**Доклади на Българската академия на науките**
**Comptes rendus de l'Académie bulgare des Sciences**
*Tome 57, No 9, 2004*

*BIOLOGIE*
*Biologie moléculaire*

# GLYCATION OF PROTEINS IN *ESCHERICHIA COLI.* INTERFERENCE OF STRAIN DIVERSITY AND GROWTH CONDITIONS WITH GLYCATION

**R. Dimitrova, R. Mironova, I. Ivanov**

(*Submitted by Academician E. Golovinsky on May 19, 2004*)

**Abstract**

Reducing sugars react nonenzymatically with protein amino groups through a series of reactions starting with formation of Schiff bases and Amadori products and finishing with advanced glycation end products (AGEs). These late glycation products (AGEs) are considered as important pathophysiological agents in diabetes-related diseases. Glycation is considered as a slow reaction occurring over a period of weeks and affecting long-lived proteins in long living organisms. There is scarce information about glycation in lower eukaryotes and until recently none about glycation in prokaryotes. In our previous studies we have demonstrated unequivocally that glycation takes place also in bacteria and that recombinant hIFN-$\gamma$ isolated from two *E. coli* strains was glycated to different extent. To check whether the latter was due to different glycating activity of the bacteria used, in this study we investigated the ability of several *E. coli* strains to accumulate early and late (AGEs) glycation products in their proteins. The obtained results showed that the accumulation of these products was different depending on both bacterial strain genotype and bacterial growth phase.

**Key words:** glycation, growth phase, *Escherichia coli*, proteins

**Introduction.** Glycation starts as a condensation reaction between reducing sugars and primary amino groups in proteins, passes through ketosamines (Amadori products) and completes with formation of complex heterocyclic compounds designated collectively as advanced glycation end products (AGEs) [1-3]. Until recently the main

This work was supported by the International Centre for Genetic Engineering and Biotechnology (Trieste, Italy) under Contract BG02/008.

objects for studying glycation were long living eukaryotes including man. Applying mass-spectral and immunochemical methods, however, we have found that glycation takes place also in organisms with a very short life span such as bacteria [4]. Up to now neither the chemical nature of the glycating compounds nor the internal (physiological) and external (environmental) factors affecting this process are well known.

Unlike enzymatic glycosylation, which is designed by nature to make proteins functional and stable, glycation is destructive for biopolymers. In a recent study we have shown that recombinant human interferon-$\gamma$ (rhIFN-$\gamma$) preparations isolated from two strains of *Escherichia coli* K12 had different stability and biological activity [4,5]. We proved that this was related with the different rate of glycation of hIFN-$\gamma$. This finding implied that different bacterial strains might have different glycation activity and tempted us to investigate in more details the interference of bacterial strain diversity (i.e. bacterial genotype) on glycation of proteins in *Escherichia coli.*

**Materials and methods.** *Escherichia coli* K12 strains used in this study and their genotype are listed in Table 1.

To isolate total bacterial protein, LB medium (250 ml) was inoculated (1:100 v/v) with overnight bacterial culture and cultivated at 37 °C in a metabolic shaker. Bacteria were harvested by centrifugation at 4 °C for 20 min and the cell pellet was suspended in 1 ml 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.14 M NaCl and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). $MgCl_2$ was added to a final concentration of 10 mM and the lysates were incubated at 37 °C for 1 h in the presence of DNase I and RNase A (50 $\mu$g/ml each) and lysozyme (20 $\mu$g/ml). Low molecular mass material was removed by gel filtration on Sephadex G25 and the protein fraction (representing total bacterial protein) was used for analysis.

Early (Amadori) glycation products were determined by the TBA (thiobarbituric acid) method [6]. To this end 1-ml protein solutions (5 mg/ml) were mixed with 0.5 ml 1.0 N oxalic acid and incubated at 100 °C in a dry thermostat for 5 h. The samples were chilled in ice, 0.5 ml ice cold 40% trichloroacetic acid was added and 10 min later the protein precipitates were removed by centrifugation. Supernatants (1.5 ml) were mixed with 0.5 ml 0.05 M 2-thiobarbituric acid, incubated consecutively at 37 °C for 15 min and 20 min at room temperature and measured at $\lambda = 443$ nm. The content of Amadori products (in $\mu$M fructose equivalents per mg protein) was determined using a standard curve built by fructose.

Non-fluorescent AGEs were detected by ELISA using a monoclonal antibody against N-carboxymethyl-lysine (CML) conjugated with horseradish peroxidase. The content of CML (in mg/ml) was measured by using a standard curve built with in vitro glycated bovine serum albumin as a reference.

**Results and discussion.** STRAIN DIVERSITY AND GLYCATION OF PROTEINS IN *E. coli.* In a preliminary study we have shown that recombinant hIFN-$\gamma$ preparations isolated from two strains of *E. coli* K12 (LE392 and XL1) were glycated to a different extent, which implied a different glycating activity of these two host strains [4,5]. Bearing in mind that glycation is unfavourable for recombinant proteins and that the host *E. coli* strains might have different glycating potentials, we designed to investigate in this study the most frequently used in the recombinant DNA practice *E. coli* K12 strains (see Table 1) for their glycating activity. To this end total bacterial protein was analysed for content of both early (Amadori) and advanced glycation end products. Amadori products (ketosamines) were determined by the TBA method using a standard referent curve built by fructose. The content of AGEs was measured by ELISA using a monoclonal antibody specific to the most common advanced glycation end product N-carboxymethyl-lysine (CML). We used in the latter case in vitro glycated bovine serum albumin (BSA) as a reference. The results from both analyses are presented in Fig. 1*A* and *B*.

Table 1

*Escherichia coli* K12 strains and their genotype

| Strain | Genotype | Phenotype (antibiotic resistance |
|---|---|---|
| AB 1157 | *F-thr-1 leu-6 thi-1 sup E44 lac Y1 mal A1 ls gal K2 ara-14 xyl-5 mtl-1 pro A2 his-4 arg E3 str-31 tsx-33 sup-37* | Penicillin Streptomycin Erythromycin |
| AB 1886 | *F-thr-1 leu-6 thi-1 lac Y1 gal K2 ara-14 xyl-5 mtl-1 pro A2 his-4 arg E3 str-31 tsx-33 sup-37 uvr A6* | Ampicillin Erythromycin |
| AB 2463 | *F-thr-1 leu-6 thi-1 lac Y1 gal K2 ara-14 xyl-5 mtl-1 pro A2 his-4 arg E3 str-31 tsx-33 sup-37 rec A13* | Penicillin Ampicillin Streptomycin Erythromycin (Erm) |
| 33W1485 | *F+ lac+* | – |
| 2340 | *F+ lac- str$^r$* | Streptomycin |
| LE392o LE392n | *hsdR514($r_k^- m_k^+$) supE44 supF58 lacY galK2 galT22 metB1 trp55* | – |
| DH1 | *recA1 endA1 thi-1 hsdR17 supE44 gyrA96* | – |
| HB101 | *pro leu thi lacY hsd20 ($hsdS_B^-$ endA recA rpsL20(str$^r$) ara-14 galK2 xyl-5 mtl-1 supE44* | Streptomycin |
| XL1 Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F′ proAB lacI$^q$ ZΔM15 Tn10 tet$^r$* | Tetracycline |

Note: LE392o and LE392n are two isolates of the strain *E. coli* LE392

As it is seen from Fig. 1*A*, the variations in content of Amadori products in the protein samples isolated from the different *E. coli* strains was negligible although some strains such as AB1157 and LE392o showed always some higher level of early glycation products in comparison with the other strains. Unlike Amadori products, the content of AGEs (CML) was remarkably different. The highest content of CML was registered in the proteins isolated from the strains LE392o, followed by LE392n, DH1, 33W1485 and AB1157. The lowest content of CML was found in the protein sample of XL1. This result correlated with our recent observation showing that hIFN-$\gamma$ isolated from *E. coli* XL1 was much more active and stable (did not undergo degradation and covalent

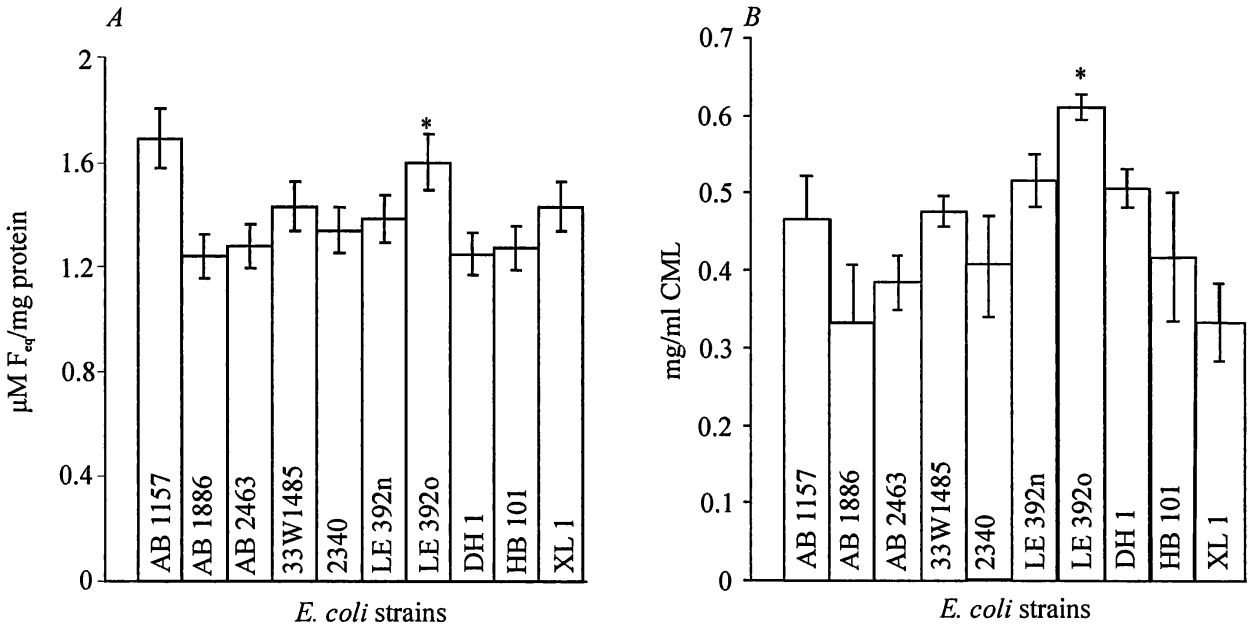


Fig. 1. Glycation products in total protein of *Escherichia coli* K12 strains cultivated in LB. *A*) Early glycation (Amadori) products: $F_{eq}$, fructose equivalents as determined from $A_{443}$ and a standard curve built with fructose; *B*) Non-fluorescent AGEs (CML) as determined by ELISA

dimerization on storage) compared to the preparation obtained from the strain *E. coli* LE392 [4,5].

GROWTH PHASE AND GLYCATION. The effect of growth phase on glycation was studied using the strain *E. coli* AB1157. To this end bacteria were grown in LB medium under standard conditions and cells were collected at three different growth stages – middle and late exponential, and stationary phase. Total bacterial protein was isolated and analysed for content of both Amadori products and CML. As it is shown in Fig. 2*A*, the differences in content of Amadori products in the three protein samples were insignificant, although there was a visible tendency to an increase in the level of early glycation products from the middle to the stationary growth phases. The differences in content of CML, however, were much more impressive. As it is seen in Fig. 2*B*, the content of AGEs (CML) in the protein sample isolated from the cells in stationary phase was almost three times higher than that of the middle exponential phase. This result indicates that although AGEs were formed in *E. coli* cells during the whole growth cycle, they were accumulated in bacterial protein mainly during the stationary phase.

The fact that the content of Amadori products varied negligibly between the strains whereas the variation of AGEs was remarkable, raised the question: Since AGEs (including CML) were late derivatives of the early glycation (Amadori) products, why the level of AGEs should be different for the different *E. coli* strains. To explain this, two hypotheses could be considered: 1) The reactivity of the primary glycating agents in the strains is different which means that the rate of conversion of the Amadori products into AGEs will be different; 2) Bacteria have "AGEs cleaning system" stripping AGEs from bacterial biopolymers. This system might work with different efficiency which predetermines the different content of AGEs in the strains.

The second hypothesis is consistent with our preliminary results on the presence of 3-deoxyglucosone (3-DG) in *E. coli* extracts [4]. 3-DG is a product of conversion of

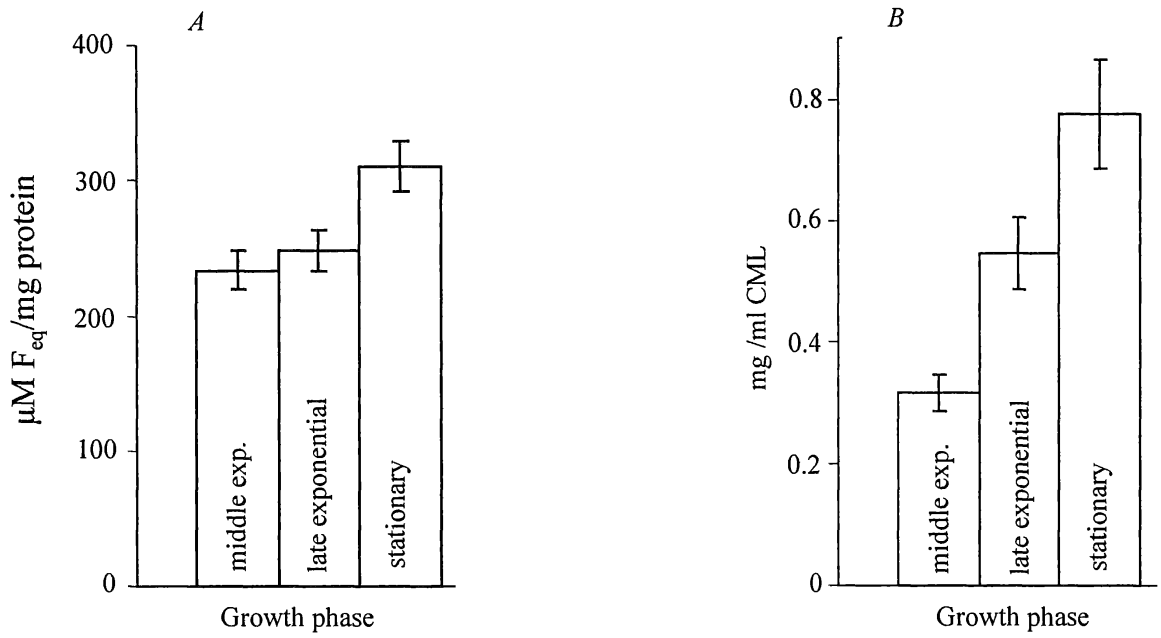


Fig. 2. Glycation products in total protein of *E. coli* AB1157 cells cultivated in LB medium and collected at different growth phase stages. *A*) Early glycation (Amadori) products: $F_{eq}$, fructose equivalents as determined from $A_{443}$ and a standard curve built with fructose; *B*) Non-fluorescent AGEs (CML) as determined by ELISA

Amadori products and belongs to the very reactive dicarbonyl glycation compounds. It has originally been found in diabetic patients [7] and we showed that 3-DG exists also in *E. coli* [4]. In this study we reported that the content of 3-DG in culture medium (the supernatant remaining after harvesting the cells) was much higher than that in the cell lysates. This implies the existence of some (unknown) mechanism for exporting 3-DG out of the cell.

Another protective mechanism against glycation could be the new enzyme amadoriase found in many prokaryotes including *E. coli* [8]. This enzyme can hydrolyze ketosamines (Amadori products) and is supposed to participate in the utilization of non-conventional carbon sources such as fructoselysine (a typical Amadori product). We are tempted to predict a new (hypothetical) role of this and other (unknown) related enzymes in removing early and late glycation products from bacterial proteins.

## REFERENCES

[1] Brownlee M., A. Cerami, H. Vlassara. N. Engl. J. Med., **318**, 1988, 1315–1321. [2] Brownlee M. Annu. Rev. Med., **46**, 1995, 223–234. [3] Monnier V. M., A. Cerami. Science, **211**, 1981, 491–493. [4] Mironova R., T. Niwa, H. Hayashi, R. Dimitrova, I. Ivanov. Mol. Microbiol., **39**, 2001, 1061–1068. [5] Mironova R., T. Niwa, R. Dimitrova, M. Boyanova, I. Ivanov. J. Biol. Chem., **278**, 2003, 51068–51074. [6] Fluckiger R., P. M.

GALLOP. Methods Enzymol., **106**, 1984, 77–87. [7] NIWA T., N. TAKEDA, H. YOSHIZUMI, A. TATEMATSU, M. OHARA, S. TOMIYAMA, K. NIIMURA. Biochem. Biophys. Res. Commun., **196**, 1993, 837–843. [8] WIAME E., G. DELPIERRE, F. COLLARD, E. VAN SCHAFTINGEN. J. Biol. Chem., **277**, 2002, 42523–42529.

*Institute of Molecular Biology*
*Bulgarian Academy of Sciences*
*1113 Sofia, Bulgaria*
*e-mail*: iviv@obzor.bio21.bas.bg