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SITE DIRECTED MUTAGENESIS OF TWO CYSTEINE RESIDUES IN THE E. COLI OGT O^6-ALKYLGUANINE DNA ALKYLTRANSFERASE PROTEIN

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The E. coli ogt O^6-alkylguanine-DNA alkyltransferase has two cysteine residues positioned identically with respect to cysteines in the E. coli ada O^6-alkylguanine-DNA alkyltransferase. In order to assess their function, these residues were each substituted by a glycine to generate altered forms of the ogt protein. Mutagenesis of cysteine-139, located within a 'PCHRV' region of homology, eliminated functional activity confirming that this residue is the methyl-accepting cysteine in the active site of the protein. Substitution of cysteine 102 within the sequence 'LRTIPCG' had little effect on the ogt protein activity demonstrating that this cysteine is not directly involved with the transfer of O^6-methylguanine adducts.

Monofunctional alkylating agents produce the toxic and mutagenic lesions, O^6-alkylguanine and O^4-alkylthymine in DNA (1). O^6-alkylguanine-DNA alkyltransferase (ATase) is a ubiquitous protein which can specifically repair these adducts by the direct transfer of the alkyl group to a cysteine residue within ATases (2). E. coli has two genes, ada (3,4) and ogt (5) which each encode an ATase enzyme. The ogt gene constitutively expresses a 19 kDa protein which constitutes the majority of the ATase measurable in wild type E. coli (6) whereas the ada gene is inducible by sub-lethal doses of alkylating agents (7) and encodes a 39 kDa ATase protein. This protein is cleaved in vivo into a 20 kDa peptide which is capable of repairing S-stereo-isomers of methylphosphotriesters and an 18 kDa peptide which is responsible for the repair of O^6-alkylguanine and O^4-alkylthymine adducts in DNA (4). The ogt ATase amino acid sequence is 28% homologous to the 18 kDa subfragment of the ada protein and 49% homologous to the 94 carboxyterminal amino acids

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Abbreviations

ATase, O^6-alkylguanine-DNA alkyltransferase (E.C.2.1.1.63); IPTG, Isopropylthio-β-Galactoside; MNU, N-methyl-N-nitrosourea.

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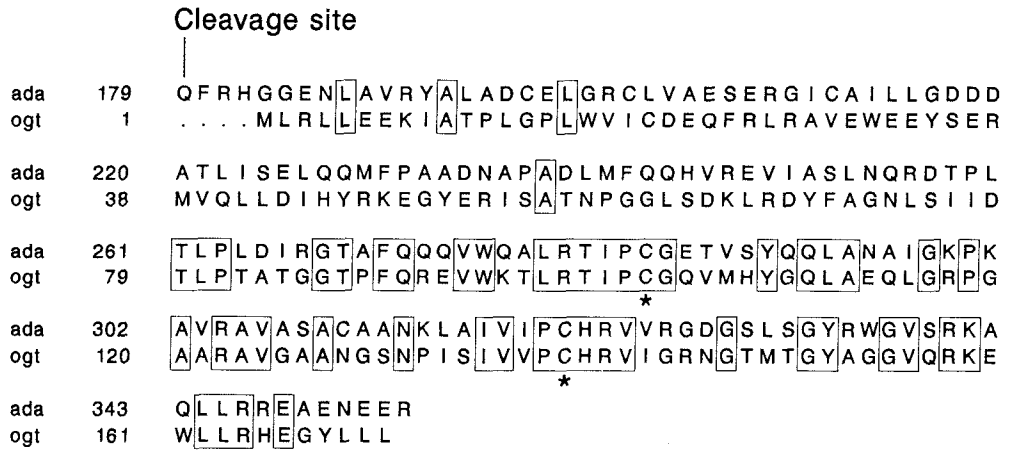

Figure 1

A comparison of the amino acid sequences of the ogt ATase and the 18 kDa subfragment of the ada ATase. The regions of homology are boxed and the cysteine residues which were substituted are marked (*).

(Fig. 1). In particular, there are two regions of homology which contain cysteine residues; a heptameric (LRTIPCG), and a pentameric (PCHRV) sequence separated by 38 amino acids in each protein (Fig. 1). Cysteine residue 321 in the ada protein has been identified as the alkyl-acceptor cysteine (8) and therefore the corresponding residue in the ogt protein (cysteine-139) is presumed to have the same role. However, as we have previously suggested (5), the two cysteine residues could generate a di-sulphide bridge forming a loop of 38 amino acids through which DNA may pass.

In this paper, we describe the construction of genes which encode altered forms of the ogt protein in which the two cysteine residues in question have been substituted by glycines. The specific activities of these mutant enzymes in crude extracts have been measured in order to determine the importance of each cysteine in generating functionally active protein.

Materials and Methods

Recombinant DNA Techniques

All recombinant DNA manipulations employed standard procedures (9).

Site Directed Mutagenesis. The ogt gene sequence (5) was available as a 650 bp EcoR1 fragment which was ligated into M13 mp19 (Boehringer Mannheim) in order to generate single stranded DNA. Site directed mutagenesis was performed using the Bio-Rad Mutagene in vitro mutagenesis kit (10). Oligonucleotides were synthesized on a DuPont 3000 Coder using standard phosphoramidite chemistry and purified by urea polyacrylamide gel electrophoresis.

Preparation of Bacterial Cell Extracts. A 2 ml aliquot of the bacterial culture was harvested by centrifugation at 14,000 x g for one minute at 4°C and the pellet resuspended in 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 3 mM dithiothreitol. Following disruption by sonication on ice for two periods of 10 seconds each at 12 μM peak to peak distance, phenylmethylsulphonyl fluoride in ethanol was added to a final concentration of 0.5 mM and cellular debris was removed by centrifugation (10,000 x g for 10 minutes at 4°C). Supernatants were used for protein estimation (Bio-Rad) and for ATase assay.

Alkyltransferase Assay. This assay has been previously described (4). Briefly, cell extracts were incubated with calf-thymus DNA that had been methylated in vitro by reaction with [^{3}H]MNU. Following incubation with the substrate DNA for 2 hours at 37°C, the DNA was hydrolysed in 1 M perchloric acid at 75°C for 40 minutes and labelled proteins were recovered by centrifugation and quantitated by liquid scintillation counting. The amount of protein in the cell extracts was determined and the ATase specific activity was calculated as fmole/mg of total protein.

Production of anti-ogt Polyclonal Antibodies. The ogt gene was ligated into the bacterial expression vector pET-3a (11) under the control of a T7 RNA polymerase dependent promoter. Following transformation into E. coli BL21-pLys-E which expressed T7 RNA polymerase under the control of the lacUV5 promoter (11), colonies were expanded and one was selected for highest level expression of ogt ATase. The ogt protein was purified from the bacteria as previously described (12) by chromatography on double stranded DNA cellulose and Sephacryl S-200 HR. Purified protein, 550 μg, was emulsified with incomplete Freund's adjuvant (Sigma) and injected subcutaneously at two-weekly intervals into the dorsal flanks of two half-lop eared rabbits over a period of 2 months. Blood was taken from ear veins and following removal of coagulated red cells by centrifugation (3,000 x g, 15 minutes at 4°C) the resulting serum was stored at -70°C.

Western Analysis. Polyacrylamide gel electrophoresis was performed using the Bio-Rad Mini Protean II system and the resulting gels were electroblotted to Hybond-C nitrocellulose (Amersham) using Bio-Rad electroblotting apparatus. The filters were preabsorbed by incubation with an extract of E. coli JRG1728 (a gift from J.R. Guest) from which the ogt gene has been deleted and then incubated with a 1:1000 dilution of the anti-ogt polyclonal antibody. The second antibody used was swine-anti-rabbit IgG (Dako Ltd) which was followed by an incubation with a peroxidase anti-peroxidase complex raised in rabbits (Dako Ltd). The peroxidase substrate consisted of 0.5 mg/ml 3',3'-Diaminobenzidine -4 HCl, 0.03% hydrogen peroxide in a 50 mM tris, 10 mM imidazole buffer, pH 7.5.

Results

Site-directed mutagenesis was performed on two cysteine residues within the ogt protein. The oligonucleotides synthesized for the mutagenesis and the amino acid substitutions made are shown in Fig. 2. The oligonucleotides introduced novel restriction endonuclease recognition sites in order that the presence of the desired mutations could be confirmed. The ogt gene was mutated to encode three altered proteins: 1 with a glycine residue substituting cysteine-102, 2 with a glycine residue substituting cysteine-139 and 3 in which both cysteines were substituted for glycines. Glycine was chosen for the substitutions since it has similar physico-chemical properties to cysteine (13).

residue 97					102			105
Leu - Arg	- Thr	- Ile	- Pro	- Cys	- Gly	- Gln	- Val	
CTA CGC	ACT	ATC	CCC	TGC	GGG	CAG	GTA	
				- Gly -				
Oligonucleotide used:	CT	ATC	CCC	GGG	GGG	CAG	G	

Sma I site created

residue	136			139			142
	Val - Val	- Pro	- Cys	- His	- Arg	- Val	
	GTC GTA	CCT	TGC	CAT	CGG	GTT	
			- Gly -				
Oligonucleotide used:	GTC GTA	CCT	GGC	CAT	CG		

Bal I site created

Figure 2

The oligonucleotides used for mutagenesis and the amino acid substitutions generated.

Following mutagenesis, the EcoR1 fragments containing the mutagenized ogt genes were each ligated into the bacterial expression vector pUC8.1 (a gift from J. Brennand, 14). These constructs, which generated fusion proteins consisting of additional 18 amino acids derived from the plasmid encoded β-galactosidase and 5' sequences of the ogt gene (Fig. 3), were transformed into E. coli JM101. Protein expression was induced by addition of 10 mM IPTG to the growth medium at A_{600} 0.6 and incubation continued for a further 90 minutes. ATase activity was assayed prior to and following IPTG induction (Fig. 4).

The specific activity of the ATase produced by the wild-type ogt fusion protein was 3,300 fmole/mg and this was substantially increased to 14,000 following IPTG induction. Substitution of cysteine-102 (protein 1) with a glycine residue resulted in ATase activity comparable to wild-type; 2,800 fmole/mg uninduced and 13,000 fmole/mg following IPTG induction. In contrast, clones 2 and 3, each containing proteins with mutated cysteine-139

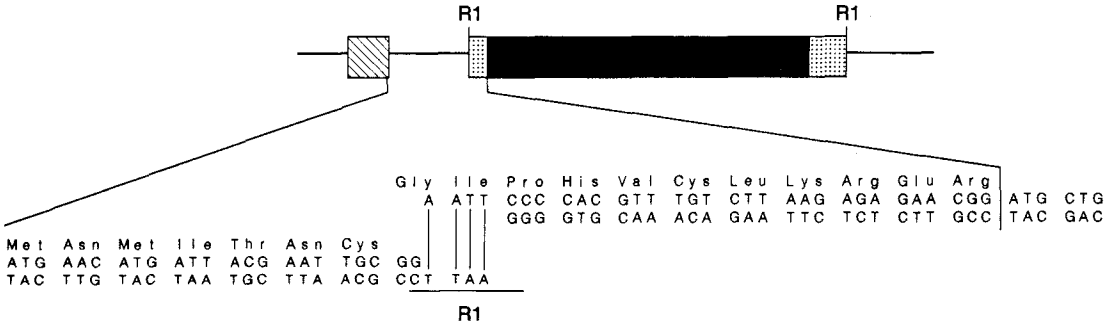

Figure 3

The amino terminus of the fusion protein produced with the ogt ATase when expressed in pUC8.1 from the lacZ promoter. The ogt protein coding sequence is shaded the noncoding sequence is dotted and the lacZ promoter is solid. R1; EcoR1 restriction endonuclease recognition site.

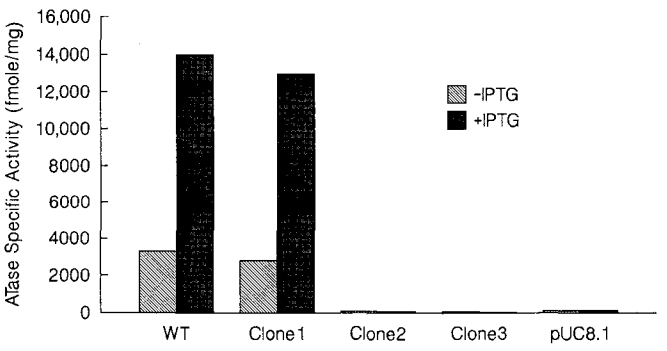

Figure 4

Histogram showing the ATase specific activities of JM101 extracts containing plasmids expressing the wild-type (WT) and mutant ogt proteins. Clone 1 - mutated cysteine 102, clone 2 - mutated cysteine 139, clone 3 - mutated cysteines 102 and 139.

residues, expressed very low levels of ATase; <150 fmole/mg prior to and following induction.

To investigate the possibility that the slightly reduced ATase specific activity of protein 1 was due to a slower rate of action of the mutant ATase enzyme on the substrate DNA, a fixed amount of either wild-type ogt ATase or protein 1 were incubated with excess [^{3}H]-MNU treated DNA for increasing lengths of time in a standard ATase assay. However, no difference in the reaction rates of the two enzymes was seen (Fig. 5), indicating that this was not the reason for the lower ATase activities.

The presence of the wild type and mutated ogt fusion proteins in extracts of JM101 E. coli was confirmed by Western analysis using ogt polyclonal antibodies (Fig. 6). The ogt

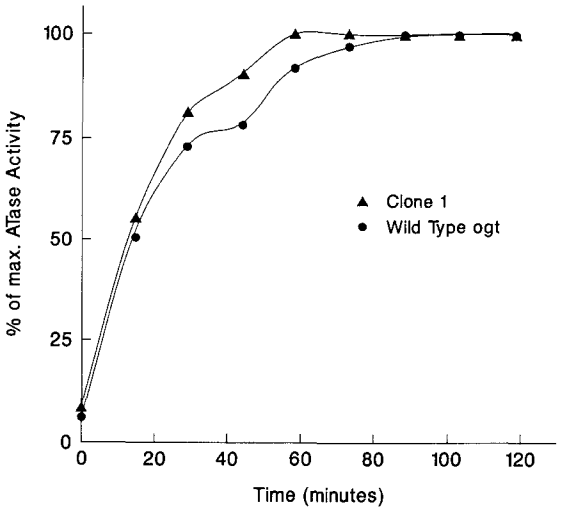

Figure 5

The rate of action of wild-type and protein 1 on [^{3}H]-MNU treated substrate DNA.

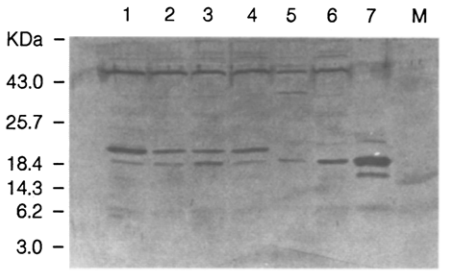

Figure 6

Western analysis of the wild-type and mutagenized ogt proteins probed with anti-ogt polyclonal antibodies. Lanes 1-6 are all extracts of JM101 which had been transformed with different constructs. 1 - Wild-type ogt in pUC8.1; 2 - Clone 1 (mutated Cysteine-102); 3 - Clone 2 (mutated cysteine 139); 4 - Clone 3 (mutated cysteines 102 and 139); 5 - pUC8.1; 6 - JM101 alone; 7 - Double stranded DNA cellulose purified ogt ATase; M - Protein molecular weight markers, not visible.

ATase encoded by the host E. coli ogt gene was represented by a 19 kDa protein band in every lane. A larger 21.5 kDa band corresponding to the plasmid-encoded fusion protein (Fig. 3) was also present in every lane containing extracts of JM101 harboring the pUC8.1-ogt constructs.

Discussion

The results of these site directed mutagenesis studies indicate that cysteine residue 139 is the alkyl-accepting cysteine residue in the ogt ATase. This is not surprising since the protein sequences of ten ATase enzymes are now known and nine contain the same pentameric region of homology (PCHRV), at the active-site (see reference 15 for a recent comparison of all the known ATase sequences except for the Chinese hamster (16)). The cysteine residue within this sequence has actually been confirmed as the alkyl-accepting cysteine residue for the ada (8,17), the human (18) and the mouse (19) ATase enzymes. Substitution of cysteine residue 102 had little effect, if any, on the activity of the repair protein demonstrating that if the two cysteine residues are involved in the formation of a di-sulphide bridge, as suggested in the introduction, its structure is not essential for the removal of O^6-methylguanine from DNA. The heptameric (LRTIPCG) sequence, containing cysteine-102, is unique to the two E. coli enzymes and because these are the only two ATases known to efficiently repair O^4-alkylthymine (12,20), it is possible that this region may be involved in the repair of this adduct.

Acknowledgments

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