EFFECT OF ACETYLSALICYLIC ACID ON GLYCATION AND MUTABILITY OF ESCHERICHIA COLI CHROMOSOMAL DNA

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ABSTRACT

Recently, we have shown that the process of non-enzymatic glycosylation (glycation) affects Escherichia coli chromosomal DNA under physiological conditions in vivo. We have further demonstrated that chromosomal DNA glycation is a source of spontaneous mutations in E. coli. The present study reveals that inhibition of glycation does not necessarily lead to suppression of DNA mutability. We tested the effect of acetylsalicylic acid (ASA) on chromosomal DNA glycation and spontaneous mutagenesis in E. coli. At a concentration of 0.1 mM in the growth medium ASA proved to be non-toxic and inhibits significantly glycation of E. coli chromosomal DNA. At a concentration of 5 mM ASA partially suppressed bacterial growth but had negligible impact on DNA glycation. Most importantly, at both concentrations tested ASA caused an increase in the frequency of spontaneous reversions from Arg to Arg’ phenotype. In addition, we observed an accelerated hydrolysis of DNA in ASA solutions as compared to that in solutions of hydrochloric acid at the same pH. The latter result suggests a possible interaction of ASA with DNA, which explains the observed genotoxicity of ASA.

Keywords: acetyl salicylic acid, ASA, glycation, non-enzymatic glycosylation, inhibition of glycation, spontaneous mutagenesis, E. coli

Abbreviations:
AGEs - advanced glycation end products
AGE-BSA – glycated bovine serum albumin
ASA - acetyl salicylic acid
ccc- covalently closed circular plasmid
CM - carboxymethyl
NBT - nitroblue tetrazolium
SD - standard deviations

Introduction

The non-enzymatic glycosylation (glycation) is a reaction between reducing sugars and amino groups containing compounds, including proteins, DNA and amino-lipids. Two well defined stages are distinguished in the glycation process – early and advanced. The early stage includes the reversible formation of Schiff bases between the sugar carbonyl and amino groups in proteins, nucleic acids, etc., followed by a rearrangement of the Schiff bases to significantly more stable aldamines (Amadori products) or ketamines (Heyns products). In the advanced stage of glycation, the Amadori and Heyns products undergo a series of chemical transformations (enolisation, oxidation, dehydration) ending up with the formation of advanced glycation end products (AGEs). AGEs are stable covalent adducts and their accumulation in long-lived proteins in vivo is associated with a number of diseases including diabetes and Alzheimer’s disease.

The relation of glycation with human pathogenesis has prompted an extensive search for compounds with potential inhibitory effect on glycation (13). Many chemicals have been tested to date, however, their impact on DNA glycation and mutability has been overlooked. Acetylsalicylic acid (ASA, aspirin) is the first compound shown to prevent glycation. It is a widely used drug belonging to the group of non-steroid anti-inflammatory agents. In the recent years ASA was found to inhibit glycation of proteins in vitro (1, 7, 19) as well as in vivo (2, 4). Early experiments have shown that in the presence of ASA proteins become irreversibly acetylated. On the other hand, ASA was found to interact with DNA (17). All these data prompted us to investigate the effect of ASA on DNA glycation and to determine the effect of an eventual effect of ASA on mutability.

Materials and Methods

Bacterial strains and growth media

The following E. coli K12 strain was used in this study: AB1157 (F' thr-1 leu-6 proA2 his-4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44). The strain was purchased form the National Bank for Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria). The rich Luria-Bertani (LB) medium contained 1% protein hydrolysate, 0.5% yeast extract and 0.5% NaCl adjusted to pH 7.4. The M9G minimal medium was composed of 0.1% glucose as a carbon source, 0.5 µg/ml thiamine, 1 mM MgSO4 and the appropriate amino acids at a final concentration of 40 µg/ml. The minimal salt agar (1.5%) contained 1% protein hydrolysate, 0.5% yeast extract and 0.5% NaCl adjusted to pH 7.4. The M9G minimal medium was composed of 0.1% glucose as a carbon source, 0.5 µg/ml thiamine, 1 mM MgSO4 and the appropriate amino acids at a final concentration of 40 µg/ml. The minimal salt agar (1.5%) for selection of Arg’ prototrophic mutants was prepared with the same medium devoid of arginine.

Isolation of E. coli chromosomal DNA

E. coli cells were collected from 125 OD596 bacterial cell culture by centrifugation and resuspended in 8 ml of 25 mM Tris HCl pH 8.0, 10 mM EDTA, 0.14 M NaCl and 50 mM glucose. SDS
and Proteinase K were added to final concentrations of 1% and 250 µg/ml respectively, the suspension was adjusted to 10 ml and incubated at 37°C for 40 min. After two phenol extractions DNA was ethanol precipitated and dissolved in 1 ml of 10 mM Tris HCl pH 8.0, 1 mM EDTA (TE buffer) containing 100 µg RNase A and 20 units RNase T1. Following 30 min incubation at 37 °C, Proteinase K was added to a final concentration 100 µg/ml and the incubation continued for additional 30 min. The samples were extracted with phenol, ethanol precipitated and DNA dissolved in TE at a final concentration of 5 mg/ml. DNA was sonicated to an average fragment size below 1300 bp and its quality was estimated by the A260/A280 spectral ratio (2 ± 0.1).

**NBT reduction assay**

Early glycation products in DNA were determined by nitroblue tetrazolium (NBT) reduction assay (8) with some modifications as previously described (12). Briefly, the DNA samples (100 µl) were mixed with 1 ml 100 mM sodium carbonate buffer (pH 10.8) containing 0.25 mM NBT and incubated at 37°C for 5 hours. Absorbance at 525 nm was read against distilled water and the content of early glycation products was determined using an extinction coefficient of 12 640 cm⁻¹ M⁻¹ for monoformazan (a product formed by reduction of NBT with early glycation adducts).

**Fluorescence spectroscopy**

Fluorescence measurements were carried out on a Shimadzu model RF-5000 fluorescence spectrophotometer. DNA samples at an approximate concentration of 5 mg/ml were sonicated in an ultrasonic homogenizer (Model CP 50, Cole-Parmer Instrument Co.) three times for 1 min (50% duty cycle at 80% power). In the comparative assay, DNA samples were serially diluted and fluorescence was measured at λex = 360 nm and λem = 440 nm. The fluorescent intensity was plotted versus the concentration of DNA and the obtained graph was used for exact calculation of the specific fluorescence related to 5 mg/ml DNA. The Raman fluorescence of the solvent (λex = 370 nm and λem = 420 nm) was subtracted from that of the DNA because of its interference with the sample spectrum at low DNA concentrations.

**Competitive ELISA**

Glycated bovine serum albumin (AGE-BSA) was prepared by incubating 10 mg/ml BSA with 0.5 M glucose in a water solution for three months at 37°C. Microtiter plates were coated over night at 37°C with 100 µl per well AGE-BSA solution at a concentration of 10 µg/ml in 50 mM sodium carbonate (pH 9.6). The coupling solution was then discarded and replaced with 100 µl 2% BSA in PBS. After blocking for 3 h at 37°C, the plates were washed twice with 0.05% Tween 80 in PBS and once with PBS. DNA or AGE-BSA referent samples, dissolved in PBS (50 µl per well) were mixed with 50 µl horseradish peroxidase labeled anti-CML antibody (16), diluted 1:30 in assay buffer (0.25% BSA in PBS). Competition reaction was carried out at 37°C for 3 h and after washing as above, color was developed with 100 µl o-phenylenediamine dissolved at 0.15 mg/ml in 0.2 M K2HPO4 citrate buffer pH 6, 0.02% H2O2. The reaction was stopped with 100 µl 0.8 M H2SO4 and the absorption at 490 nm was read using an ELISA plate reader. Data were processed by a 4P logistic curve fit. The immunoreactivity of DNA to the anti-CML antibody was expressed in AGE-BSA equivalents (eq), where 1 eq was equal to the immunoreactivity of 1 µg referent AGE-BSA.

**Mutational analysis**

Overnight LB cultures were used to inoculate fresh LB medium containing ASA (0.1 mM or 5 mM) at a starting concentration of 0.1 OD690. Control cultures were prepared without ASA. The cultures were grown at 37°C to a stationary phase and cells were collected by centrifugation. The cell pellet was washed twice with non-supplemented M9G medium and resuspended in the same medium at approximately 2 OD690/ml. Samples of 200 µl were spread on selective M9G plates (no arginine) and the number of Arg⁺ colonies was scored after 3 days of growth at 37°C. To estimate the number of viable cells, serial dilutions of the concentrated cell stock were made on LB plates. The frequency of spontaneous mutations was expressed as number of revertants per 10⁴ cells. Two experiments on separate cultures were carried out each in duplicate and the standard deviations (SD) were calculated.

**Results and Discussion**

**Acetyl salicylic acid inhibits glycation of E. coli chromosomal DNA**

The inhibitors of glycation, including ASA, reveal their effect at high concentrations (usually in the millimolar range) (3, 10) and therefore one can suggested that at such concentrations ASA might be toxic to the E. coli cells. For this reason, we first tested ASA for toxicity. The drug was added to rich Luria-Bertani (LB) medium at three different concentrations (0.1 mM, 5 mM and 10 mM) and its effect on bacterial growth was studied on the E. coli strain AB1157. Fig. 1 demonstrates that 10 mM ASA is lethal, 5 mM partly affects bacterial growth and 0.1 mM is non-toxic. Based on this result, chromosomal DNA was isolated from E. coli AB1157 cells grown to a stationary phase in LB medium supplemented with either 0.1 or 5 mM ASA and the level of glycation was estimated by three different methods: a) NBT reduction assay (to measure the early glycation products, i.e. fructoseamines); b) ELISA (to evaluation the immunoreactivity of DNA to an anti-CML antibody, which is an indirect measure of the content of carboxymethyl residues in DNA) and c) Fluorescence spectroscopy (to quantify the fluorescent adducts in DNA). The results presented in Fig. 2 clearly illustrate the anti-glycation activity of ASA. The non-toxic concentration of 0.1 mM ASA slightly affected the accumulation of fluorescent adducts (Fig. 2C) but significantly inhibited the formation of fructoseamines (Fig. 2A) and CM residues in DNA (Fig. 2B). Surprisingly, the 5 mM ASA proved to be less efficient glycation inhibitor than the 0.1 mM and this could be attributed to the ASA toxicity. The finding in

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Fig. 1 that 5 mM ASA partially inhibits bacterial growth could be explained by perturbation in bacterial metabolism at this concentration to neutralize the drug anti-glycation activity.

**Acetyl salicylic acid enhances the E. coli mutability**

Previously, we presented experimental evidence in favor of the link between DNA glycation and spontaneous mutagenesis in *E. coli* (12). If glycation contributes to mutability, the inhibition of glycation should decrease the spontaneous mutation rate. With this in mind, we determined the frequency of spontaneous reversions to arginine prototrophy (*argE* to *Arg*) in *E. coli* AB1157 grown in the presence of ASA. The arginine auxotrophy of this strain (*argE*) is due to mutation in the chromosomal N-acetylornithinase gene. The Gln-338 codon CAA in this gene is converted to nonsense ochre (TAA) triplet (9, 11). The reversion to arginine prototrophy in the N-acetylornithinase gene could be due to either base substitution at A:T base pairs in the ochre codon (back mutations) or to G:C→A:T transition and A:T→T:A, G:C→T:A transversions in several tRNA genes creating suppressor mutations (18). Another line of experimental evidence indicates that treatment of *E. coli* with glyoxal induces point mutations in the *lacI* gene by all types of base-pair substitutions with G:C→A:T transitions and G:C→T:A transversions being predominant (14). Because endogenous glycation in *E. coli* may have similar consequences, the *Arg* reversion appears appropriate for testing the effect of ASA on DNA mutability.

Although ASA inhibited glycation (Fig. 2) it had an opposite effect on the rate of spontaneous mutations. The reversion frequency to arginine prototrophy increased with increasing ASA concentration (Fig.3). What is the reason for the enhanced mutability, when bacteria were grown in media supplemented with ASA? One could assume that the presence of ASA in the growth medium is stressful for bacteria and triggers mechanisms of adaptive mutagenesis (5, 6). However, we could not rule out *a priori* the possibility that ASA exhibits...
direct mutagenic effect by interacting with DNA. Next experiments were designed to address this issue.

**Fig. 3.** Effect of ASA on *E. coli* chromosomal DNA mutability
Rate of spontaneous reversions to Arg$^+$ phenotype of control *E. coli* cells (no ASA) (●) and of cells grown in the presence of 0.1 mM (▲) or 5 mM (▲) ASA. Data are means ±SD (n=4).

**At high concentrations ASA may interact with DNA**
In order to explore the proposed interaction between ASA and DNA, plasmid pUC18 DNA was incubated with different ASA concentrations. Agarose gel electrophoresis was performed to reveal a conversion of the covalently closed circular (ccc) plasmid DNA into a relaxed form, indicative of an eventual ASA hydrolytic activity. We did not observe any DNA hydrolytic activity of ASA within 24 hours of plasmid DNA incubation at 37°C at none of the tested concentrations (**Fig. 4**). Early experiments have shown that ASA binds weakly to DNA, most probably through hydrogen bonds (17). Later studies using Raman and infrared spectroscopy confirmed this observation by demonstrating that ASA forms a complex with DNA (15). At a low ASA/DNA molar ration (1:40), ASA interacted mainly with the phosphate backbone and A:T base pairs. In this interaction adenine and thymine took part through the N-7 and O-2 atoms respectively, which are not normally involved in Watson-Crick hydrogen bonding. By stepwise increasing the ASA concentration with respect to DNA up to 2:1 molar ratio, in the reaction with ASA were involved also other DNA bases, guanine (through N-7) and cytosine. Alterations of the B-DNA structure towards A-DNA and helix destabilization were also observed. The authors suggested that ASA interacted with DNA through both carboxylic anion CO$^-$ and acetyl group (COOCH$_3$) donor atoms with those of the DNA backbone phosphate groups and DNA bases (directly or indirectly via H$_2$O molecules) (15). The weak interaction of ASA with DNA could explain why we failed to observe relaxation of the plasmid DNA. The probable interaction of ASA remained hidden in the plasmid relaxation assay used in our study.

**Fig. 4.** Effect of ASA on the electrophoretic profile of pUC18 plasmid DNA
Plasmid DNA treated with 10 mM (1), 5 mM (2), 0 mM (3), 0.1 mM (4) and 0.01 mM (5) ASA, pH 7.0.

**Fig. 5.** Effect of ASA and HCl at pH 3.4 on the electrophoretic profile of pUC18 plasmid DNA
Plasmid DNA a concentration of 1 mg/ml was treated for 4 h (A) or 30 h (B) at 37°C with 2 mM HCl (1) or 10 mM ASA (2); C – control (no treated) plasmid DNA.

In the above experiments ASA solutions were adjusted to pH 7.0 with NaOH, whereby the labile ester group in ASA is hydrolyzed to form sodium acetate and sodium salicylate. In order to avoid deacetylation of ASA, we incubated plasmid pUC18 DNA in an acidic (not titrated) 10 mM ASA solution.
The low pH of this solution (3.4) made acidic hydrolysis of plasmid DNA unavoidable. To evaluate the effect of the acidic DNA hydrolysis per se, we incubated the plasmid DNA also in 2 mM HCl solution having the same pH (3.4). Incubations were carried out at 37 °C for either 4 or 30 hours and plasmid DNA was analyzed by agarose gel electrophoresis. As shown in Fig. 5, the plasmid DNA hydrolysis was accelerated in the ASA solution. This result indicates that plasmid DNA decay is not simply due to acidic hydrolysis. Most probably, the structural alterations in DNA including helix distortions (15) render DNA more susceptible to hydrolysis. What is the exact mechanism of ASA-DNA interaction, how this interaction hinders DNA glycation but increases DNA mutability – these are important issues to be addressed in the future. It is worth mentioning that the therapeutic blood concentration of ASA is about 1 mM. In our experiments, at this concentration ASA caused an increase in the frequency of spontaneous mutations in E. coli. Although our results, obtained with bacteria, could not be extrapolated directly to humans, they imply that glycation inhibitors with the potential to interact with DNA deserve special concerns when applied at high concentrations in vivo.

Conclusions
This study provides evidence for the impact of ASA on DNA glycation and mutability. Aspirin, a worldwide used medicine, was found to inhibit DNA glycation but to enhance spontaneous mutagenesis in E. coli. Because of that, application of ASA at high concentrations in vivo for suppression of glycation may be risky. It turns out that ASA inhibits DNA glycation by interacting with DNA and is thus potentially mutagenic.

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REFERENCES