Inducible alkyltransferase DNA repair proteins in the filamentous fungus *Aspergillus nidulans*

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ABSTRACT

We have investigated the response of the filamentous fungus *Aspergillus nidulans* to low, non-killing, doses of the alkylating agent MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). Such treatment causes a substantial induction of DNA alkyltransferase activity, with the specific activity in treated cells increasing up to one hundred-fold. Fluorography reveals the two main inducible species as proteins of 18.5kDa and 21kDa, both of which have activity primarily against O⁶-methylguanine (O⁶-MeG) lesions. In addition, two other alkyltransferase proteins can also be detected. One, of MW 16kDa, is expressed in non-treated cells, but is not induced to the same extent as the 18.5 and 21kDa proteins. The other, a protein of 19.5kDa, is highly inducible and can only be detected in treated cells. Unlike the other three proteins, it acts primarily against methyl-phosphotriester (Me-PT) lesions. This is the first instance in which an MePT alkyltransferase has been detected in a eukaryotic organism and, coupled with the high level of induction of the O⁶-MeG alkyltransferase enzymes, this indicates that a control system similar to the bacterial adaptive response may be present in filamentous fungi.

INTRODUCTION

Amongst the widespread battery of cellular responses to stress are a number of highly inducible systems capable of repairing particular types of cell damage. The heat shock response provides a good example of a widely distributed system, appearing in organisms as diverse as *Escherichia coli* and man (reviewed by Lindquist, (1)). Given the universal nature of their substrate, DNA repair processes might also be expected to display a commonality of mechanism, and indeed there are striking similarities in the processes of excision repair and the constitutive repair of alkylation damage in both prokaryotes and eukaryotes. However, in eukaryotic cells, there appears to be no equivalent

to the highly inducible SOS system of *E.coli*, nor is there any reported equivalent of the highly inducible 'adaptive response' to alkylation damage. Instead, there appears to be a general low level induction of many repair enzymes, in response to DNA damage of almost any kind.

The 'adaptive response' in bacterial cells was initially observed following exposure of *E.coli* to sub-toxic levels of alkylating agents, and leads to increased resistance to both the killing and mutagenic properties of such compounds (2; reviewed by Lindahl et al (3)). The gene regulating this response, *ada*, has been cloned and characterized (4,5), and is known to encode a 39kDa protein species with two active sites involved in the repair of alkylated-DNA lesions (6). One active site, located towards the carboxy-terminus, is involved in the repair of alkyl lesions from O⁶-alkylguanine (O⁶-AlkG) and O⁴-alkylthymine (O⁴-AlkT), which are the primary mutagenic products of alkylating agents such as MNNG (7). The second site, located in the amino-terminal domain of the enzyme, is responsible for the removal of alkyl-groups from the S-stereoisomers of alkylphosphotriesters (Alk-PT), lesions which apparently have no deleterious biological effects, but which act to trigger the induction of the repair system.

In each case, the repair of alkyl lesions occurs by transfer of the alkyl group from the DNA to a cysteine residue in the protein. Because of the nature of this reaction, the *ada* protein is designated a DNA alkyltransferase (ATase). Transfer of an alkyl group to the C-terminal site results in the inactivation of the O⁶-AlkG repair capacity of the protein (8), while transfer to the N-terminal site results in conversion of the protein to a strong transcriptional activator (9). The activated protein binds to a conserved sequence, the *ada* box, found associated with the regulatory regions of two genes, *ada* itself, and *alkA*, the gene encoding the inducible 3-methyladenine DNA glycosylase II enzyme (9,10). The *alkA* glycosylase is responsible for the removal of several different alkylation lesions from DNA, including 3-methyladenine and a number of other minor methylated bases. Two other genes, of unknown function, are also part of the adaptive response, namely *aidB* and *alkB* which form small operons with *alkA* and *ada* respectively.

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In addition to the Ada protein, a constitutive O^6 -ATase protein exists in *E. coli*, the product of the *ogt* gene (11). This protein has a molecular weight of 19 kDa and repairs O^6 -AlkG and O^4 -AlkT lesions (12).

The adaptive response has also been extensively studied in the gram positive bacterium *Bacillus subtilis*, which exhibits a range of alkyltransferase proteins similar to those found in *E. coli* (13). The genes encoding these proteins have been cloned and their products characterised as *Dat1*, a 22 kDa constitutive protein analogous to the *Ogt* protein (14); an inducible O^6 -AlkG ATase, *AdaB*, having a molecular weight of 22 kDa, and *AdaA*, a 27 kDa Alk-PT ATase (15). In this organism, the alkylated form of the Alk-PT ATase also promotes increased transcription of the *ada* operon, although the *E. coli ada* box sequence is not conserved (15). Other differences are also evident in the organisation of the *B. subtilis* operon compared to *E. coli*. In *E. coli* the *alkB* gene is found downstream of the *ada* gene (4) and no equivalent gene has been found in *B. subtilis*. The *adaA* and *adaB* genes of *B. subtilis* overlap by 11 bp (15), and no such overlap is observed between *ada* and *alkB* in *E. coli*.

O^6 -ATase activities have been demonstrated in many mammalian species from both tissues and cultured cells (examples are given in (16)), and are all of similar molecular weight (between 20–27kDa; reviewed in (17)). The genes have been cloned for human O^6 -ATase (18) and rat O^6 -ATase (19), and there is a high degree of conservation of sequence at the active sites of the prokaryotic and eukaryotic enzymes. However, only small increases in eukaryotic alkyltransferase activity have been observed following pretreatment with a range of DNA damaging agents (reviewed in (16)) and it is believed that induction of ATase activity in mammalian species does not occur to the high levels that are observed in prokaryotes.

No adaptive response is apparent in the lower eukaryote *Saccharomyces cerevisiae* (20), although a partially inducible 3-methyladenine DNA glycosylase has been identified (21,22), and a constitutive alkyltransferase activity has been isolated from exponentially growing cells (23). This activity has been characterised as being specific for the repair of O^6 -AlkG and O^4 -AlkT lesions; it has a molecular weight of 25 kDa, is heat-labile, having a half life of 4 minutes at 37°C, and is constitutively expressed. Pretreatment of yeast with small quantities of MNNG brought about a decrease rather than an increase in the activity detected in cells (23,24).

An improved growth response in the presence of MNNG has been observed in the filamentous fungus, *Aspergillus nidulans*, following pre-exposure to a non-killing dose (25), and DNA alkyltransferase activity can be detected in this organism (26). We report here that this activity is highly inducible, and can remove alkyl groups from both O^6 -MeG and Me-PT lesions. The presence of both highly inducible alkyltransferase activity, and the activity against alkylphosphotriesters strongly suggests that a control system analogous to the *E. coli* and *B. subtilis* adaptive response may exist in this filamentous fungus.

MATERIALS AND METHODS

Strains

Wild type *Aspergillus nidulans* strain L20 (wA3; *pabaA1*) was used throughout. The organism was cultured at 37°C.

Media

Growth media were as described by Pontecorvo *et al.*, (27) and Clutterbuck (28). MNNG was made as a stock solution at 1

mg/ml in ethanol and stored at –20°C for no longer than two weeks. This solution was added directly to media to the desired concentration.

Preparation of protein extracts

Quantities of mycelium (> 30g) were harvested from 2.5 litres of supplemented MM following growth at 37°C in an orbital incubator for 24 hours. The mycelium was collected by passing the growth medium through a fine nylon mesh and rinsing the mycelium with an equal volume of distilled water. The mycelium was pressed dry, weighed, frozen in liquid nitrogen and then ground to a fine powder, under liquid nitrogen, in a pre-chilled mortar and pestle. The powdered mycelium was added to extraction buffer (Buffer E; 50 mM TRIS-HCl pH 7.5, 1 mM EDTA, 6 mM β -mercaptoethanol) containing protease inhibitors Phenylsulphonylfluoride (PMSF) and Benzamidine at a final concentration of 0.5 mM each. The volume of Buffer E used was approximately 4 ml per gram of pressed dry mycelium. The suspension was kept on ice and stirred constantly for 30 minutes. The mycelial debris was then removed by centrifugation at 18,000 rpm in a Beckman JA20 rotor at 4°C for 30 minutes. The supernatant was retained and the nucleic acids removed from solution by addition of 1/10th volume of 10% streptomycin sulphate (Sigma). The solution was stirred on ice for 30 minutes and the precipitate removed by centrifugation at 14,000 rpm, 4°C for 10 minutes. The supernatant was taken and ammonium sulphate (BDH) added to the solution on ice, over a period of two hours, to a final concentration of 60% saturation (0.36 g/ml). The precipitate was collected by centrifugation at 14,000 rpm, 4°C, for 15 minutes and dissolved in a final volume of 15 ml of Buffer E. This was dialysed against 4 litres of Buffer E overnight. Following dialysis, any precipitate remaining was removed and this crude protein extract (Fraction 1) was assayed to determine O^6 -ATase activity.

DNA cellulose affinity chromatography

1 g of double stranded DNA cellulose (Sigma) was resuspended in Buffer E and packed into a small column (1 cm×15 cm and extensively washed with buffer before use. The protein extract was passed through the column and 2 ml fractions collected. Any

Figure 1. Assay of constitutive alkyltransferase activity in *Aspergillus nidulans* strain L20. **Panel A:** Assay of crude extract (Fraction I) proteins. Increasing volumes of Fraction I extract were incubated with 10μg [3 H]-methylated substrate DNA, as described in Materials and Methods. Concentration of proteins in Fraction was 21.1 mg/ml. ATase specific activity was 1.6 fmol/mg. **Panel B:** Assay of DNA-cellulose pooled fractions (Fraction II). Increasing volumes of Fraction II were assayed as above (○), and activity was compared to that displayed by *E. coli* Ada protein (●), prepared from cells containing the Ada overproducing plasmid pO62HX (36), as described by Potter *et al* (30).

unbound protein was removed by washing with Buffer E until the 280 nm absorbance reading of the filtrate reached background. The alkyltransferase activity bound to the DNA cellulose was removed by passing Buffer E containing 0.25 ml NaCl over the column. The quantity of protein in each fraction was measured by absorbance at 280 nm and fractions containing active alkyltransferase were determined by assaying 50 μ l of each fraction.

Induction of *O*⁶-ATase activity

Two protocols were used for the induction of *O*⁶-ATase activity. A 17 hour induction period was first analysed to approximate the long 20 hour adaptation period found to be optimal for the induction of the adaptive response in *A. nidulans* (25). Due to limitations in growing cultures for greater than 24 hours and achieving the optimum isolation of protein, it was determined that 17 hours was the longest period in which the mycelium could be adapted. A 3 hours exposure to MNNG was also used, as this represented the minimum time in which adaptation of *A. nidulans* mycelium could be demonstrated.

17 hour MNNG induction

L20 conidia were inoculated into 2.5 litres of supplemented MM (10 $\times$ 250 ml flasks) and grown for a period of 7 hours to allow the spores to germinate. An inducing dose of MNNG (final concn. 0.5 μ g/ml) was then added to each flask and incubated for a further 17 hours. The decontamination of any residual MNNG was achieved using a 1/10th volume of 40% sodium thiosulphate solution and the flasks incubated for a further 30 minutes before the mycelium was harvested as described above.

3 hour MNNG induction

Conidia from strain L20 were inoculated into supplemented MM and incubated for 21 hours. MNNG was added to each culture to a final concentration of 0.5 μ g/ml and the mycelium incubated for a further 3 hours. The MNNG was decontaminated as described above.

Figure 2. Assay of constitutive alkyltransferase activity in *A. nidulans* strain L20. Fraction II proteins were assayed with modified substrates containing primarily either *O*⁶-MeG (○) or Me-PT lesions (●). Modified substrates were made by pretreatment of the normal methylated substrate with purified Ada fragments, as described by Potter *et al.* (30), and outlined in the text. Assay conditions were as described in Materials and Methods. The concentration of proteins in Fraction II was 0.21 mg/ml, and specific activity against non-modified methylated substrate was 1152 fmol/mg.

Assay of *O*⁶-ATase activity

Protein extracts were assayed for alkyltransferase activity using the method of Margison *et al.*, (5) with the following modifications: the final reaction volume was made to 950 μ l and the samples were incubated at 37°C for 40 minutes. The reaction was terminated by the addition of BSA (50 μ l of a 20 mg/ml solution) and TCA (400 μ l of a 4 M solution, followed by 1 ml of a 1 M solution).

Preparation of ATase samples for analysis by fluorography

Fraction II protein samples were incubated with 10 μ g substrate DNA. The volume of sample to be labelled was determined from assay as the volume capable of just producing substrate limiting conditions, (indicated in the assay as the maximum quantity of radioactivity to be incorporated into the protein fraction). This ensured that the protein contained a maximal amount of radiolabel in the protein fraction to be resolved by PAGE.

PAGE loading buffer was added to the samples and the samples were loaded directly on to the PAGE gel. Larger sample volumes were reduced under vacuum before the addition of loading buffer.

Polyacrylamide Gel Electrophoresis

Protein extracts were resolved by the SDS-PAGE method of Laemmli (29), using a 4% stacking and 15% resolving gel. All gels were run with cooling. Typical run conditions consisted of 25 mA to allow samples to pass through the stacking portion of the gel and 35 mA whilst samples passed through the resolving section of the gel.

Fluorography

In initial experiments, resolved PAGE gels were electroblotted to nitrocellulose membrane (Hybond-C, Amersham International) using Bio-Rad trans blot equipment. The transfer buffer consisted of 25 mM TRIS, 192 mM glycine, 20% Methanol and transfer was conducted overnight at 40 V. Later experiments involved transfer of proteins to Zeta-probe membrane (Bio-Rad) using Bio-Rad SD-Trans blot equipment. The transfer buffer, when using this particular membrane, did not require the addition of methanol.

Figure 3. Fluorograph of Fraction II proteins from uninduced *A. nidulans* L20, reacted with [³H]-methylated substrate. Assay conditions and fluorography procedures are described in Materials and Methods. The right hand lane contains MW markers of the sizes indicated. Lane 1 contains purified *E. coli ogt* protein, reacted with the substrate, while Lane 2 contains the reacted *A. nidulans* proteins. The *ogt* protein was purified as described by Wilkinson *et al.* (12).

Following transfer, the membrane was dried and soaked in scintillation fluid (Optiphase Safe, LKB), then wrapped in Saran before being exposed against X-ray film at -70°C .

RESULTS

Characterisation of constitutive O^6 -ATase activity

Using soluble enzyme preparations from non-adapted *A. nidulans* mycelium (Fraction I), the specific activity of the constitutive alkyltransferase was observed to be ~ 2 fmol/mg, expressed as the quantity of methyl lesions repaired per milligram of protein (Figure 1A). The small amount of ATase activity present in Fraction I extracts required that partial purification by DNA cellulose affinity chromatography was performed before any further analysis of the activity was possible. Following chromatography, the fractions containing active ATase protein were pooled (Fraction II) and assayed (Figure 1B). Under substrate limiting conditions, a plateau level was achieved that was consistent with the repair of only one alkyl lesion from the substrate DNA. It seemed likely that this would be O^6 -MeG, since all known constitutive ATase activities isolated to date have been involved in the repair of this lesion, for example the *ogt* protein from *E. coli* (11), the *dat1* protein from *B. subtilis* (13), and the MTase protein from *S. cerevisiae* (23). To determine whether only the repair of O^6 -MeG lesions was occurring, Fraction II was assayed with modified substrate DNA (30). These modified substrates were prepared by incubation of normal substrate DNA with purified fragments from the Ada protein. Subsequent recovery of the DNA produced substrate that contained either only O^6 -MeG (incubated with the amino terminal Ada fragment) or only Me-PT lesions (incubated with the carboxyl terminal Ada fragment). Assay of Fraction II derived from uninduced *A. nidulans* with these substrates confirmed that only the repair of O^6 -AlkG lesions was significant (Figure 2).

The molecular weight of the activity present in Fraction II was determined by fluorography. Fraction II protein aliquots were incubated with $10\mu\text{g}$ of normal substrate DNA and the extract resolved on PAGE gels. Two labelled protein species were detected, whose sizes were calculated to be 18 and 16.5 kDa respectively (Figure 3). At present it is not known whether the 16.5 kDa protein is a separate species or if it is a processed form of the 18 kDa protein species. To distinguish between them we

use the prefix *Anat* (*Aspergillus nidulans* alkyltransferase). We designate the 18 kDa protein species as *Anat-1* and the smaller, 16.5 kDa band, *Anat-2*.

Induction of O^6 -ATase activity in *A. nidulans*

Having characterised the constitutive O^6 -ATase activity in *A. nidulans*, the possible induction of this activity was determined following exposure of mycelium in liquid culture to MNNG. Two protocols were used, based upon the observed optimal conditions for adaptation (25).

Adaptation of mycelium with MNNG for 17 hours

Initially, mycelial cultures were induced by exposure to low doses of MNNG for seventeen hours. Assay of Fraction I extracts indicated that an approximately 3 fold increase in specific activity, to 6 fmol/mg had occurred (data not shown). The ATase activity in the MNNG induced Fraction I was partially purified by DNA affinity chromatography. Assay of the pooled active fractions (Fraction II) with modified substrate DNA indicated that the repair of only O^6 -MeG lesions was occurring (Figure 4). To determine if the induction procedure had resulted in the production of new ATase protein species or up-regulation of any existing species, the extract was subjected to analysis by

Figure 5. Fluorograph of Fraction II proteins from 17hr adapted *A. nidulans* L20, reacted with $[^3\text{H}]$ -methylated substrate. Assay conditions and fluorography procedures are described in Materials and Methods. Lane contains MW markers of the sizes indicated. Lane 2 contains Fraction II proteins prepared from non-adapted mycelium, Lane 3 the proteins from the adapted mycelium. The autoradiogram was exposed for a relatively short period of two weeks.

Figure 4. Assay of Fraction II proteins prepared from L20 mycelium exposed to an adapting dose for 17hrs. The activity was assayed with modified substrates containing primarily either O^6 -MeG ($\circ$) or Me-PT lesions ($\bullet$). The Fraction II preparation had a protein concentration of 0.45 mg/ml and a specific activity of 769 fmol/mg against unmodified methylated substrate.

Figure 6. Comparison of alkyltransferase activity in Fraction I proteins prepared from non-adapted L20 mycelium ($\bullet$) or mycelium adapted for a period of three hours ($\circ$). Assay conditions, using standard non-modified substrate, are described in Materials and Methods. Protein concentrations of the Fraction I preparations were 21.1 mg/ml and 19 mg/ml respectively. Calculated specific activities are 1.6 fmol/mg, non-induced, and 186 fmol/mg, induced.

fluorography (Figure 5). This indicated that a new protein species was present whose molecular weight was approximately 21 kDa (*Anat-3*). This species could represent the production of an entirely novel protein species, or could be a differently processed form of any of the previously identified proteins. The short exposure of this fluorograph, approximately 2 weeks, indicated that *Anat-2* was absent. However, following longer exposure (2 months) this protein species was detected (results not shown). The altered ratio of the *Anat-1* and *Anat-2* proteins in the 17-hour adapted extracts may indicate that *Anat-2* is fully constitutive in its expression, and the 18 kDa *Anat-1* species is inducible over a high constitutive basal level. Less likely is the possibility that reduced quantities of *Anat-2* are produced following adaptation.

Adaptation of mycelium with MNNG for 3 hours

The effect of a much shorter exposure time of mycelium to MNNG was next determined. The assay of Fraction I proteins from 3 hour-adapted mycelium revealed that a large increase in activity had occurred (Figure 6). The specific activity of this

Figure 7. Assay of Fraction II proteins of *A. nidulans* L20 prepared from 3-hour adapted mycelium. Modified substrates were used containing primarily either O⁶-MeG (○) or Me-PT lesions (●). Details of the assay and substrate preparation are given in Materials and Methods. Protein concentration of the Fraction II preparation was 0.49 mg/ml, and specific activity against non-modified methylated substrate was 5594 fmol/mg.

Figure 8. Fluorograph of Fraction II proteins from three hour adapted *A. nidulans* L20, reacted with [³H]-methylated substrate, as described in Materials and Methods. The right lane contains MW markers of the sizes indicated. Lane 1 contains *E. coli* Ada protein, prepared as described in the legend to Figure 1. Both the full size (38kD) and processed fragments (~18kD) can be seen. Lane 2 contains the *A. nidulans* Fraction II proteins, of 21kDa, 19.5kDa, 18kDa and 16.5kDa. The diffuse band at ~14kDa is assumed to represent degradation products.

extract was calculated to be 186 fmol/mg, an approximately 100 fold increase in alkyltransferase activity compared to uninduced extracts. In a duplicate experiment, a specific activity of 210 fm/mg was obtained. The plateau level reached with the three-hour-adapted extracts was significantly greater than that achieved with either uninduced or seventeen-hour-adapted extracts, and was consistent with the repair of more than one type of lesion. One obvious explanation would be the repair of significant quantities of Me-PT lesions in addition to the repair of O⁶-MeG.

The assay of pooled Fraction II proteins, following affinity chromatography of the 3 hour MNNG induced extracts, indicated that this apparent dual activity was retained in the fraction bound to the column (data not shown).

When these Fraction II samples were subjected to assay with modified substrate DNA, the results indicated that indeed both O⁶-MeG and Me-PT lesions were being repaired (Figure 7). The quantity of Me-PT lesions repaired was not quite as great as that achieved by the N-terminal region from the Ada protein (not shown) but was still highly significant. This may reflect an as yet uncharacterized variation in substrate specificity compared to the Ada protein.

Having demonstrated that repair of Me-PT lesions was occurring, Fraction II proteins were analysed by fluorography (Figure 8). The resulting fluorograph indicated that in addition to the ATase proteins already characterised (*Anat-1* to -3), another protein species of approximately 19.5 kDa was present in the 3-hour induced sample (*Anat-4*).

To determine if this new protein species was responsible for the repair of Alk-PT lesions, Fraction II proteins were prepared for fluorography by incubation with both types of modified substrate DNA. The fluorograph, shown in Figure 9, clearly demonstrates that the repair of O⁶-AlkG lesions was effected by the proteins *Anat-1* to -3 and that Me-PT lesions were repaired primarily by the *Anat-4* protein.

DISCUSSION

We have demonstrated that treatment of *A. nidulans* with low doses of MNNG for a period of three hours results in an increase in alkyltransferase activity by approximately one hundred fold. Uninduced cells contain a low level of activity (~2fm/mg in crude extracts), which acts against O⁶AlkG lesions. Two distinct protein species (*Anat-1* and *Anat-2*) can be shown to be

Figure 9. Fluorograph of Fraction II proteins from three-hour adapted *A. nidulans* L20 reacted with unmodified [³H]-methylated substrate (Lane 2); modified substrate containing primarily O⁶-MeG lesions (Lane 3), and modified substrate containing primarily Me-PT lesions (Lane 4). Lane 1 contains MW markers of the sizes indicated.

responsible for this activity, having molecular weights of approximately 16.5 and 18kDa respectively.

In cells exposed to low doses of MNNG for three hours, the specific activity of the alkyltransferases in crude extracts rises to ~200 fm/mg, and activity against both O⁶-MeG and Me-PT lesions can be detected. Four protein species have been identified in these extracts of treated cells as having alkyltransferase activity. These include the *Anat-1* and *Anat-2* proteins identified in non-induced cells, and also two others, *Anat-3* and *Anat-4*, which have molecular weights of 21 and 19.5kDa respectively. The *Anat-3* and *Anat-4* species were not detected in non-treated cells, and are therefore presumed to be highly inducible species, present only in small quantity unless the cells are damaged by alkylating agents. The molar ratio of the 16.5 and 18kDa species was also substantially altered in the treated cells, with the 18.5kDa species being present in considerable excess. In untreated cells, these two proteins appear to be present in approximately equal amounts, as judged by the density of the bands in the fluorograph. This suggests very strongly that expression of the 18kDa *Anat-1* species is greatly increased following MNNG treatment, while expression of the 16.5kDa *Anat-2* species is increased much less, if at all. In induced cells, the quantities of the 18.5kDa *Anat-1* and the 21kDa *Anat-3* proteins appeared to be approximately equal, as judged by the density of the fluorograph bands.

When tested against specific substrates containing primarily O⁶-MeG or Me-PT lesions, we obtained clear evidence that the enzyme preparations from induced cells had significant activity against methyl phosphotriesters. Fluorography revealed that this activity was primarily associated with the inducible 19.5kDa *Anat-4* species, whilst the other three proteins had activity only against O⁶-MeG lesions. The efficiency of removal of Me-PT lesions was rather less than that displayed by the *E.coli ada* protein, and this may indicate somewhat different substrate requirements for the two enzymes. The ability to detect the *Anat-4* Me-PT alkyltransferase was particularly sensitive to the induction period. If exposure to the low levels of MNNG was continued for seventeen hours rather than three, we observed a low level of induction of total transferase activity (~3fold). Analysis of the proteins involved revealed induction of *Anat-1* and *Anat-3*, but no *Anat-4* could be detected. It seems possible that the failure to detect this species under these circumstances simply reflects the complete inactivation of the protein in the presence of excess substrate.

The coincident occurrence of highly inducible alkyltransferase enzymes and the presence of methylphosphotriester alkyltransferase activity provides a strong indication that a control system similar to that found in prokaryotes may also exist in filamentous fungi. The key step to the induction of the adaptive response in both *E.coli* and *B.subtilis* is the activation of the positive control protein by the transfer of a methyl group from methyl phosphotriesters. In *E.coli*, this involves one of the two active sites on the Ada protein, while in *B.subtilis*, the O⁶-MeG and Me-PT alkyltransferase activities reside in quite separate proteins. Clearly, the situation in *Aspergillus* resembles more closely the *B.subtilis* system rather than *E.coli*, since the Me-PT alkyltransferase appears distinct from the O⁶-MeG activity. The possibility cannot be excluded that the 19.5kDa *Anat-4* is a processed form of the 21kDa *Anat-3* protein. However, if this were the case, the *Anat-3* protein would also be expected to show significant activity against Me-PT lesions, and this is not so. A further similarity with the *B.subtilis* system lies in the observation that the Me-PT alkyltransferase is produced in rather smaller

amounts than the O⁶-MeG alkyltransferase (13). In *B.subtilis*, the explanation for this phenomenon lies in the fact that transcription of *adaB* initiates within the *adaA* gene, resulting in the production of higher levels of AdaB than AdaA protein. It is possible that a similar mechanism exists in *A.nidulans*.

A conundrum still exists as to why some eukaryotic organisms should have a highly inducible adaptive response, while others do not. In fact, a parallel situation also exists amongst the prokaryotes. The great majority of bacterial species tested to date all appear to contain an inducible adaptive response, while just a small number do not (reviewed by Sedgwick and Vaughan, (31)). The bacterial species with the most dramatic adaptive response are however all soil-living organisms, namely *Pseudomonas aeruginosa*, *Xanthomonas maltophilia*, and *Bacillus subtilis* (32,33). *B.subtilis* also has a very high level of constitutively expressed DNA alkyltransferase, and it seems clear that all of these soil living organisms must require such protection mechanisms because they are routinely exposed to high levels of environmental alkylating agents. The precise nature of such agents is difficult to determine, but a number of obvious candidates have been suggested and include antibiotics such as streptozotocin, produced by *Streptomyces achromogenes* (34,35). Other possibilities include methylurea and methylnitroguanidine, which occur in decaying matter and can be activated as methylating agents by nitrosation, and also methyl chloride which is produced in large quantities, both by biomass burning and by biological synthesis by a number of microorganisms, including fungal species (reviewed by Sedgwick and Vaughan, (31)).

Given that the filamentous fungi usually exploit a soil habitat, it would perhaps be expected that these eukaryotes would have a high requirement for an extremely efficient mechanism for the repair of alkylation damage. Whether they are the only eukaryotes to possess such a system remains to be determined, but clearly other soil-dwelling species should warrant further study.

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