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2-Hydrazinobenzothiazole-based etheno-adduct repair protocol (HERP): A method for quantitative determination of direct repair of etheno-bases

Gururaj Shivange, Naveena Kodipelli, Roy Anindya *

Department of Biotechnology, Indian Institute of Technology Hyderabad, Ordnance Factory Estate, Yeddumailaram, 502205 Hyderabad, India

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ABSTRACT

Etheno-DNA adducts are mutagenic and lead to genomic instability. Enzymes belonging to Fe(II)/2-oxoglutarate-dependent dioxygenase family repair etheno-DNA adducts by directly removing alkyl chain as glyoxal. Presently there is no simple method to assess repair reaction of etheno-adducts. We have developed a rapid and sensitive assay for studying etheno-DNA adduct repair by Fe(II)/2-oxoglutarate-dependent dioxygenases. Using AlkB as model Fe(II)/2-oxoglutarate-dependent dioxygenases, we performed *in vitro* repair of etheno-adducts containing DNA and detected glyoxal by reacting with 2-hydrazinobenzothiazole which forms complex yellow color compound with distinct absorption spectrum with a peak absorption at 365 nm. We refer this method as 2-*h*ydrazinobenzothiazole-based *e*theno-adduct *r*epair *p*rotocol or HERP. Our novel approach for determining repair of etheno-adducts containing DNA overcomes several drawbacks of currently available radioisotope-based assay.

1. Introduction

Oxidative stress and chronic inflammation induce endogenous lipid peroxidation derived aldehydes which reacts with DNA and RNA to form etheno (ε)-adducts such as 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG) [1]. Besides, ε-adducts are also generated by reaction with vinyl chloride or 2-chloroacetaldehyde [2]. All etheno-adducts are mutagenic. In *Escherichia coli*, εA residues mainly lead to εA-T to G-C transitions and εA-T to T-A transversions, εC produces mostly εC-G to A-T transversions and εC-G to T-A transitions, N^2,3-εG forms N^2,3-εG-C to A-T transitions, and 1,N^2-εG leads to G-C to A-T transitions [1]. Due to their deleterious mutagenic nature, organisms developed repair mechanisms to remove etheno-adducts. Two proteins are involved in removal of etheno-adducts in *E. coli*; *viz.* DNA glycosylase AlkA and Fe(II)/2-oxoglutarate(2OG)-dependent dioxygenase AlkB [3–5]. Like other members of this family, AlkB requires oxygen and non-heme iron (Fe^{II}) as cofactors and 2OG as co-substrate to carry out oxidative repair of εA and εC resulting in the formation of succinate and CO_2. The alkyl chain is spontaneously released as glyoxal [6,7]. Interestingly, there are nine AlkB homologs present in human [8,9]. Among these, only human AlkB homolog-2 (hALKBH2) was reported to be specific for εA and εC repair and like bacterial AlkB, alkyl chain is removed as glyoxal [10]. However, little is known about involvement of other homologs in etheno-base (ε-base) repair. Lack of a convenient and rapid *in vitro* biochemical assay further limited the study of DNA repair activity of the AlkB homologs. Removal of ε-base by AlkB and hALKBH2 were detected by complex methods involving radiolabeled DNA, or using gas chromatography-coupled HPLC or mass spectrometry [6,10]. Although accurate, these methods are discontinuous, time-consuming, involve hazardous radioactivity handling, and require highly specialized equipment. In order to simplify the analysis of ε-adduct repair enzymes, we developed a micro-titer plate-based method, that we describe as HERP (2-*h*ydrazinobenzothiazole-based *e*theno-adduct *r*epair *p*rotocol), a rapid and accurate assay for studying etheno-adduct repair by Fe(II)/2-oxoglutarate-dependent dioxygenases. This assay involves UV–vis spectroscopic detection of glyoxal generated during repair

* Corresponding author. Tel.: +91 40 23016083; fax: +91 40 23016032.
E-mail address: anindya@iith.ac.in (R. Anindya).

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of ε-adducts by Fe(II)/2-oxoglutarate-dependent dioxygenase AlkB. To our knowledge, this is the first report of single-step colorimetric assay to detect ε-base repair.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

Recombinant AlkB was produced in *E. coli* by cloning AlkB gene into pGex-6p1 expression vector (GE-healthcare) at BamHI and SalI sites [11]. To verify specificity of oxidative de-alkylation reaction by AlkB, another *E. coli* Fe(II)-2OG-dependent dioxygenases *tauD*, encoding taurine hydroxylase [12], were cloned into the same vector. GST tagged AlkB and TauD were purified using Glutathione Sepharose 4-FastFlow (GE-healthcare).

2.2. Preparation of cell extract

Wild type and *alkB* mutant strains HK82 (Generous gift from Dr. Hans Krokan, Norwegian University of Science and Technology [13]) cells were disrupted by sonication in lysis buffer (10 mM Tris–HCl, pH 8, 400 mM KCl, 2 mM EDTA, 40% (v/v) glycerol, 0.2% (v/v) triton, 2 mM DTT). After removing the cell debris by centrifugation (18,000 × *g*, 30 min) supernatant was dialyzed against buffer containing 20 mM Tris–HCl, pH 8.0, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT) and analyzed by SDS-PAGE.

2.3. Glyoxal detection by 2-hydrazinobenzothiazole

100 mM stock of 2-hydrazinobenzothiazole (Alfa Aesar, A18006) was prepared by adding 33.042 mg of 2-hydrazinobenzothiazole into 200 μl of concentrated HCl. Solution was incubated at 55 °C for 1 h to dissolve completely. The final volume was adjusted to 2 ml by water and kept heating until the crystals dissolved completely. To determine specificity of glyoxal to 2-hydrazinobenzothiazole, 100 μl of 100 μM of formaldehyde, acetaldehyde, propanal and glyoxal (SRL Chemicals, 072735) present in were added to equal volume of 2-hydrazinobenzothiazole to make final volume of 200 μl. Reaction was incubating at 45 °C for 15–20 min for complex formation. The completion of reaction was detected by formation of pale yellow color. Absorbance of this compound was monitored by Molecular Devices SpectraMax M5 microplate reader. A standard curve of glyoxal was prepared using various concentrations of glyoxal ranging from 0.05 to 1.0 μM. 50 μl of glyoxal and 150 μl 2-hydrazinobenzothiazole were mixed to make final volume of 200 μl and detected as described before by using microplate reader. Using this standard plot, the concentration of glyoxal was determined using the equation: $c_g = [(A_{365} - 5 \times 10^{-4})10^3]/(0.039)$ (where c_g is the glyoxal concentration in nM, A_{365} is the peak absorbance at 365 nm due to 2-hydrazinobenzothiazole reaction; *Y*-intercept at $X = 0$ was 5×10^{-4} and slope of the standard plot was 0.039).

2.4. Chloroacetaldehyde treatment of DNA and generation of etheno-DNA adducts

Long single stranded DNA was purchased from Sigma Aldrich (D8899, Mw = 1.64×10^7 Da, 5×10^4 base, 41.9 mol% guanine–cytosine (G–C) content). We modified the previously reported method to generate etheno-DNA adducts (ε-DNA) [14]. Briefly, 40 μg of large single stranded DNA from calf thymus (Sigma, D8899) was treated with 300 mM of chloroacetaldehyde (Sigma, 317276) in presence of 0.3 M sodium acetate (pH 7.0) at 37 °C for 14 h in a total volume of 400 μl. The ε-DNA was not purified directly by using ethanol precipitation as it resulted poor yield. Therefore, excess chloroacetaldehyde was removed by dialysis against TE buffer (10 mM Tris. pH 8.0, 1 mM EDTA) using Spectra/Por dialysis membrane (MWCO: 3.500). Then the damaged DNA was precipitated by adding 0.3 M sodium acetate pH 5.5 and 2 volume of ice-cold ethanol. The precipitated ε-DNA was washed with 70% ethanol and finally dissolved in molecular grade water to a concentration of 500 ng/μl. To generate oligonucleotide ε-DNA, desalted oligonucleotides were purchased from Sigma–Aldrich. The oligonucleotide selected for etheno-adduct generation was 40-mer oligo-A (5′-A_{40}-3′) and treated with the chloroacetaldehyde following the same method as before.

2.5. HERP (2-hydrazinobenzothiazole-based etheno-adduct repair protocol)

For AlkB mediated repair, ε-DNA was incubated with purified recombinant AlkB (1 μM) in reaction buffer containing 20 mM Tris pH 8.0, 200 μM 2OG, 2 mM L-ascorbate and 20 μM $Fe(NH_4)_2(SO_4)_2$ in a final volume of 50 μl [11]. Repair reaction was carried out at 30 °C for 1 h in a 96-well microtiter plate. At the completion of the repair reaction, 150 μl of 2-hydrazinobenzothiazole was added to the repair reaction to make final volume of 200 μl. Since 2-hydrazinobenzothiazole was dissolved in concentrated (11 N) hydrochloric acid solution, addition of 2-hydrazinobenzothiazole stopped the AlkB activity. Reaction was incubated at 45 °C for 15–20 min for pale yellow color formation. Then absorption spectra were monitored by UV–vis spectrophotometer using Molecular Devices SpectraMax M5 microplate reader. In order to determine effect of AlkB concentration, 250 ng/μl (5 μM) of ε-DNA was incubated with 0.125–1.0 μM of AlkB in 100 μl reaction volume at 30 °C for 1 h. The effect of ε-DNA concentration on AlkB was analyzed by using 1 μM AlkB and 25–150 ng/μl of ε-DNA. To carry out repair using cell extract, 2.5 μg and 5 μg of CAA-treated long single-stranded DNA was incubated with wild type and *alkB* mutant cell extract in presence of repair buffer. Glyoxal release was detected by 2-hydrazinobenzothiazole as before.

2.6. Steady-state repair kinetics

Steady-state kinetic parameters for εA repair were measured using 40-mer oligo-εA. Chemically synthesized 40-mer oligonucleotide was treated with chloroacetaldehyde as described above. The initial velocity of εA repair reaction was determined using the equation $v = c_g/t$ (where c_g is the concentration of glyoxal produced in nM and t is the reaction time in min). Substrate concentration was determined by using the equation derived from Fig. 1D; Molar substrate concentration was determined using the equation $[\varepsilon A] = 40 \times s$ (where [εA] is the molar concentration of 1,N^6-ethenoadenine (εA) and s is the molar concentration of 40-mer oligo-A). Kinetic parameters such as V_{max} (the maximum value of reaction velocity) and K_M (εA concentration at which the reaction velocity is half-maximal) were obtained from Michaelis–Menten equation and transformation to a Hanes–Woolf plot of [εA]/*v versus* [εA]. The K_M value was derived from the intercepts of regression line. Efficiency of enzyme reaction, the turnover number was determined from the following equation: $k_{cat} = V_{max}/[E_i]$ (where $[E_i]$ is the molar concentration of AlkB).

3. Results and discussion

3.1. Detection of glyoxal

With the aim of developing an *in vitro* DNA repair assay for AlkB like Fe(II)/2-oxoglutarate-dependent dioxygenases we focused on a method to detect the reaction product glyoxal.