

In Vitro Proteolytic Cleavage of the *Escherichia coli* Ada Protein by the *ompT* Gene Product

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Down regulation of the adaptive response to alkylation damage in *Escherichia coli* has been proposed to occur by proteolytic cleavage of the regulatory Ada protein. In this paper, it is shown that proteolysis of the Ada protein as observed in cell extracts is caused by the *ompT* gene product. This protease, however, was not involved in switching off the adaptive response in vivo.

The Ada protein of *Escherichia coli* regulates the expression of at least four genes, *ada*, *alkA*, *alkB*, and *aidB*, which are induced by exposure of cells to methylating agents (11). Two of the above-mentioned gene products are known to repair deleterious covalently alkylated lesions in DNA and so protect the cells against the mutagenicity and toxicity of alkylating agents. The regulatory Ada protein itself repairs the highly mutagenic lesions, *O*⁶-methylguanine and *O*⁴-methylthymine, and also an innocuous lesion, the S stereoisomer of methylphosphotriesters. The protein transfers the methyl groups from these adducts onto two of its own cysteine residues, cysteine 69 and cysteine 321 (3, 18). Self-methylation of the Ada protein at cysteine 69 by repair of a methylphosphotriester converts it into a strong positive activator of gene expression (21). This methylated protein binds to the promoters of the inducible genes and stimulates their transcription (14, 21).

The existence of a mechanism for switching off the adaptive response is uncertain. The methylated Ada protein is not actively demethylated, and there is no genetic evidence for a repressor of the response. The methylated Ada protein may be simply diluted out by growth after removal of the alkylating agent. However, the 39-kilodalton (kDa) Ada protein is sensitive to cleavage by an endogenous proteolytic activity in cell extracts (22). Two major proteolytic fragments are formed, an N-terminal polypeptide of 20 kDa and a C-terminal polypeptide of 19 kDa. Both fragments remain active in DNA repair (18). The N-terminal fragment, self-methylated at cysteine 69, can bind to the promoter of the *ada* gene in vitro but is unable to induce its transcription (17a). Proteolysis of the Ada protein, or its methylated form, has therefore been proposed as a possible means of down regulating the adaptive response (for a review, see reference 11). Competition of the methylated N-terminal fragment with any remaining intact methylated protein in binding the promoters of the inducible genes would accelerate their switch off. Indeed, synthesis of N-terminal fragments of the Ada protein from recombinant plasmids has been observed to inhibit the induction of the adaptive response (10, 19). Evidence of proteolytic cleavage of the methylated Ada protein in vivo would add support to this hypothesis. Cleavage of the unmethylated Ada protein occurs readily after cell lysis but has not been observed in living cells (20, 22).

The proteolytic activity which degrades the Ada protein in cell extracts has not been identified. It is known not to be the product of the *hflAB*, *lon*, or *recA* genes, which are associated with proteolytic activities, or of the Ada-regulated *alkB* or *aidB* genes, which have unknown functions (22). The

product of the *ompT* gene, an outer membrane protease, degrades the *E. coli* ferric enterobactin receptor (FepA protein) and T7 RNA polymerase after cell lysis (6, 15). This protease has some properties in common with the activity which degrades the Ada protein, namely, association with the cell membrane fraction, resistance to a range of protease inhibitors, and cleavage of proteins adjacent to lysine residues (6, 8, 22).

To determine whether degradation of Ada is due to the *ompT* gene product, proteolysis was examined in cell extracts of two *ompT* deletion mutants, *E. coli* K-12 UT5600 and *E. coli* B BL21, and their parental strains, *E. coli* K-12 UT2300 and *E. coli* B B707 (4, 16) (all strains were a kind gift from J. Grodberg). The cells from 20-ml exponential cultures (A_{600} of 0.5) were harvested and sonicated in 160 μ l of extraction buffer (70 mM Tris hydrochloride [pH 8], 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol) (22). The crude cell extracts were standardized to 11 mg of protein per ml. Samples (5 μ g) of purified Ada protein (13) were added to 20 μ l of cell extract and incubated at 25°C. At specified times, 50 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 2 mM EDTA, 5% β -mercaptoethanol, 10% glycerol, and bromophenol blue were added. The samples were heated at 95°C for 10 min. The Ada protein and its proteolytic fragments were resolved on a 15% polyacrylamide-sodium dodecyl sulfate gel. The polypeptides were detected immunologically by Western blotting (immunoblotting) (9, 23). The nitrocellulose membrane was probed with two anti-Ada monoclonal antibodies which interact with the two major proteolytic fragments of the Ada protein, the 20- and 19-kDa domains. Isolation of these monoclonal antibodies will be described elsewhere (J. Hall and D. Lane, unpublished data). After washing, the blots were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO). The protein bands were visualized by incubation with the peroxidase substrates 4-chloro-1-naphthol and hydrogen peroxide.

In extracts of the wild-type strains, *E. coli* K-12 UT2300 and *E. coli* B B707, the major proteolytic fragments detected by immunoblotting were the 20-kDa N-terminal and 19-kDa C-terminal domains (Fig. 1A, lane 5, and Fig. 1B, lanes 2 and 6) (18). The 20-kDa fragment was more susceptible than the 19-kDa fragment to further cleavage. After incubation for 19 h at 25°C, the 20-kDa fragment was observed in small amounts in the *E. coli* B cell extract but was completely degraded in the *E. coli* K-12 cell extract (Fig. 1A, lanes 3 and 5). This suggests that the proteolytic activity was greater in the *E. coli* K-12 strain. Little proteolysis of the Ada protein

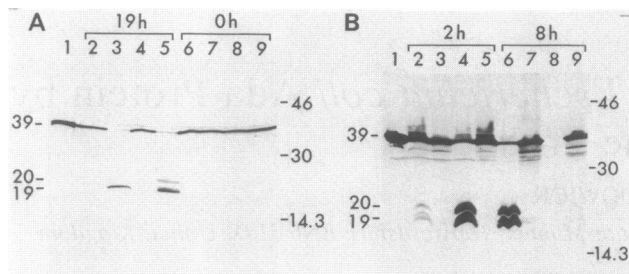


FIG. 1. Proteolytic cleavage of the Ada protein in cell extracts of strains which lack or overproduce the OmpT protease. Ada protein was added to cell extracts of various strains and incubated at 25°C. (A) The strains used were UT5600 (*E. coli* K-12 Δ ompT) (lanes 2 and 6), UT2300 (lanes 3 and 7), BL21 (*E. coli* B Δ ompT) (lanes 4 and 8), and B707 (lanes 5 and 9). Incubations were for 0 and 19 h as indicated above the lanes. (B) The strains were UT2300 (lanes 2 and 6), UT5600 (Δ ompT) (lanes 3 and 7), UT5600(pML19) (lanes 4 and 8), and UT5600(pML19 Δ sma) (lanes 5 and 9). Incubations were for 2 and 8 h as indicated above the lanes. Lane 1 contained the Ada protein alone. The molecular mass markers were Amersham rainbow markers with a range from 14,300 to 200,000 kDa. The Ada protein fragments were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected immunologically by using two anti-Ada monoclonal antibodies which interact with the 20- and 19-kDa domains of the Ada protein.

was observed in extracts of the two *ompT* deletion mutants, *E. coli* K-12 UT5600 and *E. coli* B BL21, even after incubation for 19 h at 25°C (Fig. 1A, lanes 2 and 4). These strains have large deletions, and it was therefore necessary to verify that the lack of proteolytic activity was due to deletion of the *ompT* gene and not an adjacent gene. Proteolysis of the Ada protein was thus examined in the deletion mutant UT5600 transformed with the hybrid plasmid, pML19 or pML19 Δ sma (these plasmids were constructed by M. Lundrigan and obtained from J. Grodberg). The plasmid pML19 is a derivative of pUC19 containing a 2-kilobase insert of *E. coli* DNA carrying the *ompT* gene. The plasmid pML19 Δ sma has a 300-base-pair deletion from within the *ompT* coding sequence (5, 6). This deletion greatly reduces the plasmid-encoded protease activity, although it does not completely eliminate it (6). The Ada protein was readily cleaved in cell extracts of UT5600(pML19); in fact, after 8 h at 25°C even the 19-kDa domain had been completely degraded (Fig. 1B, lanes 4 and 8). This extensive cleavage was greater than that observed in extracts of the wild-type strain, UT2300 (Fig. 1B, lane 6), and was presumably due to overproduction of the outer membrane protease from multiple copies of the *ompT* gene. T7 RNA polymerase is also very sensitive to degradation in extracts of strains carrying pML19 (6). In extracts of UT5600(pML19 Δ sma), there was little or no degradation of the Ada protein (Fig. 1B, lane 9). These observations show that the proteolytic degradation of Ada protein in cell extracts is caused by the *ompT*-encoded protease.

To determine whether the OmpT protease plays a role in down regulating the adaptive response *in vivo*, the induction and decay of the response were compared in strains UT2300 and UT5600 (Δ ompT). Expression of the adaptive response was monitored by measuring the autoregulated *ada* gene product. The Ada protein was assayed at intervals during the induction period and for several hours after removing the inducing agent. The response was induced by exposing exponential cultures (A_{540} of 0.1) grown in M9 minimal salts medium and 0.1% Casamino Acids at 37°C to 1 μ g of

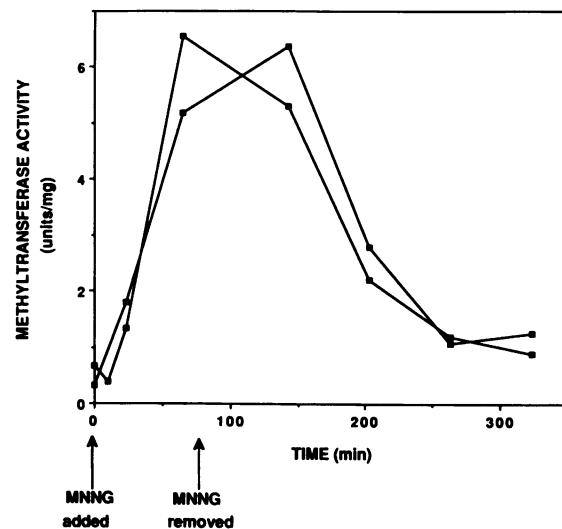


FIG. 2. Induction and decay of O^6 -methylguanine-DNA methyltransferase activity in strains UT2300 (□) and UT5600 (Δ ompT) (■).

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) per ml for 1 h. To remove the inducing agent, the cultures were centrifuged and resuspended in fresh growth medium. At hourly intervals, the cultures were diluted twofold to allow exponential growth to continue. Also, at intervals, 10-ml culture samples were centrifuged, washed in phosphate-buffered saline, and suspended in 100 μ l of extraction buffer. The cells were lysed by sonication and the cell debris was removed by centrifugation (23). The Ada protein was assayed by monitoring the demethylation of O^6 -methylguanine in [H^3]MNNG-treated DNA (10^4 cpm per pmol of O^6 -methylguanine) as described previously (2).

The Ada protein was induced to the same cellular levels, and after the removal of MNNG, the cellular levels decreased at the same rates in the wild-type and *ompT* deletion strains (Fig. 2). The OmpT protease is, therefore, not required for down regulation of the adaptive response. After 3 h, the methyltransferase activity returned to its noninduced level, which accounts for the previously observed decay of cellular resistance to the mutagenicity of MNNG (17).

The *E. coli* UvrB protein is also cleaved in cell extracts by a heat-inducible endogenous protease (1, 7). The cleavage sites in the UvrB and Ada proteins are preceded by a short sequence of homology, and it has been suggested that they are cleaved by the same enzyme (22). The OmpT protein is, in fact, synthesized in a temperature-dependent manner (12) and may therefore be the heat-inducible activity which cleaves the UvrB protein. The sites cleaved in Ada and UvrB occur between lysine-glutamine and lysine-alanine residues, respectively (22). The prevalent OmpT cutting sites in T7 RNA polymerase occur between consecutive basic residues, lysine-arginine or two lysine residues (6). The T7 RNA polymerase cleavage sites do not have homology with the Ada and UvrB cleavage sites. The specificity of the OmpT protease may therefore be less restrictive than previously suggested and it is possibly a trypsinlike enzyme in that it cleaves adjacent to lysine and arginine residues. Cleavage of the Fep protein by "protein a," now known to be OmpT, is in fact inhibited by benzamide, a trypsin inhibitor (8, 15). Cleavage of the Ada protein by the OmpT protease occurs within an exposed region which is very

sensitive to a number of reagent proteases, including trypsin (18). The limited digestion of Ada protein by a low OmpT activity may therefore be due to this structural feature. The Ada protein was, in fact, degraded extensively in cell extracts containing higher OmpT activity (Fig. 1B).

Degradation of the Ada protein, the Fep protein, and T7 RNA polymerase by the OmpT protease on cell lysis has caused considerable difficulties in purifying these proteins intact particularly because of the resistance of the protease to standard protease inhibitors. Initial attempts to isolate the Ada protein resulted in the purification of the active C-terminal domain (2). The *ompT* deletion mutants may therefore prove to be excellent strains for use in the purification of proteins which show instability on cell lysis.

In this paper, it is shown that the OmpT outer membrane protease which cleaves the Ada protein *in vitro* is not involved in down regulation of the adaptive response *in vivo*. In fact, proteolysis of the unmethylated Ada protein has not been observed *in vivo* up to 3 h after its induction (unpublished data of immunoblotting experiments; 20). It is still possible that a proteolytic activity, other than OmpT, which specifically cleaves the methylated form of the Ada protein could be involved in a mechanism of down regulation of this response.

ADDENDUM IN PROOF

Recently, the heat-inducible UvrB protease has been found to be absent in *ompT* deletion mutants (P. R. Caron and L. Grossman, *Nucleic Acids Res.* **20**:9641–9650, 1988; and personal communication).

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