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PROTEIN AMADORIASE ACTIVITY OF THE *ESCHERICHIA COLI* K-12 GLYCOLYTIC ENZYME PHOSPHOGLUCOSE ISOMERASE

Elitsa Boteva, Konstantin Doychev, Rositsa Tsekovska, Yordan Handzhyski, Ivan Ivanov, Roumyana Mironova[#]

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Abstract

The Maillard reaction (glycation) is a spontaneous non-enzymatic reaction between primary amines and carbonyl compounds, which affects proteins and DNA of both pro- and eukaryotes. In recent studies, we have shown that the glycolytic enzyme of *Escherichia coli* phosphoglucose isomerase (PGI) catalyzes in vitro the deglycation of DNA modified with glucose 6-phosphate (G6P)-derived Amadori products (APs). APs are early products of the Maillard reaction, which are formed not only on DNA but also on other amines including proteins. The aim of the current study was to test the *E. coli* PGI for protein deglycation (amadoriase) activity. To this end, we used chicken lysozyme glycated with G6P as a model protein. Treatment of the glycated lysozyme with protein extract from an *E. coli* PGI proficient but not deficient strain resulted in the release of G6P, which was indicative of PGI protein amadoriase activity. G6P-derived APs represent fructose 6-phosphate (F6P) residues bound to free amino groups of the model protein and because of that we compared the kinetic constants of the *E. coli* PGI for the glycated lysozyme and for free F6P. PGI demonstrated nearly two times higher affinity to the glycated lysozyme (K'_m = 0.06 mM) than to free F6P (K'_m = 0.1 mM). However, the apparent catalytic constant of the enzyme with the glycated lysozyme (K'_{cat} = 93

[#]Corresponding author.

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s^{-1}) was eight times lower than with F6P (K'_{cat} = 736 s^{-1}). Future studies are expected to shed light on the physiological relevance of the PGI protein amadoriase activity we report here.

Key words: phosphoglucose isomerase, amadoriase, *Escherichia coli*

Introduction. The MAILLARD reaction (glycation) [1] is a non-enzymatic reaction between reducing sugars and amino groups in proteins, lipids and DNA [2–5]. In the early stage of this reaction, the amine nitrogen acts as a nucleophile to attack the electrophilic carbon atom of the sugar carbonyl group. As a result, with aldose sugars reversible aldimines (Schiff's bases) are formed, which then rearrange less reversibly to ketoamines called Amadori products (APs) (Fig. 1). Schiff's bases (SBs) and APs undergo further spontaneous transformations, often through a lot of intermediate stages, to form stable final derivatives designated as advanced glycation end products (AGEs).

In living cells, both proteins and DNA are affected by the Maillard reaction [2,5]. The accumulation of early and advanced glycation adducts in these life-essential amines usually impedes their physiological functions. Therefore, organisms have evolved diverse defense mechanisms against glycation. Enzymes called amadoriases (deglycases) for repair of APs-modified proteins have been discovered two decades ago [6,7]. These enzymes employ different catalytic mechanisms to repair APs-modified amines. Amadoriases with kinase [6,7], oxidase [8,9] and hydrolase [10] activities have been described. The human DJ-1 deglycase and its *Escherichia coli* homologues HchA (Hsp31),YhbO and YajL repair amino acids and proteins glycated by glyoxals [11,12]. Also, the same enzymes have been reported to repair glycated guanine nucleotides and nucleic acids as well [13]. The primary glycation adducts repaired by DJ-1 and its bacterial ho-

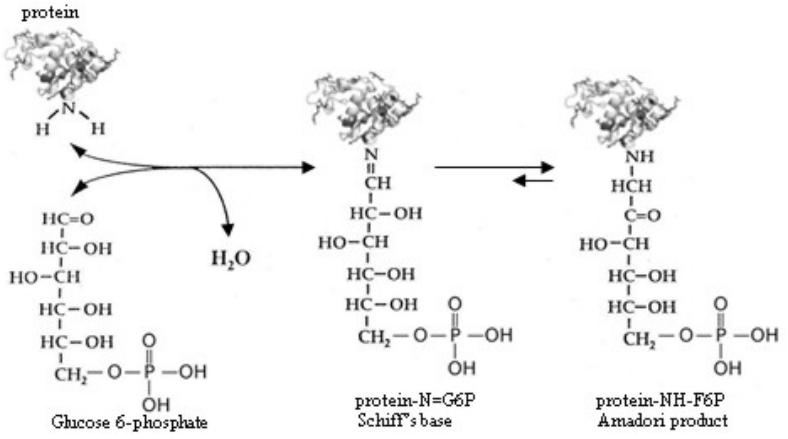

Fig. 1. Early stage of the Maillard reaction initiated on proteins by glucose 6-phosphate

mologues are aminocarbinols rather than APs and because of that these enzymes cannot be defined as amadoriases.

Previously, we have demonstrated that the glycolytic enzyme of *E. coli* phosphoglucose isomerase (PGI) exhibits DNA amadoriase activity in vitro. We have shown that PGI deglycates a single stranded DNA oligonucleotide and its hybrid duplex with a peptide nucleic acid [14] as well as high molecular mass double stranded DNA [15] modified with glucose 6-phosphate (G6P)-derived APs. Our studies revealed that besides phosphorylation, oxidation and hydrolysis, amadoriases can utilize also an isomerization mechanism for deglycation. In support of this finding, the *E. coli* FrlB amadoriase, which deglycates fructoselysine 6-phosphate, has been reported to share homology with the isomerase domain of the enzyme glucosamine 6-phosphate synthase [10]. In the present study, we used chicken lysozyme modified with G6P-derived APs to test the *E. coli* PGI for broader substrate specificity including proteins. We report data on the apparent kinetic constants of the *E. coli* PGI for glycated lysozyme as compared to the respective kinetic constants of the enzyme for free fructose 6-phosphate (F6P).

Materials and methods. ***Preparation of glycated chicken lysozyme.*** Chicken lysozyme from egg white (Serva 28260) with a concentration of 10 mg/ml was incubated for 22 days with 0.1 M G6P in deionized water at 37 °C. Before incubation the reaction mixture was filter (0.2 µm) sterilized. The glycated lysozyme was purified from the unreacted G6P in two steps including gel filtration chromatography and ultrafiltration. The gel filtration was done on a PD-10 Sephadex G25-M column (Pharmacia 17-0851-01). The column was equilibrated with buffer A (50 mM Tris HCl pH 8, 3.3 mM $MgCl_2$) and then the incubation mixture of lysozyme with G6P was loaded. The elution was done with buffer A and the eluted protein was further purified from the unreacted G6P using Amicon Ultra filters with 3 kDa cutoff. Ultrafiltration included three consecutive centrifugations at 14 000 g for 30 min at 4 °C, between which the sample was washed with buffer A. The lysozyme concentration in the final preparation was measured by the bicinchoninic acid protein assay.

Quantitation of Amadori products in lysozyme. The nitroblue tetrazolium (NBT) reduction assay [16] was performed to determine the concentration of APs in lysozyme. NBT (Sigma N6639) was dissolved at a final concentration of 0.02% in 0.1 M Na_3PO_4 pH 12 to produce the working solution. Ten microliters of each sample were pipetted onto a microplate in duplicate and mixed with 0.1 ml of the NBT reagent. Absorption was measured at a wavelength of 550 nm. Standard curve was prepared with 1-deoxy, 1-morpholinofructose mixed with 40 mg/ml bovine serum albumin, and used to calculate the concentration of APs in the lysozyme samples.

PGI activity assay. The assay was performed according to Bergmeyer [17] in a volume of 0.5 ml containing 50 mM Tris-HCl pH 8.0, 0.67 mM $NADP^+$, 3.3 mM $MgCl_2$ and varying concentrations of the respective substrates as indi-

cated in the Results section. First, 1 unit of Type VII G6P-dehydrogenase from *S. cerevisiae* (Sigma-Aldrich G7877) was added, and the incubation was carried out at ambient temperature until no increase in the absorbance at 340 nm was observed. Then, the isomerization reaction was initiated by the addition of 8 µg protein extract from an *E. coli* PGI deficient or proficient strain with PGI specific activity of 1.25 U/mg in the latter case. The two bacterial strains and the preparation of protein extracts therefrom are described elsewhere [14]. An absorption coefficient of 6.22 mM^{-1} cm^{-1} for NADPH at 340 nm was used to calculate the increase in NADPH concentration over time. For calculation of the apparent catalytic constants (K'_{cat}), a specific activity of the *E. coli* PGI of 342 U/mg and a molecular mass of 120 kDa were applied [18].

Results and discussions. Glycation affects mostly the free (non-protected) N-terminus of proteins, and the ε-NH_2 and guanidine groups of lysine (Lys) and arginine (Arg) residues, respectively. We used chicken lysozyme to prepare G6P-modified protein substrate for PGI. Lysozyme (E.C. 3.2.1.17) is a glycoside hydrolase with bactericidal and bacteriostatic activity, which comprises about 3.5% of the chicken egg white [19]. The positive charge of the protein (pI 9.63) is contributed mostly by Lys and Arg residues that constitute totally 12.3% of all 147 amino acids in the lysozyme polypeptide chain of 16.2 kDa. As a result of glycation with G6P we obtained lysozyme with a specific concentration of APs equaling to 300 nmol per mg protein. The glycated lysozyme is designated throughout the text as lysozyme-NH-F6P since G6P-derived APs represent F6P-resudues linked to the free NH_2-groups of the protein (Fig. 1). We first performed the PGI assay with either lysozyme-NH-F6P or free F6P as substrates using a protein extract from the *E. coli* PGI deficient strain. In both cases the enzyme test was negative, which indicates that there was no interference of the bacterial proteins with the assay. Next, we carried out the PGI assay with a protein extract from the *E. coli* PGI proficient strain and lysozyme-NH-F6P with increasing concentrations of the F6P residues (APs) in the range from 0.05 mM to 0.8 mM. In this case the enzyme test proved to be positive, and the graphs with the initial velocities (V_0) of the enzyme reactions at different APs concentrations in lysozyme-NH-F6P are shown in Fig. 2.

The PGI enzyme assay is coupled to a G6P-dehydrogenase reaction (see the Methods section). This means that free G6P must be released from lysozyme-NH-F6P in order reduction of $NADP^+$ to NADPH to occur. In other words, Fig. 2 demonstrates that *E. coli* PGI exhibits lysozyme-NH-F6P amadoriase activity. We suppose that the release of G6P from lysozyme-NH-F6P is preceded by PGI catalyzed isomerization of the ketoamine lysozyme-NH-F6P to the aldimine lysozyme = N-G6P (Fig. 1). The proposed mechanism is very similar to that of the *E. coli* lysine amadoriase FrlB, which was shown to share homology with the isomerase domain of the enzyme glucosamine-6-phosphate synthase [10]. It appears that the amadoriase activity of both enzymes, PGI and FrlB, is tightly

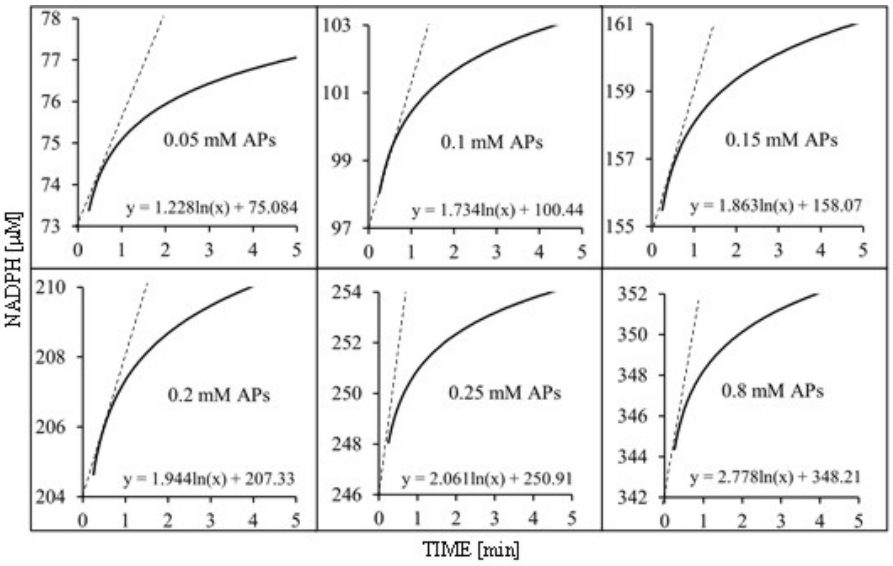

Fig. 2. Initial velocities of the release of G6P from lysozyme-NH-F6P catalyzed by *E. coli* PGI at APs concentrations as indicated in the middle of the panels

linked to their isomerase functionality, which may serve to reverse the poorly reversible step from APs to SBs in the early stage of the Maillard reaction (Fig. 1). Then, spontaneous or PGI catalyzed hydrolysis of SBs may take place to liberate the glycated lysozyme from the G6P-modification.

We used as a control in our experiments the source (non-glycated) chicken lysozyme, which also proved to contain APs at about six time lower concentration (45 nmol per mg protein) than the glycated sample. This observation is not surprising because glycation occurs also in vivo. In addition, chicken eggs, where lysozyme is purified from, are usually stored for a month. Therefore, even at the low sugar concentration in eggs ($< 1\%$) formation of APs in commercial lysozyme preparations is expected to take place during storage. Given the above indicated low specific content of APs (45 nmol/mg), we could perform the PGI assay with the control lysozyme at APs maximal concentration of 0.26 mM. At this substrate concentration we measured an initial velocity of the PGI reaction $V_0 = 0.27$ µM NADPH/min. At a similar APs concentration in the glycated lysozyme (0.25 mM) the initial velocity of the reaction was $V_0 = 2.06$ µM NADPH/min (Fig. 2), i. e. over seven times higher. This means that perhaps only a small fraction of the pre-formed APs in the egg white lysozyme are derived from G6P. Note that the NBT assay is measuring the total concentration of APs, which could be derived not only from G6P but also from other aldoses.

In order to determine the apparent Michaelis–Menten constant (K'_m) of the *E. coli* PGI for the lysozyme-NH-F6P substrate we built the Lineweaver–Burk plot

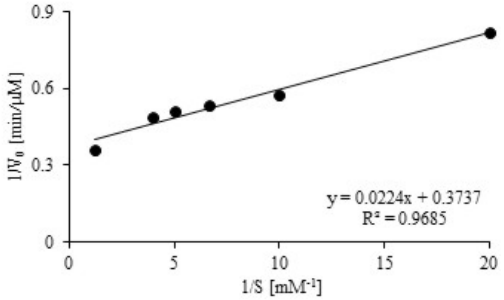

Fig. 3. Lineweaver–Burk plot for calculation of the apparent Michaelis–Menten constant of the *E. coli* PGI for the lysozyme-NH-F6P substrate

(Fig. 3) using the data presented in Fig. 2. From the Lineweaver–Burk plot we determined the values for $V_{max} = 2.8$ µM/min and for $K'_m = 0.06$ mM. We also calculated the kinetic constants $K'_{cat} = 93\ s^{-1}$ and $K_{cat}/K'_m = 1.6 \times 10^6\ M^{-1}s^{-1}$ of the enzyme for the lysozyme-NH-F6P substrate.

PGI catalyzes the isomerization of G6P to F6P in the second step of glycolysis. The reaction is reversible with an equilibrium constant of the forward reaction $K_{eq} = 0.3$ [20]. This K_{eq} value indicates that the PGI catalyzed reaction is slightly favourable in the reverse direction that is isomerization of F6P to G6P, which as we suppose (see above) is involved in deglycation. In fact, the PGI activity assay is based on the reverse reaction [17] and we went on determining the kinetic constants of the *E. coli* PGI for free (unbound to lysozyme) F6P. The enzyme reactions were performed with F6P concentration in the range from 0.01 mM to 3.2 mM, and the initial velocities of these reactions (Fig. 4A) were used to build the Lineweaver–Burk plot (Fig. 4B) for calculation of K'_m of the *E. coli* PGI for free F6P. For V_{max} we calculated a value of 22.12 µM/min and for K'_m a value of 0.1 mM. The catalytic constant and the enzyme efficiency of PGI for the F6P substrate were found to be $K'_{cat} = 736\ s^{-1}$ and $K_{cat}/K'_m = 7.4 \times 10^6\ M^{-1}s^{-1}$, respectively.

The above data show that the *E. coli* PGI has about twice higher affinity to F6P linked to lysozyme ($K'_m = 0.06$ mM) than to free F6P ($K'_m = 0.1$ mM). This could be due to movement constraints imposed on F6P when immobilized on lysozyme in the form of APs. The catalytic constant of PGI with the glycated lysozyme, however, was nearly 8 times lower than that with free F6P. We suppose that unlike free F6P, in the case of lysozyme-NH-F6P the F6P-modified amino acids might occupy various local structures on the protein surface because of its structural heterogeneity. Thus, some of the glycated amino acid residues could fail to properly accommodate their F6P-residue in the PGI active site. In addition, besides an isomerization step, deglycation includes also a hydrolytic reaction needed to break down the SBs to free G6P and deglycated (repaired)

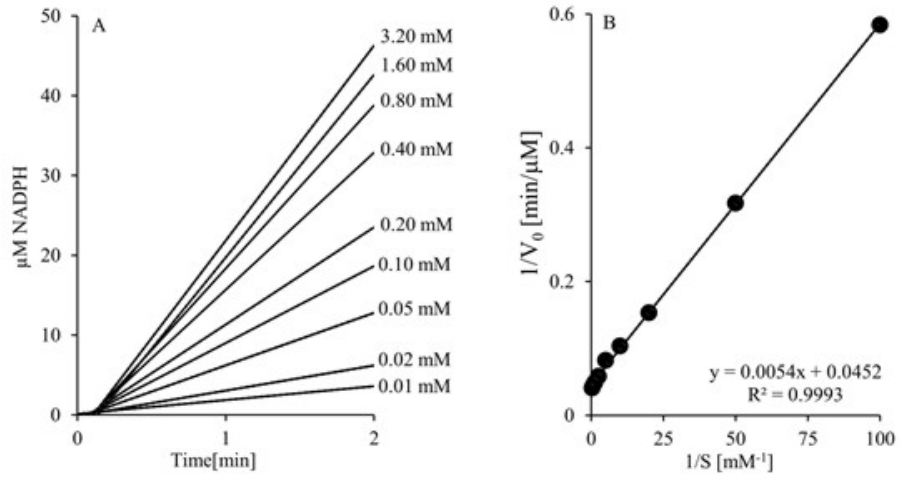

Fig. 4. Initial velocities (A) and Lineweaver–Burk plot (B) for calculation of the apparent Michaelis–Menten constant of the *E. coli* PGI for F6P

protein (Fig. 1). These specificities of the deglycation reaction could explain the nearly five time lower catalytic efficiency of the *E. coli* PGI with the glycated lysozyme ($K_{cat}/K'_m = 1.6\times10^5$ $M^{-1}s^{-1}$) than with free F6P ($K_{cat}/K'_m = 7.4\times10^6$ $M^{-1}s^{-1}$). They also pose the question about the physiological relevance of the PGI amadoriase activity, which will be a direction of future investigations.

The *E. coli* PGI enzyme was purified and characterized from crude extracts 40 years ago [18]. Two native PGI species were detected in *E. coli* differing by molecular mass, abundance and total electronegative charge. The major species comprised more than 90%, had an apparent molecular mass of about 125 kDa, and consisted of two 59 kDa subunits. The minor species had an apparent size of 230 kDa and was proposed to result from dimerization of the major species, most probably caused by a negatively charged post-translational modification of the subunits. The reasons for the existence of two PGI species in *E. coli* remain to date unknown and in the light of the current study represent yet another direction for future investigations.

Conclusions. Treatment of chicken lysozyme modified with G6P-derived APs with a protein extract from an *E. coli* PGI proficient but not deficient strain resulted in the release of G6P. This result reveals a yet unknown secondary PGI activity of a protein amadoriase. Despite the higher affinity of PGI to the glycated lysozyme than to free F6P, however, the enzyme performance with the glycated lysozyme was about five times lower than with F6P. Future studies are needed to explore PGI for protein amadoriase activity in vivo as well as to determine the specific functions of the two PGI forms discovered in *E. coli*.

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Institute of Molecular Biology "Roumen Tsanev"
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 21
1113 Sofia, Bulgaria
e-mail: eli.boteva@gmail.com
konstantindoychev@gmail.com
rcekovska@gmail.com
iivanov@bio21.bas.bg
folie@abv.bg
rumym@bio21.bas.bg