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GLYCATION OF PROTEINS IN *ESCHERICHIA COLI*. DETECTION OF GLYCATING COMPOUNDS USING HISTONE H1 AS A SUBSTRATE

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Abstract

Glycation is a condensation reaction between reducing sugars and primary amino groups in proteins. It proceeds in many stages leading to formation of reversible Schiff bases, Amadori products and finally to irreversible products called advanced glycation end products (AGEs). Glycation is extensively studied in relation to diabetes and aging. Because of the common belief that it is a slow process under physiological conditions affecting long-lived proteins, until recently glycation has not been studied in prokaryotes. Our recent data, however, have shown that glycation takes place also in bacteria. The aim of this study is to check whether bacterial cytosol contains glycating compounds. Using histone H1 as a substrate for glycation, we show that incubation of histone H1 (placed in dialysis bags) in bacterial lysates leads to accumulation of both early and late (AGEs) glycation products. This means that the glycating compounds of *Escherichia coli* are low molecular mass substances dissolved in bacterial cytoplasm.

Key words: *Escherichia coli*, glycation, histone H1

Introduction. Glycation is a non-enzymatic condensation reaction between reducing sugars and primary amino groups in proteins (Maillard reaction). It proceeds throughout many stages, starting with formation of aldimines (Schiff bases) and ending up with complex heterocyclic compounds designated collectively as advanced glycation end products (AGEs) [1-3]. The Maillard reaction has been originally studied in food chemistry in relation to "food browning" occurring during thermal processing of foods rich in both proteins and carbohydrates. Later on AGEs were found in blood proteins (haemoglobin, lens crystallin, etc.) of diabetic patients [4-8]. This was an indication that non-enzymatic glycosylation was also possible under physiological conditions (in vivo). Because of its importance to medicine, glycation is most extensively studied in humans. There are only few reports on glycation in lower eukaryotes [9,10] and, until re-

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cently, no report on glycation in prokaryotes. Applying specific monoclonal antibodies against AGEs, fluorescent spectroscopy, mass-spectrometry and other adequate methods for protein research, we have shown undoubtedly that glycation takes place also in *Escherichia coli* [11,12]. Although the obtained results allow us to assume that the site of glycation in bacteria is bacterial cytoplasm, this has not been proven yet.

To check whether the cytoplasm of *Escherichia coli* contains chemically reactive glycating compounds, we used in this study the Lys/Arg rich histone H1 as a substrate for detecting AGEs after treatment with bacterial lysates.

Materials and methods. Histone H1 was extracted from rat liver by direct homogenization in 5% perchloric acid. After centrifugation at 1500 g for 10 min, the protein was precipitated by trichloroacetic acid (20% final concentration) for 1 h at 4 °C. Precipitate was washed twice with cold ethanol/diethyl ether, dried in vacuum and stored at −20 °C.

Protein-free bacterial lysates were prepared as follows: 1 litre LB or M9 (minimal) medium was inoculated with overnight *E. coli* LE392 cell culture and grown for 16 h at 37 °C. Bacteria were harvested by centrifugation and the cell pellet was resuspended in 200 ml TS buffer (10 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, 0.1 mM phenylmethanesulphonyl fluoride). The cells were disrupted by ultrasonication and the lysates were cleared at 3000 g for 20 min. To remove proteins, bentonite was added to 5% final concentration and after stirring at room temperature for 1 h the lysates were centrifuged and used for further experiments.

To assay bacterial lysates for glycation activity, histone H1 samples in TS buffer (1 mg/ml) were placed in benzoylated dialysis tubings (with a molecular mass cut-off of 2 kDa) and dialysed overnight at 37 °C against protein-free bacterial lysates. The samples were then dialysed against TS buffer for 24 h and stored at 4 °C.

Early glycation (Amadori) products in protein samples were determined by the TBA (thiobarbituric acid) method [13]. To this end 1-ml histone H1 solutions (5 mg/ml) were mixed with 0.5 ml 1.0 N oxalic acid and incubated at 100 °C in 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 $\lambda = 443$ńm. The content of Amadori products (in μM fructose equivalents per mg protein) was determined using a standard curve built by pure fructose.

Fluorescent AGEs in histone H1 were determined by fluorescence measurement on Shimadzu RF-5000 spectrophotometer at $\lambda_{ex} = 365$ nm and $\lambda_{em} = 443$ nm and non-fluorescent AGEs were detected by ELISA using a monoclonal antibody against N-carboxymethyl-lysine (CML). The content of CML in the protein samples was quantified by a standard curve built by using in vitro glycated bovine serum albumin as a referent.

The destructive effect of glycation on histone H1 was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels according to LAEMMLI [14].

Results and discussion. Prokaryotic organisms have been ignored for a long time from studies on non-enzymatic glycosylation because of the common belief that glycation is a very slow process. It requires prolonged (up to several weeks) incubation of proteins with reducing sugars to obtain detectable amounts of AGEs which, of course, is much longer than the life span of bacteria. Our recent studies, however, unequivocally demonstrated that in spite of the short life span of *E. coli* (20–30 min) its proteins were extensively glycated [11]. This means that glycating agents of *E. coli* are more reactive in comparison with those of the higher eukaryotes including man. Since glycation affects intracellular proteins (both bacterial and recombinant) we assume that the bacterial glycating compounds are endogenous products and, therefore, they are supposed to be dissolved in bacterial cytoplasm.

This study aims to prove that the cytoplasm of *Escherichia coli* (in the form of clear bacterial lysate) contains reactive glycating compounds. To this end we choose to use histone H1 as a substrate of glycation for three reasons: i) It is rich in Lys and Arg, and glycation is known to affect the side chain amino groups mainly of these two amino acids; ii) As a eukaryotic nuclear protein, histone H1 is less glycated in comparison to other eukaryotic and prokaryotic cytoplasmic proteins, and, therefore, its natural "AGEs background" is very low; iii) The procedure for isolation of histone H1 is simple and destructive for most of the non-histone proteins (see Materials and methods), which reduces the risk of contamination with cellular proteases or other unwanted enzymes.

To prevent unpredictable interactions of histone H1 with bacterial cytosolic proteins, two precautions were taken: 1) Bacterial lysates were deproteinized under mild conditions using bentonite (a native colloidal aluminium silicate), which is known to adsorb proteins but not sugars and other low molecular mass substances, and, 2) The substrate protein was exposed to the deproteinized bacterial lysates via dialyses membranes with a molecular mass cut-off of 2 kDa.

In our experiments histone samples were treated with bacterial lysates overnight and after extensive dialysis against buffer (to remove the lysate's components) were incubated at 4 °C for up to one month. Taking into consideration that glycation products are two types – early and late (AGEs), and that the late products are fluorescent and non-fluorescent, the histone samples were analysed for all these glycation products.

Early glycation (Amadori) products were determined by TBA method in histone H1 samples dialysed 24 h against cell lysates obtained from bacteria cultivated in LB (rich) and M9 (minimal) growth media. As it is shown in Fig. 1, Amadori products were formed in both media, although, surprisingly, their content in LB medium was higher.

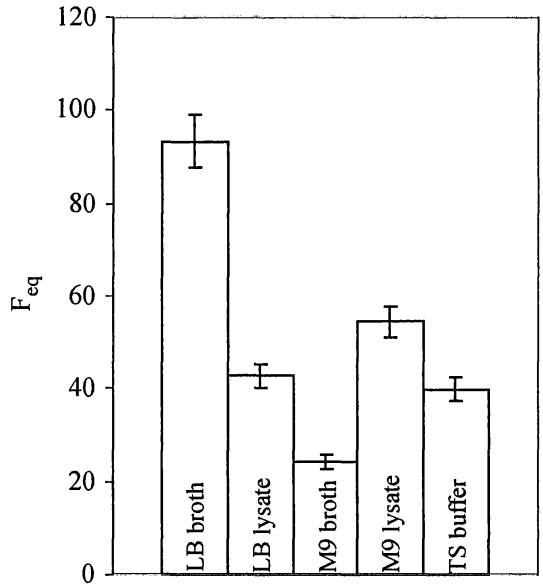

Fig. 1. Early (Amadori) products in histone H1 dialysed against LB and M9 broths or deproteinized lysates from *Escherichia coli* cultured in the same broths. F_{eq}: μM fructose equivalents as determined by A_{443} and a standard curve built with fructose

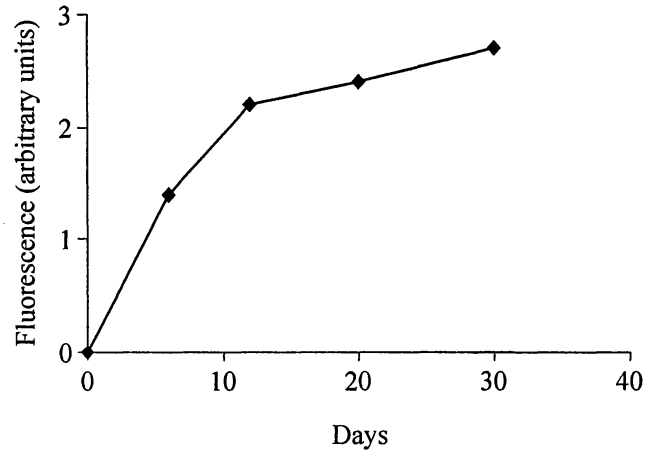

Fig. 2. Fluorescent AGEs in histone H1 dialysed against deproteinized *Escherichia coli* lysates. AGEs were determined by fluorescence measurements at $\lambda_{ex} = 365\,nm$ and $\lambda_{em} = 443\,nm$. FI: Fluorescence intensity in arbitrary units (ordinate) represents the difference between FI of the treated and untreated (control) samples

Accumulation of fluorescent AGEs in bacterial lysate treated histone H1 samples during storage at 4 °C was quantified by fluorescence measurements at $\lambda_{ex} = 365\,nm$ and $\lambda_{em} = 443\,nm$ (typical conditions for AGEs). The results presented in Fig. 2 indicate that AGEs gradually accumulated in the substrate protein during the first 5–10 days of storage, after which their content remained constant.

Non-fluorescent AGEs were determined by ELISA using a monoclonal antibody against the most typical non-fluorescent advanced glycation end product – N-carboxymethyl-lisine (CML). As it is seen from Fig. 3, CML was found in both protein samples treated with either LB or M9 bacterial lysates. This result also shows that in spite of the different content of early glycation products in samples treated with LB and M9 lysates (see Fig. 1), the content of CML (representing late AGEs) is the same.

In the paper cited above [11] we describe the observation that recombinant human interferon-γ isolated from *E. coli* undergoes severe molecular changes, such as non-enzymatic cleavage and covalent dimerization, and proves that this process is related to the glycation of the protein. To check whether histone H1 (as a substrate of bacterial glycation) is subjected to similar alterations, 20-day-old histone preparations pretreated with bacterial lysates were analysed by SDS-polyacrylamide gel electrophoresis. Figure 4 demonstrates that these preparations (unlike controls) were totally changed. They contained both truncated and oligomeric forms of histone H1 which was another indication that this protein was glycated.

The results presented in this paper indicate that histone H1 treated with deproteinized bacterial lysates contains both early (Amadori) and late (AGEs) products of glycation and is subjected to dramatic molecular changes including degradation and polymerization. This means that glycating agents of *E. coli* are low molecular mass substances dissolved in bacterial cytosol. The fact that these substances exist in bacterial cells cultured both in rich (LB) and minimal (M9) media means that they are endogenous products, most probably related to the conventional bacterial metabolism. Their identification is beyond the scope of this study and will be a subject of forthcoming investigations.

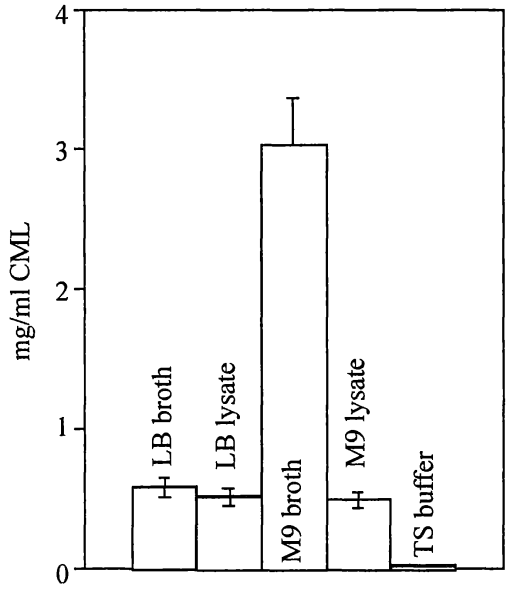

Fig. 3. Non-fluorescent AGEs (CML) in histone H1 dialysed against LB and M9 broths or deproteinized lysates from *Escherichia coli* cultured in the same broths

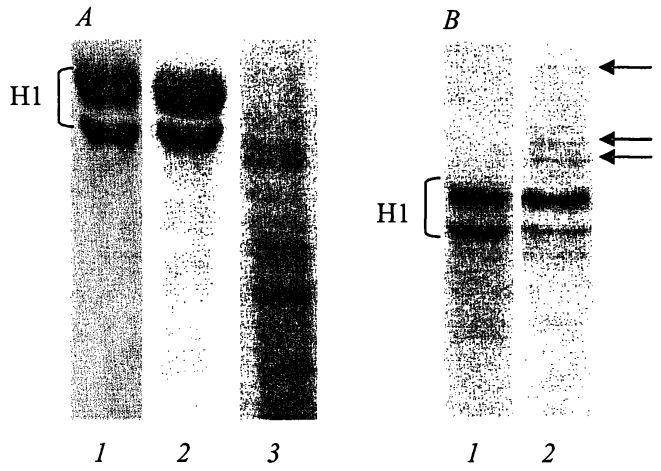

Fig. 4. SDS-PAGE analysis of histone H1 treated with deproteinized bacterial lysates after storage at 4 °C: *A*. Degradation products in histone H1 preparations after 0 (*2*) and 20 days (*3*) of incubation; control sample (untreated protein) (*1*). *B*. Overrun gel to see oligomeric forms of histone H1: 20 days (*2*) and control sample (*1*)

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] Bunn H. F., R. Shapiro, M. McManus, L. Garrick,

M. J. McDonald, P. M. Gallop, K. H. Gabbay. J. Biol. Chem., **254**, 1979, 3892–3898. [5] Stevens A. J. Am. Optom. Assoc., **69**, 1998, 519–530. [6] Day J. F., S. R. Thorpe, J. W. Baynes. J. Biol. Chem., **254**, 1979, 595–597. [7] Sasaki J., G. L. Cottam. Biochem. Biophys. Res. Commun., **104**, 1982, 977–983. [8] Hedrick C. C., S. R. Thorpe, M. X. Fu, C. M. Harper, J. Yoo, S. M. Kim, H. Wong, A. L. Peters. Diabetologia, **43**, 2000, 312–320. [9] Colas B., Y. Boulanger. FEBS Lett., **163**, 1983, 175–180. [10] Zahner D., V. Liemans, F. Malaisse-Lagae, W. J. Malaisse. Diabetes Res., **12**, 1989, 165–168. [11] Mironova R., T. Niwa, H. Hayashi, R. Dimitrova, I. Ivanov. Mol. Microbiol., **39**, 2001, 1061–1068. [12] Mironova R., T. Niwa, R. Dimitrova, M. Boyanova, I. Ivanov. J. Biol. Chem., **278**, 2003, 51068–51074. [13] Fluckiger R., P. M. Gallop. Methods Enzymol., 106, 1984, 77–87. [14] Laemmli U. Nature, **227**, 1970, 680–685.

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