1,N^6-α-hydroxypropanoadenine, the acrolein adduct to adenine, is a substrate for AlkB dioxygenase

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Summary

Combination of *in vivo, in vitro* and *in silico* studies shows that acrolein adduct to adenine is mutagenic and is effectively repaired by AlkB dioxygenase. Cationic form of R isomer of HPA is strongly favored in AlkB active centre.

Abstract

1,N^6-α-hydroxypropanoadenine (HPA) is an exocyclic DNA adduct of acrolein – an environmental pollutant and endocellular oxidative stress product. *E. coli* AlkB dioxygenase belongs to the superfamily of α-ketoglutarate (αKG)- and iron-dependent dioxygenases which remove alkyl lesions from bases *via* an oxidative mechanism, thereby restoring native DNA structure. Here, we provide *in vivo* and *in vitro* evidence that HPA is mutagenic and is effectively repaired by AlkB dioxygenase. HPA generated in plasmid DNA caused A→C and A→T transversions and, less frequently, A→G transitions. The lesion was efficiently repaired by purified AlkB protein; the optimal pH, Fe(II) and αKG concentrations for this reaction were determinated. *In vitro* kinetic data show that the protonated form of HPA is preferentially repaired by AlkB, albeit the reaction is stereoselective. Moreover, the number of reaction cycles carried out by an AlkB molecule remains limited. Molecular modeling of the T(HPA)T/AlkB complex demonstrated that the R stereoisomer in the equatorial conformation of the HPA hydroxyl group is strongly preferred, while the S one seems to be susceptible to AlkB-directed oxidative hydroxylation only when HPA adopts the *syn* conformation around the glycosidic bond. In addition to the biochemical activity assays, substrate binding to the protein was monitored by differential scanning fluorimetry allowing

1

the identification of the active protein form with cofactor and cosubstrate bound and monitoring substrate binding. In contrast FTO, a human AlkB homolog, failed bind an ssDNA trimer carrying HPA.

Short title

Acrolein adduct to adenine is an AlkB substrate

Keywords

1,N^6-α-hydroxypropanoadenine, AlkB dioxygenase, acrolein, mutagenesis, DNA repair, substrate binding

Introduction

Acrolein (ACR), a highly reactive α,β-unsaturated aldehyde, is a ubiquitous environmental contaminant formed during incomplete combustion and overcooking of various food products, it is also used for the synthesis of diverse organic compounds, including methionine and the refrigerant methyl chloride. The general population is exposed to ACR *via* smoking, second-hand tobacco smoke inhalation and exposure to wood or plastics smoke. The distribution of the ACR–DNA adduct in the TP53 gene has been shown to coincide with the p53 mutational pattern in cigarette smoke-related lung cancer (1). Firefighters and population living or working in areas with heavy automotive traffic are exposed to higher levels of ACR by inhalation of ambient air. Ample data indicate that ACR causes irritation of many organs (for review see (2)). It is formed during the metabolism of the anticancer drug cyclophosphamide and appears to be involved in the bladder toxicity of this drug (3, 4) and has been shown to initiate tumor growth in the rat bladder (5). Acrolein can be also generated endogenously as a by-product of lipid peroxidation (6). Its interactions with diverse cellular molecules including amino acids, proteins and nucleic acids have been extensively studied (reviewed recently in (7)). ACR is strongly mutagenic in the DNA-repair-deficient xeroderma pigmentosum fibroblasts but not in normal human fibroblasts and has also been found to cause an inhibitory effect on DNA repair. It seems that this effect is through its direct interaction with DNA repair proteins (8).

Acrolein mainly forms exocyclic adducts to DNA (9), mostly at guanine residues - 1,N^2-α-hydroxypropanoguanine and 1,N^2-γ-hydroxypropanoguanine (HPGs) (10). The C5 cytosine methylation at CpG sequences greatly enhances the ACR–dG adduction at those sites. *E. coli*

nucleotide excision repair (NER) enzymes, UvrA, UvrB and UvrC, working as UvrABC nuclease, can incise HPGs specifically and quantitatively (1).

In addition to the guanine adducts, 1,N^6-α-hydroxypropanoadenine (HPA) (see scheme in Figure 3 for structure) and 3,N^4-α-hydroxypropanocytosine (HPC) are minor cyclic acrolein adducts found in DNA. The only (to the best of our knowledge) work describing possible mutagenicity of HPA is reference (11) where 22% of base substitutions induced by ACR in human fibroblasts were found at A·T sites. The formation of this adduct was also shown in rat liver epithelial RL34 cells exposed to acrolein and in rats exposed to Fe^{3+}NTA, a carcinogenic iron chelate that specifically induces oxidative stress in the kidney of rodents (12).

Exposure of prokaryotic cells to sublethal doses of DNA alkylating agents induces expression of four genes, *ada*, *alkB*, *alkA* and *aidB,* forming an adaptive response system. Among the proteins induced in this system are broad substrate specificity DNA glycosylase AlkA and AlkB dioxygenase (13-16). *E. coli* AlkB is one of the most studied members of the superfamily of α-ketoglutarate (αKG)- and iron-dependent dioxygenases (17). Its homologs are present in almost all organisms including human (18, 19). A recent bioinformatic analysis of protein sequence databases has identified 1943 AlkB sequences with eight new AlkB subfamilies (20). AlkB-like DNA repair dioxygenases remove alkyl lesions from DNA bases *via* an oxidative mechanism restoring the native bases in the DNA. They require oxygen (O_2) and non-heme Fe(II) as cofactors and αKG as a cosubstrate to initiate oxidative demethylation of DNA bases resulting in the formation of succinate and CO_2. *E. coli* AlkB acts preferably on ssDNA effectively oxidizing its substrates in such a short oligodeoxynucleotides as pentamers and trimers (21). Methylated dsDNA and RNA can also be repaired by it, as well as by other proteins of the AlkB family (22-24). AlkB is a promiscuous enzyme (25). Although m^1dAMP and m^1dATP have been shown to be its minimal substrates, it seems not to remove cyclic adducts from modified deoxynucleoside triphosphates (21, 26). Its primary substrates are 1-methyladenine (m^1A) and 3-methylcytosine (m^3C) (27, 28), it can also revert bulkier DNA adducts such as ethyl, propyl and hydroxyalkyl ones (21, 29) as well as exocyclic etheno (ϵ) and ethano adducts including 1,N^6-ethenoadenine (ϵA) and 3,N^4-ethenocytosine (ϵC) (30-36). 1-methylguanine (m^1G), 3-methylthymine (m^3T) and 1,N^2-ethenoguanine are repaired much less efficiently (37, 38). It should be noted that a large number of experimental structures of AlkB and its homologs are available in PBD records, some of which can be used as a reliable template in the modeling of complexes of AlkB with new substrates. However, in most of

3

these structures Co(II) or Mn(II) are present as a catalytically silent analog of Fe(II), both binding in exactly the same way (25, 39, 40).

Recently, we have found that AlkB repairs yet another chemically distinct group of substrates - exocyclic saturated adducts: the five-membered chloroacetaldehyde (CAA) adduct $3,N^4$-α-hydroxyetanocytosine (HEC) (34) and the six-membered acrolein adduct $3,N^4$-α-hydroxypropanocytosine (HPC) (35). $1,N^6$-α-Hydroxypropanoadenine (HPA) is a new member of this group. In 2013 we provided experimental evidence for the molecular mechanism of action confirming that *E. coli* AlkB dioxygenase preferentially repairs substrates in cationic form and showed that AlkB removes efficiently hydroxypropano ring from acrolein adduct to cytosine ($3,N^4$-α-hydroxypropanocytosine) (35, 36). Singh at al. demonstrated later that ACR adducts to guanine, which are neutral at physiological pH, are also processed by AlkB, however reactions are substantially less efficient (41). Herein, we present results of a combined *in vivo*, *in vitro* and *in silico* analysis on the mutagenic properties of HPA and molecular mechanisms of its repair by AlkB, and confirm that the cationic form of HPA is preferentially repaired by AlkB. Moreover, for the first time, we evidenced the limited turnover number in AlkB-mediated repair, which for HPA was estimated to 38 ± 4 cycles.

Materials and Methods

ACR modification of plasmid DNA - pIF101, pIF105 pIF106 - Plasmids were isolated according to a standard protocol (42) and then treated with ACR for 15 min at 37°C. The incubation mixture in the volume of 0.1 mL contained: 200 μg of plasmid DNA, 0.5 M sodium acetate buffer pH 4.5, and 0.5 – 5 μL of 1 M ACR (Fluka, final concentration 5 – 50 mM, respectively). After modification DNA was precipitated twice with 2.5 volumes of ice-cold ethanol in the presence of 0.3 M sodium acetate buffer pH 5.2, washed with cold 70% ethanol, redissolved in appropriate volume of TE buffer and then frozen at -20ºC. Mock treated plasmids were prepared in the same way except that ACR was absent from the incubation mixture.

Mutagenicity assay, preparation of electrocompetent cells and electrotransformation were done according to (34). Briefly, modified and mock treated plasmids were introduced by electroporation into wt and *alkB* electrocompetent *E. coli* cells. Appropriate dilutions of the transformation mixture were spread on LB plates containing chloramphenicol (30 μg/mL) to count the total number of transformed cells and on lactose minimal plates for selection of

Lac$^+$ revertants. Mutation frequency (MF) was calculated as the number of mutants per 10^4 transformed cells.

Statistics. Since some distributions were found to be non-Gaussian according to the Anderson-Darling test (43), the statistical significance of the observed differences was assessed according to non-parametric Kruskal-Wallis H test (44). All analyses were performed using Statistica 10 (StatSoft, 2011). Null hypotheses that given distributions do not differ from each other were tested at the significance levels of $\alpha = 0.05$, 0.01 and 0.001.

Preparation of HPA-containing deoxyoligomeric substrates. To a solution of 150 nmol of the TTATT deoxypentanucleotide or TAT deoxytrinucleotide (Metabion, Martinsried, Germany) in 340 µL of 0.65 M sodium acetate pH 4.5 15 µL of 15 M ACR (95% aq. solution, Fluka) was added and the mixture was incubated for 40 min at 37°C. The reaction mixture containing completely modified oligomers was purified from ACR and reaction buffer using preparative HPLC according to (35), lyophilized and redissolved in TE buffer. The identity of the modified oligomers was ascertained by HPLC and MS analysis.

AlkB dioxygenase purification, HPLC chromatography, and ESI-TOF mass spectrometry were done exactly as we described previously (34, 35).

Size exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC-ICP-MS). The determination of amount of Mn, Fe, Ni, Cu and Zn in FTO protein was carried out by Agilent 7500a ICP Mass Spectrometer (Agilent Technologies,Tokyo, Japan) using 10 ng mL^{-1} of yttrium (^{89}Y) as an internal standard for analyzed samples and standard solution used for instrument calibration. Solutions containing the protein were more stable in 5 mM ammonium acetate pH 6.0 for external calibration curve. Curves were linear in the investigated range from 0.5 ng mL^{-1} to 100 ng mL^{-1} with r^2 above 0.999. Limit of detection (LOD) was calculated for standard deviations of 10 measurements of blank and it was found to be 0.2 – 0.7 ng mL^{-1}. ICP MS measurements conditions (nebulizer gas flow, rf power and lens voltage) were optimized daily using a standard built in procedure.

Temperature dependence of protein fluorescence - differential scanning fluorimetry (DSF) assay (45). Fluorescence data were collected on a Varian Cary Eclipse spectrofluorometer equipped with a variable-temperature cell holder, using 10-mm path-length cuvettes.

5

Emission was monitored at 345 nm (excitation at 280 nm) at temperatures increasing in the range of 20–85 ºC (95 ºC for FTO) with 1 ºC/min heating rate. The protein sample was diluted to a final concentration of 1.2 µM with 10 mM HEPES buffer pH 7.5 containing 100 mM NaCl and 2 mM DTT. Thermal unfolding of the protein was monitored either in the presence or absence of 100 µM αKG and 50 µM Mn(II) and with increasing concentrations of a potential substrate (0.6 - 6 µM).

DSF data analysis. The simplest model for thermal unfolding of a protein assuming a two-state process associated with temperature-dependent equilibrium of the folded, F, and unfolded, U, protein states was applied in the data analysis (46), together with a linear approximation of temperature-dependent fluorescence intensity for both protein forms ($F_F(T)$ and $F_U(T)$, respectively). Thermodynamic parameters were determined by fitting the model to the experimentally observed temperature-dependent intensities of fluorescence, $F(T)$, according to the following formulae:

$$F(T) = F_U(T) \cdot x_U + F_F(T) \cdot (1 - x_U) \tag{1}$$

$$F_{F/U}(T) = A_{F/U} \cdot T + B_{F/U} \tag{2}$$

$$x_U = \frac{1}{1 + e^{-\Delta G_{unf}(T)/RT}} \tag{3}$$

$$\Delta G_{unf}(T) = \Delta H_{unf}^o + \Delta C_{p,unf} \cdot (T - T_o) - T \cdot \left[\Delta S_{unf}^o + \Delta C_{p,unf} \cdot ln\left(\frac{T}{T_o}\right)\right] \tag{4}$$

where: x_U is the relative population of the unfolded protein, ΔG_{unf} is the free energy change for unfolding transition, $\Delta C_{p,unf}$ is the heat capacity change associated with the protein unfolding process (assumed temperature-independent), and ΔH_{unf}^0 and ΔS_{unf}^0 represents van't Hoff heat and entropy of unfolding at the arbitrarily selected reference temperature T_o. When the melting temperature, T_m, is selected as the reference, $\Delta H_{unf}^m = T_m \cdot \Delta S_{unf}^m$, and the equation (4) can be expressed in the form:

$$\Delta G_{unf}(T) = \Delta H_{unf}^m \cdot (1 - \frac{T}{T_m}) + \Delta C_{p,unf} \cdot \left[(T - T_m) - T \cdot ln\left(\frac{T}{T_m}\right)\right] \tag{5}$$

A numerical model based on Equation (1), combined with Equations (2), (3) and (5) was fitted to the experimental data using the optimization algorithms implemented in the Origin 9.0 package (www.originlab.com). Initial low- and high-temperature asymptotes ($A_{F/U}$ and $B_{F/U}$ parameters) were roughly pre-estimated using restricted datasets (T < 30 ºC and T > 70 ºC, respectively), and ΔH_{unf}^m and T_m were then optimized keeping all other parameters constrained ($\Delta C_{p,unf}$ fixed to 0). All data were carefully curated, and those which disagreed with neighboring ones were removed from the analyses. Finally, the whole sets of six, and

afterwards seven parameters, were optimized, albeit in some cases the latter procedure led to strongly biased estimates of $\Delta C_{p,unf}$.

AlkB assay - The reaction mixtures (20 µL) contained: appropriate 50 mM buffer, 1 mM dithiothreitiol, $Fe(NH_4)_2(SO_4)_2$, αKG at varying concentrations and 10 - 50 µM TT(HPA)TT ssDNA substrate. Reactions were started by addition of purified AlkB protein (0.1 - 0.5 µM for kinetics and 1 µM for optimization) or the component studied, allowed to proceed at 37ºC, stopped by adding 230 µL of ice-cold water and frozen at -20ºC to deactivate AlkB, and then analyzed by HPLC.

Quantum mechanical (QM) calculations. The initial geometry of 1,N^6-α-hydroxypropano-N9-methyl-adenine, which stands the model for HPA modified base, was adopted from the PDB structure of adenosine in which the sugar moiety was replaced by a methyl group. This was followed by *ab initio* analysis with the aid of the Firefly version 8.1.1 program (47). The density functional theory calculations were performed with the B3LYP functional (48) using the 6-31G(d,p) basis set (49) previously found applicable in the analysis of similar systems (35, 50, 51). The resulting geometry, together with ESP-derived atomic charges, was used to parameterize the force-field used in further molecular modeling.

Molecular modeling of AlkB - T(HPA)T complexes. Initial coordinates were adopted from the structure of T(m^1A)T bound to AlkB (pdb3i2o) (25). Structural calculations were performed with the aid of simulated annealing followed by molecular dynamics in the presence of explicit water molecules (both implemented in the YASARA Structure package) using the standard YASARA2 force field (52) extended for the nonstandard ligand by adding *ab initio* derived geometry and ESP-derived charge distribution. In accordance with the QM calculations, R and S isomers of the cyclo-hydroxypropyl group were tested in either axial or equatorial conformation of the hydroxyl group (see Figure 6 for details). For each of these four structural forms, the *syn* and *anti* conformers of the HPA glycosidic bond were analyzed.

Results and Discussion

In vivo **repair of HPA by *E. coli* AlkB**

HPA is one of the minor acrolein adducts to DNA bases. Apparently the only report considering its possible mutagenicity was published in 1998 (11). In an analysis of mutations

induced by ACR in normal human fibroblasts using *supF* shuttle vector plasmids it was found that the majority of the ACR-induced base substitutions took place at guanine or cytosine residues. However, to the authors' surprise, 22% of the base substitutions were at A·T sites. Employing dsDNA plasmid treated with acrolein at pH 7.4, which allows modification of all DNA bases, the authors were unable to identified which of the possible G·C and T·A pair derivatives were responsible for the observed substitutions. Here, for the first time, we provide evidence that the acrolein adduct to adenine is mutagenic and can be effectively repaired by AlkB dioxygenase.

To examine the *in vivo* mutagenic potency of HPA we employed the test system elaborated and successfully used in our previous studies on chloroacetaldehyde mutagenicity (33, 34). The system involves a series of pIF plasmids (53) carrying alleles of lactose operon of the CC101 - 106 strain (54) allowing monitoring of the occurrence of Lac$^+$ revertants arising by substitution mutation of a particular base. Acrolein originated from exogenous sources is known to be cytotoxic to bacteria even in relatively low doses due to its impact on cellular proteins (55). To avoid this toxic effect plasmid DNA was modified with acrolein *in vitro*. The modification reactions were performed in acidic conditions, where thymine is not modified by acrolein (56, 57), so base substitutions caused by HPA could be observed exclusively. After electrotransformation of the ACR-modified plasmids into bacteria (wt and *alkB*) their repair *in vivo* was studied. Three plasmids: pIF101, pIF105 and pIF106 indicatory for AT→CG, AT→TA and AT→GC mutations, respectively, were used. The transformation efficiency with mock-treated (control) plasmids varied between 1 x 10^6 and 2 x 10^6 cfu/μg DNA depending on the batch of competent cells and decreased with the ACR concentration used for plasmid modification, down to 2 x 10^4 for *alkB* strain and 50 mM ACR. The decreased plasmid survival results from ACR-induced lesions in the whole plasmid DNA, as we discussed in detail previously (34).

The mutation frequencies are shown in Figure 1 and in Supplementary Table S1 (see Supplementary Materials). They increased in a dose dependent manner for both strains and all the plasmids tested and were 1.5 up to nine times higher for the *alkB* strain than for the *wt*. HPA caused mainly transversions (A→T slightly more often than A→C) and less frequently, A→G transitions. Background (spontaneous) mutation frequency varied depending on the plasmid used, being the highest for the A→G substitutions, where, on the other hand, the difference between wt and *alkB* strain was the lowest.

FIGURE 1

AlkB dioxygenase is induced as part of the system of adaptive response to alkylating agents in *E. coli*. It is worth emphasizing that the observed HPA repair (Fig. 1) occurred without prior Ada response induction in the studied bacterial cells, thus indicating that AlkB dioxygenase at the constitutive level exerted observable repair activity. The protective effect of AlkB protein was previously observed for uninduced *E. coli* cells treated with CAA or bearing CAA modified plasmid by Essigmann group (32, 58) and us (33, 34). Moreover, neither CAA (59) nor ACR (Dylewska et al, in preparation) induce Ada response. Interestingly, induction of the system in the studied bacteria decreased the mutation frequency not only in the wild type, but also in the *alkB* strain (Dylewska et al, in preparation). This could indicate an involvement of another inducible protein in HPA repair and, consequently, suggests that HPA may be even more mutagenic than it is shown here.

Assembly of protein/cosubstrate/cofactor-substrate complexes monitored by differential scanning fluorimetry.

Differential scanning fluorimetry (DSF) is commonly used in screening for ligand binding to a protein, whenever the ligand binding affects the protein fluorescence, its thermal stability or heat of unfolding (45).

The DSF data recorded for AlkB in the apo form and in the presence of a cofactor (Mn(II) used as a non active Fe(II) analogue) and/or cosubstrate (αKG) demonstrated that AlkB virtually did not bind Mn(II) in the absence of αKG, while the binding of αKG alone substantially decreased the protein fluorescence (Figure 2A).

α-Ketoglutarate content, assessed with ESI-TOF mass spectrometry, was found undetectable both in all protein samples and in supernatant remaining after protein thermal degradation. Since the peak at 144.99 Da, corresponding to αKG monoanion, was clearly visible for equimolar αKG solution, samples of both proteins were considered as αKG-free.

Since the AlkB/αKG form was inactive in biochemical assays (27), the content of Fe(II) in the purified protein sample could be neglected, and the concentration of Mn(II) drives the formation of protein structure almost identical with that of active (i.e. Fe(II)-bound) form (25).

Basing on the known structures of the protein with ligands (cofactor and cosubstrate) bound, the αKG-induced changes in AlkB fluorescence should be assigned directly to quenching of Tyr 122 and Trp 178 by the proximal αKG, separated from each other by less than 4.3 Å, while the next-nearest Trp/Tyr side-chain (Trp 69) is separated by more than 6.5 Å (see Supplementary Figure S1A). In contrast to the apo form, the fluorescence of AlkB/αKG was

9

visibly affected by Mn(II), thus confirming (17, 27, 28) that co-coordination by αKG is crucial for metal binding (Figure 2A). In this case Trp 178 is mostly affected, and the protein fluorescence is decreased further. The high-temperature part of the DSF curves remains unaffected by Mn(II) or αKG, clearly demonstrating that neither of the ligands can interact with the unfolded protein. The thermodynamic parameters extracted from the DSF data, shown in Table 1, confirm that Mn(II) does not bind to the apo form of the protein. Interestingly, although αKG binding causes a substantial increase of van't Hoff heat of unfolding (770 ± 140 kJ/mol *vs*. 431 ± 32 kJ/mol for the apo form estimated at ~55 ºC), no thermal stabilization of the protein is observed. The latter results from an increase of the entropy of unfolding (2.4 ± 0.4 *vs*. 1.3 ± 0.1 J/mol/K) associated with a restricted flexibility of the bound αKG. However, further addition of Mn(II) results in a strong increase of T_m by 7.2 ± 1.8 ºC, thus confirming the formation of an active form of AlkB.

FIGURE 2

More importantly, the DSF curves recorded for the AlkB/αKG/Mn(II) complex change visibly upon further addition of a putative substrate, the deoxytrinucleotide T(HPA)T. Analysis of AlkB crystal structure (pdb3khb) (39) (Supplementary Figure S1A) and the model of AlkB complex with T(HPA)T (Figure 6) indicates that Trp 69, together with Tyr 76 and Tyr 78, may sense ligand binding. It should be however noted that the fluorescence intensity observed for both folded and unfolded AlkB forms may also be affected by absorption at 280 nm by the added deoxytrinucleotides decreasing the effective excitation. Despite that, the estimated thermodynamic parameters (T_m, ΔH_{unf}^m, $C_{p,unf}$ and ΔS_{unf}^m) consistently vary with the substrate concentration. Using the simplest model in which changes of each observable follow the equilibrium of substrate-protein binding, the observed trends allow the T(HPA)T-AlkB dissociation constant to be estimated at 1.8 ± 1.1 μM (Supplementary Figure S2). This demonstrates that even a limited set of DSF experiments allows a rough estimation of substrate affinity.

Among nine homologues of AlkB identified in human genome, ABH2 and ABH3 are considered to be involved in DNA repair (22). Interestingly, FTO dioxygenase, some of whose alleles are associated with obesity (19), has been also shown to have some DNA repair activity (60). Since no suitable biochemical assay has been established yet, the content of some bivalent metal cations (Fe, Mn, Ni, Cu, Zn) was measured using size exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC-ICP-MS)

10

technique. The concentration of any of these ions was found substantially lower than that of the protein (see Supplementary Table S2), so the FTO sample may be considered iron and manganese free.

As a preliminary step to determine the FTO substrate specificity we carried out DSF experiments analogous to those with AlkB described above (Figure 2B). According to the crystal structure of FTO (pdb3lfm) (40), Tyr 295 is the residue whose florescence is most strongly affected by the proximal αKG, while Tyr 106, Tyr 108 and Trp 270 should sense substrate binding. It must be noted, however, that due to the small difference between the fluorescence of the folded and unfolded states, T_m could only be reasonably precisely determined only for the apo form of FTO, while other DSF data allowed a qualitative analysis only. Such analysis showed that FTO binds Mn(II) in the presence of αKG, while T(HPA)T is not bound by FTO/αKG/Mn(II) (Figure 2B). The same results were obtained for other deoxynucleotide trimers, indicating that none of them can be regarded an FTO substrate (results not shown).

All these negative results are consistent with the structural alignment made for FTO (pdb3lfm) (40) and the AlkB-DNA complex (pdb4nid) (61) which showed that six-residue loop of FTO ([84]RIQGKD[89]) cannot sample DNA major grove as efficiently as the short β-turn [52]PGG[54] in the structure of AlkB does. Moreover, long loop [208]D-G[224] in FTO precludes recognition of DNA minor groove (Supplementary Figure S6).

Altogether, the result presented here show that DSF analysis can be used to monitor the assembly of protein-cofactor-cosubstrate complexes and to screen for potential substrates or competitive inhibitors, and even for a rough estimation of their binding affinities.

TABLE 1.

Repair of TT(HPA)TT by purified AlkB protein

The best AlkB substrates, m^3C and m^1A, are in a cationic form at physiological pH, whereas m^3T and m^1G, which are neutral at pH 7, are repaired very poorly (37, 62, 63). As we have shown previously (35), efficient repair of the exocyclic adducts εC, εA, HEC and HPC occurs at a pH favoring their protonated forms. Moreover, we observed a negative correlation between the pH optimal for repair of a given substrate and the logarithm of iron concentration required for a maximal efficiency of this process. We reason that, at a lower pH the imidazole ring of the histidine residues involved in Fe(II) coordination (His 131 and His 187) is protonated, which has to be compensated for by an increase of Fe(II) concentration. In

11

contrast, the optimal αKG concentration did not depend on pH since even at the most acidic conditions screened, the cosubstrate maintained its active doubly dissociated anionic form.

Here we used on HPA-containing pentadeoxynucleotide, TT(HPA)TT, as a substrate for AlkB. Using HPLC to separate the modified and unmodified (repaired) oligonucleotides we confirmed the *in vivo* observation that the modification is indeed efficiently repaired by *E. coli* AlkB dioxygenase. The optimal pH for HPA repair was found to be 7.5, slightly more than one pH unit below the pK_a of HPA (8.77 ± 0.06, see Supplementary Figure S3). This clearly indicates that the cationic form of the HPA base is preferably repaired. The optimal iron concentration was 147 ± 5 μM for repair at pH 7.5, while the optimal αKG concentration was 50 ± 7 μM (Supplementary Figure S4). Supplementary Figure S5 shows combined data on the interdependence between pH and Fe(II) concentration optimal for repair of m^3C, HPC, HEC, εA and εC adducts by AlkB protein taken from (35) together with the present result for HPA at the optimal pH of 7.5. The slope of -0.54 ± 0.03 estimated for log(Fe) vs. pH relation indicates that iron binding by AlkB is at lower pH associated with the deprotonation of the imidazole ring of both His residues involved in metal coordination (His 131 and His 187).

Mass spectrometry analysis of HPA repair by AlkB

AlkB usually acts by a direct reversal mechanism leading to regeneration of the natural base, but some exceptions have been noted. A cyclic alkanoadduct to adenine, $1,N^6$-ethanoadenine, was found to be oxidatively hydroxylated by AlkB, but the final product of this reaction was ethano ring opened N^6-(2-oxoethyl)-adenine (31). AlkB repair reactions of acrolein and malondialdehyde adducts to guanine - α-hydroxypropano-dG (α-OH-PdG), γ-hydroxypropano-dG (γ-OH-PdG), and m_1dG, respectively, are complex, involving multiple intermediates, overlapping pathways with multiple convergence points, and only for α-OH-PdG was formation of a small amount of fully dealkylated product (dG) observed (41). Bearing in mind the above observations we verified full HPA repair by AlkB by mass spectrometry (MS). As shown in Table S3, after incubation with AlkB the pentadeoxynucleotide containing HPA was converted to its unmodified (*i.e,* repaired) form. Our data indicate that the adduct to adenine studied here is repaired completely, as are other saturated hydroxyalkanoadducts - described by us previously, HEC and HPC (34, 35).

Basing on ACR reaction with adenine described in (64), the general mechanism of AlkB action (25, 27, 28, 32) and the finding that the final product released during AlkB-mediated transformation of ACR modified guanine is malondialdehyde (41) in Figure 3 we present a reaction of formation- and foregoing AlkB-mediated repair of HPA in DNA.

FIGURE 3.

HPA repair by AlkB differs qualitatively from that observed for εA

In our previous work (35) we analyzed AlkB-directed repair of the chloroacetaldehyde adduct to adenine – non-isomeric εA. We found that the reaction followed pseudo first-order kinetics during initial 10 minutes, while further reaction progress was visibly slower than expected. Here, the repair of HPA by AlkB was followed in reaction mixtures of various composition to improve the reliability of the estimated rates using the same approach as for εA.

FIGURE 4.

Initially TT(HPA)TT to TTATT conversion follows the general trend observed by us previously for TT(εA)TT→TTATT (35), as presented in Figure 4 for the reaction carried out by 0.5 µM AlkB at conditions optimized for either substrate. Interestingly, for the lowest substrate concentration (10 µM) the HPA decay during the first minute agrees perfectly with the reaction progress observed for the same concentration of εA, clearly indicating that HPA is as good a substrate for AlkB as is εA (empty triangles *vs*. empty circles in Figure 4). However, with the reaction progress the two curves diverge, and the concentration of εA decreases steadily accordingly to pseudo-first-order kinetics (dashed arrow) while the concentration of HPA reaches a plateau. The same tendency is observed at higher initial substrate concentrations, for which the higher the HPA concentration is, the slower the repair relative to that of εA. Furthermore, the fraction of HPA remaining unprocessed increases with initial substrate concentration. Taken together, these result indicate that there is a qualitative difference between the two reactions. Although εA is smaller than hydroxypropano ringed HPA, both molecules fit to the catalytic site, and the initial rates of their repair are close. However, the substantial difference concerns the size of the released by-product – glyoxal and malondialdehyde for of εA and HPA, respectively (32, 41).

The apparent initial rate of the repair of TT(HPA)TT at a low concentration is very high and virtually identical to that determined by us recently for TT(εA)TT (35), but with increasing substrate concentration the repair rate falls, and, in parallel, increases the amount of substrate remaining unprocessed. Moreover, for all the substrate concentrations the rate decreases substantially as the reaction proceeds. Altogether, it implies that each AlkB molecule is capable of processing only a limited number of substrate molecules and, consequently, the amount of active protein decreases continuously, until its full deactivation.

13

TABLE 2.

The data presented in Table 2 give a semi-quantitative insight into the HPA repair by AlkB. Thus, the initial first-order rate (v_0) decreases substantially with increasing TT(HPA)TT concentration. This clearly implies that HPA inhibits AlkB activity. It should be noted that the reaction progress extrapolated to the initial reaction time shows that a substantial amount of substrate is processed in the burst phase, during the first 20 s. However, the amount of substrate processed during this pre-steady-state period exceeds 7-10 folds AlkB concentration, clearly indicating that the initial rate of AlkB-driven TT(HPA)TT repair must be higher than the observed rate of "late" first-order kinetics (i.e. after 20 s).

Limited amount of HPA can be processed by AlkB.

Inspection of substrate concentration at very long reaction times clearly confirms that, unlike for most of previously studied substrates (35), even after 90 min a significant fraction of HPA remains unprocessed. However, a general relation between the AlkB to substrate concentration ratio and apparent limit of turnover number could be deduced. Thus, the higher protein to substrate ratio is, the larger amount of HPA is repaired (Figure 5). Interestingly, even at highest ratio only app. 86% of TT(HPA)TT is converted to TTATT. Moreover, the intercept calculated for the relation observed between % of the repair represented as a function of enzyme to substrate ratio indicates that each AlkB molecule carries out 38 ± 4 enzymatic cycles before its total inactivation. The most likely candidate for the toxic entity is malondialdehyde - the chemically active by-product, which is apparently much more reactive than glyoxal.

Taken together, kinetic data supports the following scenario. AlkB extremely efficiently repairs TT(HPA)TT, however the by-products are toxic for the protein. Thereby, after 7-10 enzymatic cycles the protein loses most of its activity, however slowly carries next 30 cycles, until its full deactivation.

FIGURE 5.

Ab initio* sampling of 1,N⁶-α-hydroxypropano-N9-methyl-adenine *low-energy states

Conformational equilibrium of HPA and relative energy of the most stable conformers were analyzed using an *ab initio* approach. 1,N⁶-α-hydroxypropano-N9-methyl-adenine was used as a general model for the base of HPA deoxynucleoside. The hydroxyl group can be attached

to Cα to form either the R or S stereoisomer (Figure 7), both of which display identical properties alone, however become non-equivalent when (deoxy)ribose is attached at N9, and can even be substantially differentiated in an asymmetric environment (e.g. in a protein binding site). Moreover, the additional six-member ring formed by α-hydroxypropane can adopt two alternative conformations with the hydroxyl group oriented either equatorially or axially, the equatorial form being favored by ~ 1.8 kcal/mol. Similar calculations were performed for cationic forms of both stereoisomers, which were found to be protonated preferentially at N^6.

FIGURE 6.

Molecular modeling of HPA location in AlkB substrate-binding pocket

Since no structures of AlkB with the studied here substrate had been published, we modeled the structure of T(HPA)T bound to AlkB. Initial coordinates of the complex were obtained by a stepwise simulated-annealing procedure in which 1-methyladenine from a complex of AlkB with DNA dodecamer (pdb3bi3) (65) was modified sequentially to $1,N^6$-dimethyladenine, $1,N^6$-propanoadenine, and eventually to the R and S stereoisomers of $1,N^6$-α-hydroxypropanoadenine (HPA). The two latter structures, each in either *syn* or *anti* conformation of glycosidic bond, were further subjected to 1-ns molecular dynamics in the presence of explicit water molecules (see Methods for details). Representative structures obtained are shown in Figure 7. Interestingly, both isomers pack perfectly inside the binding pocket, when the α-hydroxyl group adopts the equatorial conformation (R-*anti* and S-*cis*). In both cases the modified base makes stacking interactions with the side-chain of proximal Trp 69, accompanied by hydrophobic contacts with Tyr 76 and Leu 118. In both structures the proximal iron cation makes the base susceptible to a radical attack that initiates the dealkylation process. Additionally, the side-chain carboxyl group of the proximal Asp 135 recognizes the electron–deficient region at the N4 side, of which interaction is in accord with the experimentally observed preference towards a cationic form of the substrate. However, the Asp 135 side-chain is moved slightly away from the (S)-HPA form due to steric hindrance with its α-hydroxyl oxygen, which may result in preferential activity towards the R isomer of HPA. In general, despite the increased size of the α-hydroxypropano derivative compared to the original 1-methyladenine, neither stereoisomer of HPA disturbs significantly the network of intermolecular interactions observed in the crystal structure of AlkB with the DNA oligomer (65). Thus, the presented models show the molecular mechanism of HPA

15

recognition, including Trp 69 involved in a stacking interaction with the modified base and Asp 135 acting as a gate-keeper. Moreover, it should be underlined that the AlkB pocket is filled with the tricyclic ring of HPA almost completely, so the diffusion of the reaction by-product (malondialdehyde (41)) may be slowed down markedly as long as the product (adenine) occupies the binding site. Such hardly mobile reactive specie could attack on fly an AlkB residue(s) essential for the enzymatic activity, leading to its gradual inactivation, which was observed here by us to be much faster for HPA than found previously for εA (35).

FIGURE 7.

Conclusions

In this study, we provide *in vivo* and *in vitro* evidence that, the acrolein adduct to adenine, $1,N^6$-α-hydroxypropanoadenine is mutagenic and is effectively repaired by AlkB dioxygenase. As the lesion is efficiently formed *in vivo* after exposure to acrolein (12), it is likely to be harmful to living organisms.

The molecular mechanism of AlkB action was characterized using kinetic experiments and *in silico* modeling. The analysis demonstrated that a protonated form of HPA is repaired preferentially, the reaction is stereoselective, and AlkB exhibits highly limited turnover number towards its substrate.

Additionally, a differential scanning fluorimetry assay was elaborated, providing a useful tool for discrimination of protein active forms with cofactor and cosubstrate bound and for monitoring substrate binding.

Author Contribution

AMM, JP and JTK conceived the study and designed experiments; MD AMM and TP performed the experiments; JP performed MM and QM calculations; MD, AMM, JP and JTK analyzed the data; AMM and JP wrote the paper.

16

Acknowledgements

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Tables

Table 1. Thermodynamic parameters of thermal unfolding of AlkB and FTO dioxygenases in the presence of various ligands.

Table 2. Kinetic parameters estimated for AlkB-directed repair of HPA in pentadeoxynucleoside.

Figures

FIGURE 1. Mutation frequency in the *lacZ* gene induced by ACR. Frequencies of A→C, A→T and A→G mutations of the pIF101 (A), pIF105 (B) and pIF106 (C) plasmids, respectively, replicated in wild type (black circles) and AlkB-deficient (empty circles) *E. coli*. Data are represented by medians (circles) and quartiles, while min-max ranges are denoted by vertical lines. The statistical significances of the differences between groups are denoted by asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Please note that the scale for A→G substitutions (C) differs from the others.

FIGURE 2. DSF data for AlkB (A) and FTO (B) dioxygenases. Difference between *apo* and Mn(II)/αKG loaded forms, represented by empty and black circles, clearly demonstrates formation of AlkB and FTO active complexes. Solid lines represent models fitted to DSF data, parameters of which are listed in Table 1. Distribution of gray markers in panel A indicates that Mn(II) alone does not bind to *apo* AlkB (diamonds) while αKG alone does (triangles). Distribution of gray diamonds in panel B, representing DSF data recorded for FTO/Mn(II)/αKG in the presence of T(HPA)T in 1:1 or 5:1 molar ratio, shows that T(HPA)T does not bind to the protein.

FIGURE 3. Chemical structures and proposed pathway for formation- and AlkB-mediated repair of HPA.

FIGURE 4. Repair of HPA and εA byAlkB dioxygenase (0.5 µM). Both the rate of repair and the fraction of processed deoxypentamers correlate negatively with initial substrate concentration. It should be noted that the initial rate of HPA repair is at lowest substrate concentration the same as that of εA (35), albeit amount of unprocessed HPA is higher than that of εA.

FIGURE 5. Dependence of the final fraction of HPA repaired on the initial AlkB/HPA molar ratio. The fraction of unprocessed HPA decreases with an increasing enzyme : substrate ratio, but even at the highest ratios tested less than 90% of TT(HPA)TT can be repaired. Asymptotic trend observed for low enzyme : substrate ratio indicates that the limited number of HPA molecules is processed by a single AlkB molecule.

FIGURE 6. Possible stereoisomers/conformers of HPA. R (top) and S (down) configuration at Cα with either equatorial (left) or axial (right) orientation of the hydroxyl group.

FIGURE 7. HPA in the binding site of AlkB. R-*anti* (A) and S-*syn* (B) HPA was modelled into AlkB active site using the structure of m^{1}A bound to AlkB (pdb3i2o) (25) as the template. Both conformers are susceptible to the action of radicals formed close to the the metal center. In complexes with the alternative conformers (R-*syn* and S-*anti*) the exocyclic hydroxyl group points towards the metal center, thereby shielding the cyclohydroxypropane adduct from a direct radical attack. HPA with the axial orientation of the hydroxyl group (see Figure 6) does not fit the AlkB binding site. Asp135, postulated to be crucial for recognition of substrates in their cationic forms, is shown in stick representation, and Fe(II) is represented as pink sphere of appropriate radius.