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SITE-DIRECTED MUTAGENESIS OF GLUTAMIC ACID 172 TO GLUTAMINE COMPLETELY INACTIVATED HUMAN O^6-ALKYLGUANINE-DNA-ALKYLTRANSFERASE

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SUMMARY. DNA repair by O^6-alkylguanine-DNA-alkyltransferase involves the stoichiometric transfer of the O^6-alkyl group from the guanine lesion to the active-site cysteine residues of the protein. Site-directed mutagenesis of glutamic acid 172 of human O^6-alkylguanine-DNA-alkyltransferase (EC 2.1.1.63) to glutamine totally abolished the alkyltransferase activity of the protein. This suggests that glutamic acid 172 is crucial to the alkyl transfer. It may act as a general acid (as CO_2H) or base (as CO_2^-), or have a role as a component of a salt-link ($-CO_2^-.....^+N-$), vital for the structural integrity of the active site. This is the first mutational inactivation of a protein in this family of DNA repair molecules by means of a residue change outside the highly conserved pentet (PCHRV) which includes the active-site cysteine.

The presence of O^6-methylguanine in DNA has been shown to be mutagenic, toxic, clastogenic and probably carcinogenic (1-6). Cells are protected against these detrimental effects by a unique DNA repair pathway involving the transfer of the O^6-alkyl group of the DNA lesion to a cysteine residue in the active site of the O^6-alkylguanine-DNA-alkyltransferase (ATase, EC 2.1.1.63) protein (3,5,7,8). The result is conversion of this cysteine residue to a methyl sulphide. There is no biochemical method known to S-demethylate the ATase and therefore O^6-alkylguanine in DNA acts as an

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Abbreviations used are: ATase, O^6-alkylguanine-DNA-alkyltransferase (EC 2.1.1.63); hAT, human O^6-alkylguanine-DNA-alkyltransferase; hAT_{E172Q}, human O^6-alkylguanine-DNA-alkyltransferase with glutamic acid 172 mutated to glutamine; phAT, wild type hAT cDNA in pUC8.0; $phAT_{E172Q}$, hAT_{E172Q} cDNA in pUC8.0; E, glutamic acid; Q, glutamine; P, proline; C, cystine; H, histidine; R, arginine; V, valine; PCR, polymerase chain reaction.

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autoinactivating substrate. The ATases studied to date (9-12) and references therein] are relatively low in molecular mass (~22-24 kDa) and there is a marked amino acid sequence homology between the mammalian, yeast and various bacterial ATases, especially for a "core" of ~80 residues (13), from P_{80} to E_{172} using the human ATase (hAT) as a reference sequence. However, there is considerable inter-species sequence variation of the N-terminal ~80 residues and the presence of a ~20 amino acid C-terminal tail distinguishes mammalian from other sequences (13,14).

One of the anticipated features of the ATase mechanism would be the need for an active site base to accept the proton of the -SH group of the active site cysteine and an early suggestion was that the conserved histidine in the active site sequence PCHRV could provide for this in a manner analogous to papain (15). It is also likely that an active site acid is required to protonate the guanine as a leaving group (16). Such mechanistically critical amino acids would be expected to be conserved and we have therefore analysed the available ATase sequences for evidence of conservation of candidate residues (9,13). We concentrated on the core in view of the clear sequence homology of this region (13) and the fact that we had already been able to remove the C-terminal tail from hAT without loss of DNA-binding or ATase activity (14). There are only five fully conserved residues in the core which are potential proton donors or acceptors (histidine$_{146}$, tyrosine$_{158}$, tyrosine$_{114}$, lysine$_{165}$ and glutamic acid$_{172}$). The active site histidine of PCHRV has already been studied by site-directed mutagenesis and found to be replaceable by phenylalanine, methionine, asparagine or glutamine with retention of some (albeit low level) activity (17). As there are many fewer confirmed instances of tyrosine or lysine acting as proton donor or acceptor than of glutamic acid (E), we chose in the first instance to mutate E_{172} to glutamine (Q) and now report the results of this mutation on the activity of human ATase.

MATERIAL AND METHODS

Restriction Endonuclease Digestion

All restriction endonucleases were used according to the manufacturers' instuctions and in the optimised incubation buffers provided.

Two Sided Overlap PCR

Two complementary mutagenic primers spanning the target codon (172) were synthesised. Each was used in two independent amplifications in combination with the

appropriate sense or antisense primer spanning the translation start and stop codons respectively. The latter primers also introduced unique Bam HI sites immediately 5′ or 3′ to the translational start and stop codons. A typical PCR, carried out in 100μL of *Tli* DNA polymerase buffer ($MgCl_2$ free) contained 40ng of phAT template DNA, 30pmol of each primer, 200μM of each dNTP, 1.25mM $MgCl_2$ and 2.5 units of *Tli* DNA polymerase added after the initial denaturation step at 93°C. Twenty cycles of amplification were then performed (93°C, 1 min., 60°C, 1min., 74°C, 1min.). The overlap extension PCR (18) was carried out as described above except that 40ng of each of the reaction products of the two independent PCRs were used as template and only the primers spanning the start and stop codons were included in the reaction. Conditions were as described except that the annealing temperature was 50°C. The PCR product was digested with Bam HI and ligated into the appropriate site of pUC8.0 to generate $phAT_{E172Q}$.

Transformation of *E.coli* KT233

The *ada*$^-$, *ogt*$^-$ *E.coli* strain KT233 (19) were grown to OD_{600}=0.15 and made competent using hexamminecobalt chloride and dithiothreitol/DMSO as previously described (20). The transformation procedure involved heat shock at 42°C for 90 seconds. Colonies were screened for the presence of the construct containing an insert in the sense orientation by either PCR amplification of crude bacterial lysates using a vector specific and a hAT cDNA specific oligonucleotide or by digestion with the appropriate restriction endonucleases.

DNA Sequencing

Sequencing was carried out according to the protocol of Hultman *et al.* (21).

O^6-alkylguanine-DNA-alkyltransferase Assay

Sonicated extracts of pelleted bacterial cultures were prepared and assayed using [^{3}H]-methylated calf thymus DNA substrate as previously described (22).

MNNG Survival Assay

E.coli KT 233 cells harbouring phAT, $phAT_{E172Q}$ or pUC 8.0 were grown overnight in 5mL of LB containing 50μg/mL ampicillin. These were diluted in fresh LB broth to E_{600} 0.26 and incubated in the presence of freshly prepared N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG, added at concentrations from 0 to 100μg/mL) for 15 minutes at 37°C. Appropriate dilutions of the cultures were plated onto ampicillin containing LB agar plates. These were incubated at 37°C for 16h after which colonies were counted.

RESULTS AND DISCUSSION

The mutation of codon 172 from GAA to CAA, which would result in the E⇒Q alteration, also changes a Nla III restriction endonuclease recognition site. Analysis of the product of two sided overlap PCR confirmed that the mutated hAT (hAT_{E172Q}) coding sequence was refractory to cleavage by Nla III, while the wild type hAT cDNA yielded the expected digestion products of 529 bp and 130 bp (data not shown). DNA sequencing of the entire PCR product provided absolute confirmation that the G⇒C mutation converting codon 172 to encode glutamine had been effected and that no other changes had been introduced during PCR.

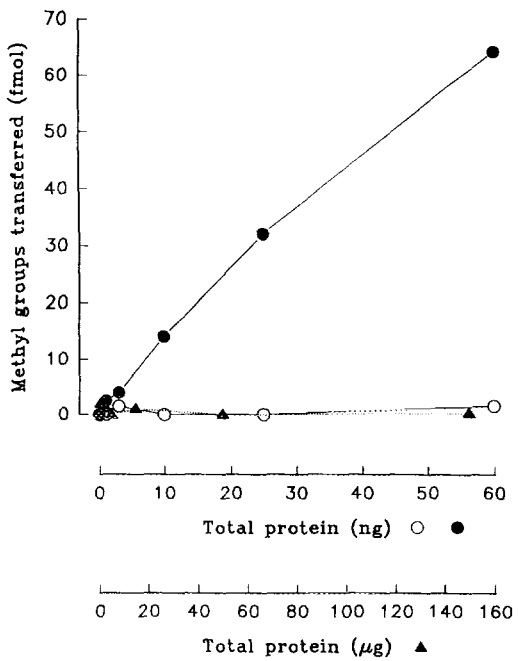

Figure 1. ATase activity in extracts of *ada*⁻, *ogt* *E.coli* KT233 harbouring phAT (●) or $phAT_{E172Q}$ of which up to 60 ng (○) or up to 150μg (▲) were assayed. Assay conditions are described in the text.

Crude extracts of *E. coli* KT233 ATase deficient clones containing wild type phAT had a specific activity of $1x10^6$ fmoles ATase/mg of protein. In contrast, extracts of *E. coli* KT233 host cells and those harbouring $phAT_{E172Q}$ had undetectable ATase activity after incubation for 2h (Fig.1), with no increase in activity on overnight incubation. Furthermore, increasing the amount of total protein in the assay from 60ng to 150μg also produced no ATase activity (Fig.1). By extrapolation, if 150μg of crude extract from wild type hAT expressing cells had been added to the assay, then $0.15x10^6$ fmoles, equivalent to ca. $2x10^6$ cpm, would have been expected to be transferred to the ATase protein. Thus, if the hAT_{E172Q} protein was as active as the hAT protein, and given that it is 25 fold less abundant, then $8x10^4$ cpm would be expected to be transferred under similar conditions. In fact, addition of 150μg of extract containing hAT_{E172Q} resulted in the transfer of <5 cpm to protein indicating that the mutated protein was at most ca. 0.003% active relative to hAT, thus demonstrating that it is essentially inactive for O^6-alkylguanine repair.

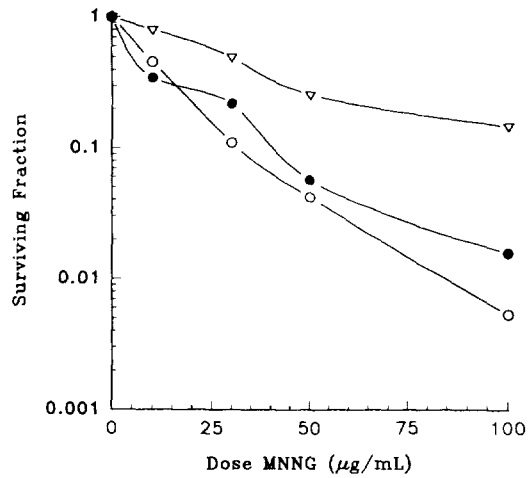

Figure 2. Effect of expression of wild type hAT and hAT_{E172Q} protein in *E.coli* KT 233 treated with varying doses of MNNG. Data are shown for cells transformed with phAT (∇), $phAT_{E172Q}$ (○) and pUC 8.0 (●).

The *in vitro* data above were confirmed by determining the effect of expression of the hAT_{E172Q} protein on the sensitivity (survival) of KT 233 cells to the methylating agent MNNG (Fig. 2). The host cells harbouring pUC 8.0 alone were sensitive to the cytotoxic effect of this agent and expression of the hAT_{E172Q} protein did not alter the sensitivity of these cells. However, expression of the wild type hAT protein in the cells conferred protection against toxicity. These data clearly demonstrate that the hAT_{E172Q} protein was not active *in vivo*.

Immunoblotting using highly specific mono and polyclonal antibodies to hAT (23), was used to confirm that a 22-24 kDa protein was expressed from $phAT_{E172Q}$, although the level of expression of hAT_{E172Q} was about 25 fold less than for the unaltered protein (data not shown).

The E_{172} residue could affect the activity of hAT in a number of ways. It could make a chemical contribution to the alkyl transfer process by acting as a general base in the CO_2^- form (e.g. to deprotonate C_{145}) or as a general acid in its CO_2H state (e.g. to protonate the leaving group). Alternatively, it might act structurally, for example to facilitate the interaction with DNA or as a component of a salt-link ($-CO_2^-\ldots{}^+NH_3-$) regulating the alkyltransferase activity as in the manner of the I_{76}-D_{194} salt-link of α-chymotrypsin (24). This

is the first mutational inactivation of a protein in this family by means of altering a residue other than the active site cysteine in the highly conserved pentet, PCHRV (17). More detailed studies of the hAT_{E172Q} protein are now in progress.

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