphotriose intermediates along the glycolytic pathway, namely dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP), through a spontaneous non-enzymatic elimination of a phosphate group from the 1,2-enediolate intermediate form of both phosphotrioses [16]. Methylglyoxal may also be formed in a paracatalytic process, where the enediol intermediate is decomposed in the catalytic center of triose phosphate isomerase [17]. Alterations in the functions of two glycolytic enzymes, triose phosphate isomerase (TIM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which control the levels of GAP and DHAP may lead to the accumulation of DHAP (Figure 1) and cause an increase in cellular MG concentration [18,19]. Lower quantities of MG are formed from the decomposition of Amadori products of the autooxidation of free monosugars, or from the oxidation of acetone as well as from L-threonine via aminoacetone [20–22]. The level of cellular AGE depends on the intracellular mechanisms of enzymatic MG detoxification in the glyoxalase or aldose reductase system, as well as on the activity of fructosamine-3-kinase and fructoselysine-3-phosphate hydrolase, the enzymes which release free hexose from proteins glycated at early steps of the Maillard reaction [23,24]. Numerous reports on the glycation of intercellular proteins indicate the susceptibility of many enzymes to modification with methylglyoxal [25–27]. Despite extensive studies on the role of glycolysis in the generation of MG as a glycation factor, data are scarce on such a modification of just glycolytic enzymes. It was shown that MG inhibits glycolysis in metastatic cells of Ehrlich tumor and leukemic leukocytes by the formation of glycated GAPDH [28,29]. Moreover, *in vitro* glycation with MG of that enzyme from rabbit muscle and also rabbit muscle lactate dehydrogenase (LDH) induces significant reductions in their catalytic

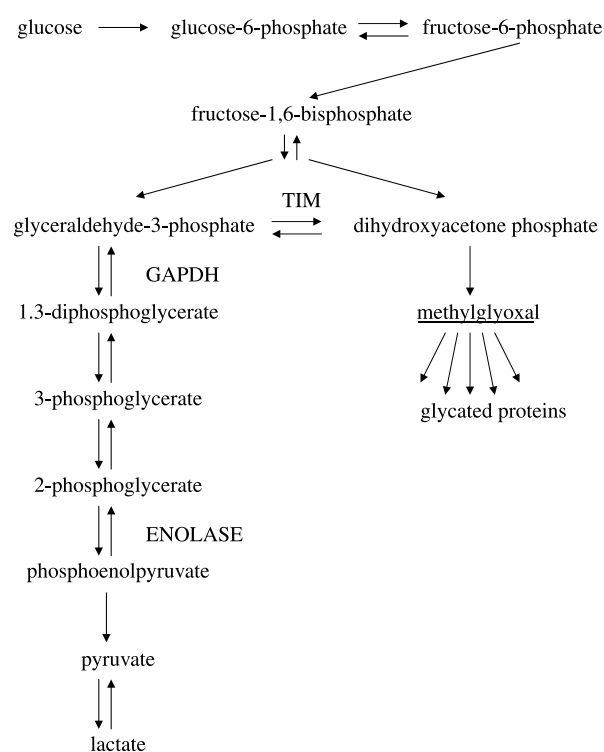


Figure 1. Formation of methylglyoxal as a by-product of the glycolytic pathway. Disturbances in the triose phosphate metabolizing enzymes TIM and GAPDH cause accumulation of dihydroxyacetone phosphate, which undergoes non-enzymatic conversion to reactive α -oxoaldehyde–methylglyoxal.

activity in a dose- and time-dependent manner [30,31]. A recent study on the modification of some glycolytic enzymes by methylglyoxal in *Saccharomyces cerevisiae* established enolase as the primary and major glycation target in yeast cells [32].

In the present study we show that human skeletal muscle enolase is *in vitro* inhibited by MG and the enzyme undergoes glycation process with the formation of high molecular weight advanced glycation end products. These AGEs are characterized by their resistance to proteolysis. The kinetic parameters of enolase, determined in the course of glycation in the presence of the glycolytic substrate 2-PGA and magnesium ions, indicate that MG diminishes enolase specific activity, although some of the enzyme's ability is maintained to catalyze the 2-PGA \leftrightarrow PEP reaction.

Materials

Methylglyoxal (40% aqueous solution) was obtained from Sigma-Aldrich and distilled before use. All other chemicals were obtained from Boehringer Mannheim GmbH and Sigma-Aldrich and were of the highest commercially available quality. Human striated muscles for enolase isolation were from histologically normal tissue obtained as postoperative material from the Department of Vascular, General, and Transplantation

Surgery of Wrocław Medical University, and approval of the study was granted by the Bioethics Commission of Wrocław Medical University.

Methods

Protein modification by methylglyoxal

Enolase from human skeletal muscle was prepared by a procedure reported previously [33] and stored in stabilizing 15 mM imidazol-HCl buffer, pH 7.0, with 50 mM NaCl, 3 mM MgSO₄, and 25% glycerol. For the experiments the enzyme samples were dialyzed against PBS buffer pH 7.34 or against 15 mM Tris-HCl pH 7.0 containing 3 mM MgSO₄. Treatment of enolase by MG was carried out at 37°C in the dark. The experiments in PBS buffer pH 7.4 were performed using 15 μM of human enolase (concentration 1.5 mg/mL, specific activity 40 U/mg) and an excess of methylglyoxal: 0.5–4.34 mM (approx. a 33 – 290 molar ratio of MG to enzyme). The high molar level of glycating factor was used for maximal saturation of accessible reactive amino-acid residues in the dimeric enolase molecule. The modification process was stopped by the addition of the 10 μL of 4 mg/mL lysine solution to bind the uncoupled methylglyoxal. Aliquots containing 5–20 μg of enolase were withdrawn at various time intervals to measure enzyme activity and for SDS/PAGE analysis.

The concentration of enolase in mg/mL was determined from absorbance at 280 nm using the absorbance coefficient $A^{0.1\%} = 0.89$, which was estimated for 1 mg of rabbit muscle enolase per 1 mL solution [1]. Enolase specific activity was measured in 2 mL test sample contained 50 mM imidazole-HCl buffer pH 6.8, 1 mM 2-PGA as a substrate, 3 mM MgSO₄ and 0.4 M KCl. The reaction was followed for 1 min at 25°C after addition of the enzyme. The formation of product PEP from 2-PGA was monitored by increase of absorbance at 240 nm. The amount of micromoles PEP was calculated from $\Delta A_{240}/\text{min}$ using value $1520 \text{ M}^{-1}\text{cm}^{-1}$ of the molar absorbance coefficient for PEP [34]. One unit of enolase activity is defined as the amount of protein which catalyzes the synthesis of 1 micromole of PEP/min under these conditions [33,35]. The specific activity was expressed in units per milligram of enzyme.

Electrophoresis of enolase advanced glycation end-products

Samples of the reaction mixtures after treatment with MG were diluted with 68 mM Tris-HCl buffer, pH 6.8, containing 1.9% SDS, 9.5% glycerol, and 5% 2-mercaptoethanol and then heated at 95°C for 5 min. An aliquot of each sample (usually 20 μg of protein) was subjected to SDS/PAGE slab gel using a Tris-glycine buffer system, pH 8.3, according to Laemmli [36].

The concentrations of stacking gel and resolving gel were 4.5% and 10%, respectively. The protein bands were fixed, stained with 0.025% Coomassie Brilliant Blue R-250 and analyzed for the molecular masses according to Witkowska et al. [33].

Effect of magnesium ions and 2-phosphoglycerate on the glycation of enolase by methylglyoxal

The treatment of human muscle-specific enolase with MG in the presence of magnesium ions as the enzyme activator and after addition of the glycolytic substrate 2-PGA was performed at optimal concentrations of both, namely 3 mM MgSO₄ and 1 mM 2-PGA. Prior to the modification experiments the samples of human enolase obviously had to be deprived of magnesium ions from the stabilizing buffer by extensive dialysis for 36 h against PBS with 5 mM EDTA at 4°C. The samples with 15 μM enolase were preincubated for 30 min. at 37°C with 1 mM 2-PGA or with 3 mM MgSO₄ or with 1 mM 2-PGA and 3 mM MgSO₄ before addition of MG. Modification was performed with 4.34 mM MG (molar excess of MG to enolase 290:1) at 37°C for 22 h. The reaction was stopped by addition of an excess of lysine.

Proteolytic digestion of enolase advanced glycation end products by trypsin

A 5-mg sample of lyophilized bovine pancreas trypsin (500 U) was dissolved in 1 mL of 15 mM Tris-HCl buffer, pH 7.3, with 10 mM CaCl₂ for digestion experiments. Enolase was modified by 4.34 mM methylglyoxal at 37°C for 19 h in PBS. The reaction was stopped by the addition of lysine solution to bind unreacted MG. A 20 μL sample containing 60 μg of glycated enolase was treated with 5 μL of protease solution at 25°C for 3 h. Aliquots of 20 μg of protein were withdrawn from the reaction mixture for SDS/PAGE analysis.

Kinetic parameters of glycated enolase

The optimum values of K_M , V_{max} , and pH were determined after 210 min of enzyme modification in PBS at 37°C with a 290:1 molar ratio of MG to protein. Modification was stopped after addition of an excess of lysine to the reaction mixture and then the samples were dialyzed against 15 mM Tris-HCl buffer, pH 7.0, with 3 mM MgSO₄. The presence of phosphate is not desired when determining enolase kinetic parameters because these ions are involved in inhibition of enzyme activity [1,38]. The catalytic reaction was initiated by the addition of 0.16 nanomole of enolase to 2 mL of the standard assay medium contained 50 mM imidazole-HCl buffer, pH 6.8, with 0.4 M KCl and 3 mM MgSO₄. The 2-PGA and PEP concentrations varied from 0.33 to 1.5 mM

and from 0.17 to 1.32 mM, adequately. Reaction was performed at 25°C for 1 min. The K_M and V_{max} values were determined from measurements of the initial rates of the catalytic reaction using the graphical method of Lineweaver-Burk [38]. The effect of pH on the activity of human muscle enolase glycosylated by methylglyoxal was studied in 2 mL samples of standard assay containing 50 mM imidazole-HCl buffer with 0.4 M KCl, 3 mM $MgSO_4$, and 1 mM 2-PGA. The pH value of standard assay varied from 6.4 to 7.4. The amount of 16 nanomole of enolase initiated the catalytic reaction.

Results

Methylglyoxal inhibits catalytic activity of human muscle-specific enolase. The enzyme incubated with methylglyoxal was susceptible to a progressive and irreversible decrease in catalytic activity. When enzyme solution at concentration 15 μM was modified with 2 mM of MG (133 molar excess of MG to protein) in 15 mM Tris-HCl buffer, pH 7.0, with 3 mM $MgSO_4$, about 65% of the initial activity remained after 180 min of the reaction at 37°C in the dark (Figure 2). The extent of enolase activity inhibition depended on the concentration of the inhibitor and also the kind of buffer used as the reaction medium. When inhibition of human muscle enolase was performed in PBS for 180 min with 2 mM, 3.1 mM and 4.34 mM MG, significant decreases in enzyme activity were observed, with 32%, 55% and 82% of the initial level, respectively (Figure 3).

By the effect of incubation of human muscle enolase with MG the enzyme activity is inhibited but also in a irreversible process the high molecular weight protein derivatives are formed as glycation products (Figure 4). After 1 h of enolase modification in PBS, only low-intensity bands of polymerized proteins were observed, whereas after longer times the appearance of high molecular weight protein bands was more evident. Increased amounts of two major protein fractions were observed, namely with M_w 120 kDa and 137 kDa and a less intensive band of protein with M_w 167 kDa, after glycation with a 290 molar excess of MG to protein.

Magnesium ions and 2-PGA influence on the glycation of enolase. Modification of human muscle enolase by methylglyoxal was subsequently extended for the presence of magnesium ions as an enolase activator and 2-PGA as glycolytic substrate in reaction mixture. For these experiments magnesium ions present in stabilizing medium were first removed by extensive dialysis for 36 h against PBS containing 5 mM EDTA and then the enzyme was treated with a 290 molar excess of methylglyoxal at 37°C for 22 h. As can be seen in Figure 5(A), lane (6), glycation of enzyme without of activator ions and glycolytic

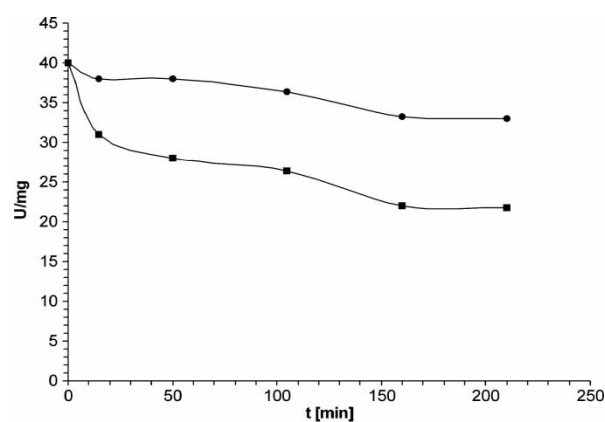


Figure 2. Inactivation of human muscle-specific enolase by methylglyoxal in 15 mM Tris-HCl buffer, pH 7.0, containing 3 mM $MgSO_4$: black squares – modified enzyme and black circles – native enzyme. Reaction mixture containing 15 μM enolase (40 U/mg) was incubated with 2 mM MG at 37°C in the dark for 210 min. Modification process was stopped by addition of 10 μL of lysine solution (4 mg/mL) to bind the noncuppled MG in reaction mixture. Aliquots containing 10 μg of protein were withdrawn at indicated time intervals for enzyme specific activity determination.

substrate leads to the formation of minute amounts of three protein aggregates with M_w of 94 kDa, 120 kDa, and 137 kDa. The enzyme maintained only 5% of the initial catalytic activity upon glycation for the first 90 min (Figure 5(B)). Preincubation of enolase with 3 mM $MgSO_4$ and then the addition of methylglyoxal facilitated the formation of AGEs (Figure 5(A), lane (3)). Methylglyoxal in the presence of magnesium ions induces the distinct formation of protein aggregates 120 kDa and a slight amount of 94 kDa and 137 kDa protein. Significant inactivation of the enzyme occurred under these conditions and an over 95% loss of initial activity occurred already after 50 min.

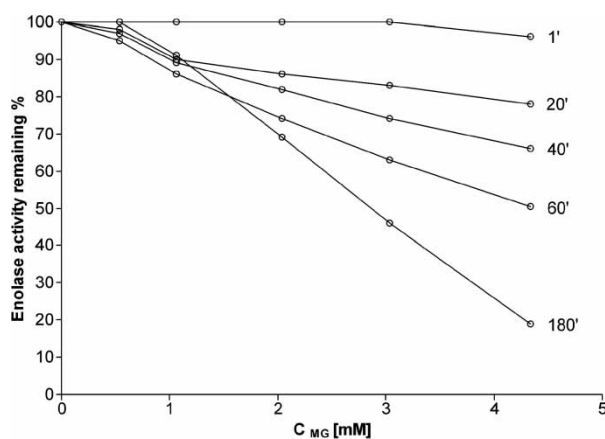


Figure 3. Inactivation of human muscle enolase by methylglyoxal in PBS. Enolase (15 μM , 40 U/mg) was incubated in PBS, pH 7.4, at 37°C in the dark for 1–180 min with different amounts of methylglyoxal: 0.54, 1.1, 2.0, 3.1, and 4.34 mM. Changes in enzyme catalytic activity are expressed in percentage of the control samples containing water instead of methylglyoxal.

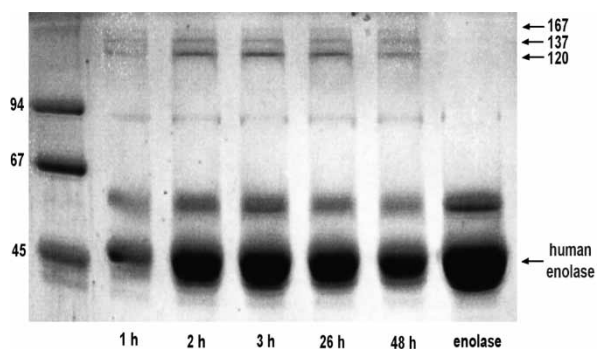


Figure 4. Formation of high molecular weight derivatives of human muscle enolase by methylglyoxal glycation. Samples of 15 μ M enolase after preincubation in PBS pH 7.4, at 37°C for 30 min were modified with 4.34 mM methylglyoxal at various times (1 h, 2 h, 3 h, 26 h, 48 h). The reaction was stopped by addition of an excess of lysine to bind unreacted methylglyoxal. Pre-stained protein standards are marked in the left lane and unmodified enolase is in the right lane. Arrows indicate enolase advanced glycation end products and unmodified enzyme.

of incubation with methylglyoxal (Figure 5(B)). The rate of inactivation of human muscle-specific enolase by MG was diminished when glycolytic substrate 2-PGA was present in the reaction mixture. As can be seen in Figure 5(B), the enzyme maintained about 40% of its activity at 50 min. of glycation by MG and over 20% after the next 40 min., despite the formation of advanced glycation end-products (Figure 5(A), lane (5)). The glycolytic substrate present in the reaction medium caused the formation of a distinct amount of 120-kDa aggregated protein, and a significant less amount of 94 kDa and 137 kDa bands. The glycation of enolase by methylglyoxal after preincubation of the enzyme with 3 mM $MgSO_4$ and 1 mM 2-PGA allowed maintaining about 50% of the initial activity at 50 min. of modification time-course (Figure 5(B)) and generated two types of AGEs (Figure 5(A), lane (4)). Distinct protein bands of 137 kDa and 120 kDa are visible, but they are of lower intensities than those formed in the presence of only 3 mM $MgSO_4$ without 2-PGA (Figure 5(A), lane (3)).

The glycation of human muscle-specific enolase creates AGEs resistant to proteolytic digestion by trypsin. The SDS/PAGE pattern reveals the presence of two protein bands at 160 kDa and 167 kDa after trypsin treatment of glycated enolase (Figure 6, lane (5)), the same pattern as for modified enolase

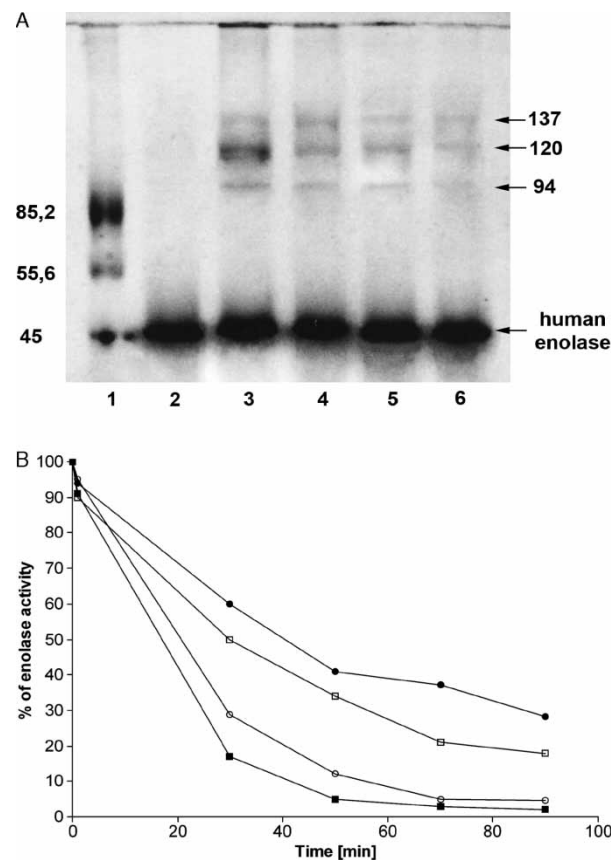


Figure 5. Effect of 3 mM $MgSO_4$ and 1 mM 2-PGA on modification of human muscle enolase by methylglyoxal. (A) SDS/PAGE pattern in 10% gel: lane (1) molecular mass protein standards: fructose-6-phosphate dehydrogenase 85.2 kDa, glutamate dehydrogenase 55.6 kDa, ovalbumin 45 kDa; lane (2) native enolase; lane (3) enolase glycated in the presence of 3 mM $MgSO_4$; lane (4) enolase modified after addition of 3 mM $MgSO_4$ with 1 mM 2-PGA; lane (5) enolase glycated in the presence of 1 mM 2-PGA; lane (6) modification of enolase without activator ions and glycolytic substrate. Arrows indicate an enolase advanced glycation end products and unmodified enzyme. (B) Rate of inactivation of enolase with methylglyoxal in the presence: black squares – of 3 mM $MgSO_4$, white squares – of 1 mM 2-PGA, black circles – of 3 mM $MgSO_4$ with 1 mM 2-PGA and white circles – without activator and glycolytic substrate.

but untreated with trypsin (Figure 6, lane (4)). The sample of native unmodified enolase did not have the high-molecular-mass derivatives (Figure 6, lane (2)), but instead was partially digested by trypsin due to the protein band of the enolase monomer at 45 kDa (Figure 6, lane (3)) which was of lower intensity than

Table I. Kinetic parameters for native and glycated human muscle enolase. Experiments were performed in conditions as described in Methods. The K_M and V_{max} values were determined from measurements of the initial rates of the catalytic reaction using the graphical method of Lineweaver-Burk.

	Substrate	K_M (mM)	V_{max} [μ mole of substrate (min^{-1}) (μ mole of enzyme) $^{-1}$]
Native enolase	2-PGA	0.20	3600
	PEP	0.58	1400
Glycated enolase	PEP	0.83	1400
	2-PGA	0.66	3600

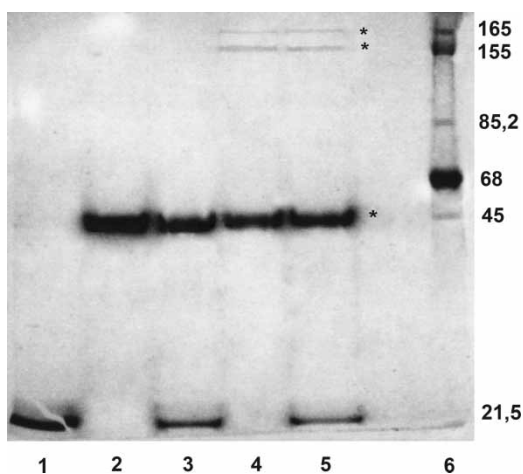


Figure 6. Susceptibility of glycation products derived from human muscle enolase to proteolytic degradation by trypsin. Electrophoretic separation of protein aggregates obtained after 19 h of reaction with MG and treated for 3 h by trypsin: lane (1) trypsin; lane (2) native enolase; lane (3) native enolase with trypsin; lane (4) enolase glycosylated 19 h by MG; lane (5) glycosylated enolase after 3 h digestion by trypsin; lane (6) molecular-mass protein standards (polymerase RNA III 165 kDa and II 155 kDa, fructose-6-phosphate kinase 85.2 kDa, BSA 68 kDa, ovalbumin 45 kDa, soybean trypsin inhibitor 21.5 kDa). Asterix indicates enolase advanced glycation end-products and unmodified enzyme.

the protein band of the same amount of undigested enolase (Figure 6, lane (2)). Trypsin demonstrated proteolytic action toward native enolase, because human enzyme have been inactivated (data not shown).

In vitro glycation of human muscle enolase by methylglyoxal altered some of the kinetic parameters of the catalyzed 2-PGA \leftrightarrow PEP reaction for the forward and backward processes. An unchanged V_{\max} value for the native and glycosylated enolase was noted, however the modification of enolase by MG

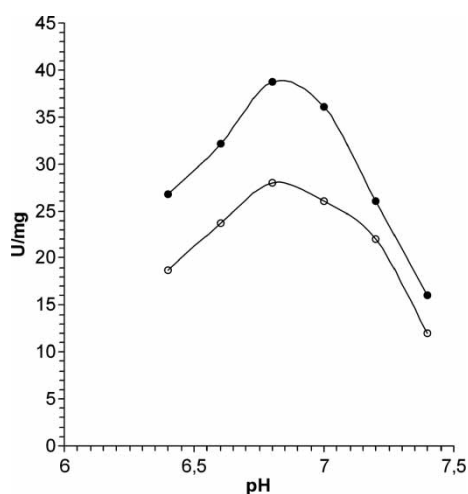


Figure 7. The pH-dependence of the catalytic activity of human muscle-specific enolase modified by methylglyoxal – white circles, compared with native enzyme – black circles.

significantly decreased its affinity to both substrates. Human muscle specific enolase modified by methylglyoxal maintained its optimum pH at 6.8, like the unmodified enzyme (Figure 7).

Discussion

Methylglyoxal may irreversibly modify various extra- and intra-cellular proteins, even when present at physiological levels, i.e. micromolar concentrations in cells. Under these conditions the majority of the generated MG is bound with a guanidine group of arginines, an amino group of lysines, and a sulfhydryl group of cysteines [39–41]. Due to the reversible reaction with the SH-group of cysteines, their modification is not a severe threat to cellular metabolism. Increasing concentrations of MG may, however, lead to irreversible modifications of arginine and lysine residues, forming advanced glycation end-products [41,42].

In previous studies we showed that specific chemical modifications of selected amino-acid residues in some muscle enolases resulted in a gradual inactivation of the enzyme; particularly the crucial roles in catalysis was found for the basic amino-acids histidine and arginine [43,44]. These results were later proved in Lebioda and Reed laboratories when the crystalline structures of yeast enolase and neuron-specific human enolase were established [2,45,46]. Selective mutagenesis of yeast enolase and its recent crystallographic studies revealed that the basic amino-acids Lys345, Lys396, His159, His373, and Arg374 in the catalytic center are important in the mechanism of the reversible transition of 2-PGA to PEP. Arginine also plays an important role in the association of enolase monomers to the dimeric structure of the native enzyme [32,47].

In these studies we showed that human muscle enolase undergoes modification by methylglyoxal. A distinct molar excess of MG, namely a 130–290 molar ratio of MG to protein, was used to modify basic amino-acid residues in the native dimeric molecule of the enzyme. Similar molar ratios were used for modifications of BSA, human Cu,Zn-superoxide dismutase, and also in the glycation of glycolytic enzymes from rabbit muscles, 3-phosphoglyceric aldehyde dehydrogenase, and lactate dehydrogenase [39,27,31].

Numerous reports indicate that interaction of MG with proteins is more effective in anionic than in cationic buffers and that the process is facilitated by phosphates and an alkaline environment of the reaction [48,49]. For these reasons we performed the modification of human muscle-specific enolase with methylglyoxal using a 100 mM phosphate buffer, pH 7.4. Then not only inhibitory effect, but more evident formation of high molecular weight enolase derivatives as a glycation products we observed.

The pH of incubating assay was not increased to a more basic because the optimal value pH for human muscle enolase activity is 6.8 [35,37]. It should be kept in mind, however, that the muscle enolase is competitively inhibited with respect to 2-PGA by inorganic phosphates in concentration range of a few dozen millimoles [37]. Therefore the first experiments on the effect of MG on human muscle enolase activity were performed at conditions favorable to the enzyme, namely 15 mM Tris-HCl buffer, pH 7.0, with 3 mM MgSO₄. The absence of phosphates allowed us to observe the enolase catalytic activity under the influence of only the glyating factor. Inactivation of the enzyme was evident under such conditions, although not so distinctly as in phosphate buffer. Moreover, glycation was less effective and high molecular weight derivatives had lower M_w and were formed more slowly.

The extent of inactivation of human muscle enolase by methylglyoxal depends on the concentration of MG and the time of reaction. The same dependence concerned the activity of the other enzymes, including the glycolytic ones [26,27,30,31]. The loss of enzymatic activity is most likely caused by the binding of MG to the lysine and arginine present in the active center and other regions of the enzyme. The geometric size of methylglyoxal enables the molecule to penetrate to the catalytic pocket of enolase and block the arginine and lysine residues important in the reversible transformation of 2-PGA to PEP. The incubation of human muscle enolase with 2 mM and 4.34 mM of MG for three hours caused a loss of 32% and 82% of the initial specific activity, respectively. Similar effects were observed for the other glycolytic enzyme, GAPDH from rabbit muscle after glycation by MG [30,31].

The inhibition of human muscle enolase by MG was found to be influenced by magnesium ions and the glycolytic substrate 2-PGA. Magnesium ions play a double role in enolases. The so-called conformational ions ensure the proper spatial structure of the active center, whereas the catalytic ions are necessary for the reaction mechanism [45,46]. They ensure the insertion of the substrate into catalytic pocket and its transformation to the reaction product [50]. Since methylglyoxal is, like the glycolytic substrate 2-PGA, a tricarbon molecule, it can get to the catalytic center.

The presence of 2-PGA substrate during human muscle enolase glycation with MG caused that the loss in catalytic activity was lower; moreover, the presence of substrate and magnesium ions maintained the enzyme activity even better. This is evidence for the protective role of the substrate in the process of modification of the protein with methylglyoxal. The phosphate residue of 2-PGA is a very important functional group for the insertion and proper orientation of the substrate to the active center of the enzyme. The phosphate group interacts with the

amino-acid residues of the catalytic pocket and conformational magnesium ions, which ensures the transformation of 2-PGA into an enol intermediate and the formation of the reaction product PEP [50].

In this study we observed that both factors, magnesium ions and 2-PGA substrate, influence the formation of diverse high molecular weight and cross-linked protein derivatives during the glycation of human muscle enolase. Prolonged incubation of the enzyme with 4.34 mM MG without magnesium ions caused less cross-linking and aggregation of the glycation products. The formation of high molecular weight AGEs occurred most effectively in the presence of magnesium ions. Probably the most relevant for the formation of these aggregates is most likely relaxing the protein conformation generated by these ions. This may facilitate the penetration of glyating factor and speed up the AGE formation.

Substrate present in the reaction mixture not only protects human muscle enolase against total loss of activity, but also impedes the formation of high molecular weight derivatives. This may be connected with the formation of more compact conformation by the protein after the binding of substrate in the active center [46,47].

The electrophoretic analysis revealed that the high molecular weight products of human muscle enolase glycation do not disappear in the presence of trypsin. The substrate specificity of trypsin involves the hydrolysis of peptide bonds close to the carboxyl group of arginine or lysine. Therefore, native enolase lost activity in the presence protease and the blocking of their basic amino-acids residues with MG prevents of protein against proteolytic action of trypsin. A similar effect was observed on digestion with trypsin followed by chymotrypsin of ribonuclease glycated with phenylglyoxal [25]. This demonstrates a general property of AGE resistance to proteolytic degradation and the tendency to form insoluble aggregates in cells and tissues [5,7].

Analysis of the kinetic parameters of human muscle enolase glycated with methylglyoxal revealed that optimum pH and reaction V_{max} was not changed, by either the glycolytic process of dehydration of 2-PGA to PEP or the reverse reaction along the gluconeogenesis pathway. Modification did, however, change the affinity of the enzyme to both substrates. For glycated enolase the K_m value for 2-PGA increased about 3.3-fold and the K_m value for PEP increased less and was 1.45-fold the value of the unmodified enzyme.

Summarizing, the susceptibility of enolase to modification by methylglyoxal indicates that glycolysis as an energy-generating metabolic pathway may be one of many cellular processes sensitive to that highly reactive glyating factor. Our experiments revealed that modification of human muscle enolase with methylglyoxal leads to a gradual inhibition of the

catalytic activity of the enzyme, with the associated formation of high molecular weight products of advanced glycation. The modification is facilitated by magnesium ions, enolase activators, but in the presence of glycolytic substrate the process is milder. The substrate plays a protective role to some extent, which leads to a less effective formation of cross-linked AGEs, allowing enolase to maintain better catalytic activity. This may have a physiological relevance because, although the glycation process of human muscle enolase generates the formation of proteolytic degradation-resistant AGEs, it retains enzymatic activity to some extent, allowing it to exert its function on the glycolytic pathway. Under such conditions, muscle cells may have the possibility to generate energy from glucose oxidation in spite of fact that the process is slower at the step catalyzed by enolase.

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