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Processing of Ada protein by two serine endoproteases Do and So from *Escherichia coli*

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Two soluble serine proteases Do and So from *Escherichia coli* were found to distinctively cleave the purified, 39 kDa Ada protein into fragments with sizes of 12–31 kDa. Protease So appears to generate a C-terminal 19 kDa polypeptide, similarly to OmpT protease. In addition, the purified 19 kDa C-terminal half of Ada protein can be further processed mainly to an 18 kDa fragment by protease So and to a 12 kDa by protease Do. These results suggest that proteases Do and So are involved in endogenous cleavage of Ada protein, which may play a role in down-regulating the adaptive response to alkylating agents.

Adaptive response; Ada protein; Protease Do; Protease So

1. INTRODUCTION

The adaptive response protects *Escherichia coli* from the mutagenic and toxic effects of alkylating agents through the induction of a DNA repair system [1]. The inducible Ada protein acts as a DNA repair enzyme and also as a transcriptional activator of the expression of its own *ada* gene [2,3]. Ada protein accepts a methyl group from O^6-methylguanine-DNA onto its cysteine residue at position 321 (Cys-321) [4]. This enzyme also repairs phosphotriesters in methylated DNA by accepting the methyl group onto its Cys-69 [4]. This methyl transfer converts Ada protein from a weak to a strong activator of transcription [5].

The 39 kDa Ada protein has been found to be readily cleaved in cell extracts or during purification into fragments with discrete sizes ranging from 16 to 27 kDa [5–7]. Efforts have been made to search for endogenous proteolytic enzyme(s) responsible for the processing of Ada protein. Proteases cleaving Ada protein have recently been isolated from both soluble and membrane fractions of *E. coli* [8–10]. However, processing of Ada protein with purified, soluble protease(s) has not yet been demonstrated. *E. coli* is known to contain 9 distinct soluble endoproteases, which are named Do, Re, Mi, Fa, So, La, Ti, Pi and Ci [11–16]. In this study, we have systematically examined the ability of these various proteases to cleave Ada protein. Two cytosolic enzymes, proteases Do and So [11,13], appear particularly active in this reaction, and the nature of this process is discussed.

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2. MATERIALS AND METHODS

[^{3}H]Methylated DNA was prepared by treating *Micrococcus luteus* DNA with [^{3}H]*N*-methyl-*N*-nitrosourea, and the methylated DNA was free of N-alkylated purines by heat treatment [17]. Intact and a 19 kDa C-terminal fragment of Ada protein were purified according to the procedure described by Nakabeppu et al. [18] but using *E. coli* K12 cells harboring plasmid pCJ24 that contains the structural *ada* gene under the *lac* promotor [19]. Proteases Do, Re, So, La and Ti were purified as described previously [11–15]. Peak II (a mixture of proteases Mi, Fa and Pi) and peak V (containing protease Ci) were obtained as described by Goldberg et al. [16].

The activity of O^6-methylguanine-DNA methyltransferase was assayed as described by Demple et al. [4]. Reaction mixtures contained 4 μg of the purified Ada protein and 10 μg of [^{3}H]methylated *M. luteus* DNA in 50 mM Tris-HCl (pH 7.8) containing 10 mM dithiothreitol, 1 mM EDTA, 5% glycerol and 50 μM spermidine. Proteolytic processing of Ada protein was assayed by incubating the reaction mixtures with 4 μg of Ada protein and proper amounts of purified or partially purified *E. coli* proteases in the same buffer. After incubating them for appropriate periods at 37°C, the reaction was terminated by adding sodium dodecyl sulfate (SDS) to give a final concentration of 2% (w/v). The samples were electrophoresed in the presence of SDS in 12.5% (w/v) polyacrylamide gels as described by Laemmli [20]. The cleavage products were visualized by silver-staining [24].

3. RESULTS

When protease Do, a multimeric enzyme with an usually high molecular weight [11], was incubated with the purified Ada protein, a number of fragments with discrete sizes were generated (fig.1A). Early in the incubation, fragments of about 30 kDa appeared. When

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Fig.1. Limited digestion of Ada protein by proteases Do (A) and So (B). Ada protein (4 μg) was incubated with each protease for various periods at 37°C. After the incubation, samples were added with 2% SDS and electrophoresed as described in the text. Protein bands were visualized by silver staining. For some unknown reason(s), both proteases were stained very poorly. Lanes a and f, 3.5 μg each of Do and So, respectively; b and g, Ada only; c–e and h–j, Ada incubated with 1 μg each of Do and So, respectively, for 30, 60 and 120 min.

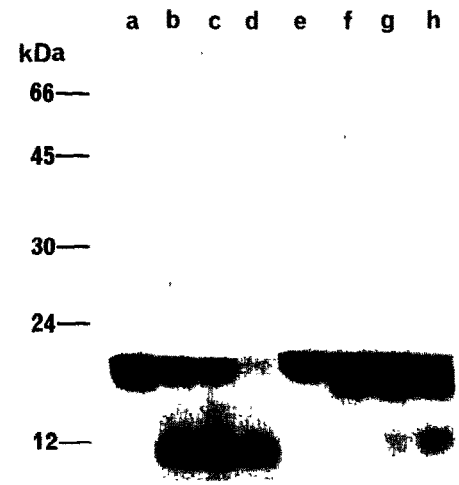

Fig.2. Cleavage of the 19 kDa C-terminal half of Ada protein by proteases Do and So. Limited digestion of the C-terminal polypeptide (4 μg) was measured as in fig.1. Lanes a and e, 19 kDa fragment alone; b–d and f–h, the fragment incubated with Do and So, respectively, for 30, 60 and 120 min.

increasing the reaction period, the larger polypeptides disappeared with concomitant accumulation of 23 kDa fragments and smaller fragments of 12–16 kDa. Protease So, a dimeric enzyme containing a 77 kDa subunit [13], also digested Ada protein at a number of sites (fig.1B). This enzyme generated major fragments of 29 kDa, 19 kDa, and 18 kDa, all of which accumulate upon increasing the incubation period. Thus, the cleavage patterns produced by these enzymes appear distinct from each other. Little or no hydrolysis of Ada protein was observed by other proteases in *E. coli* [12,14–16], including the ATP-dependent proteases La and Ti.

To examine if the limited digestion by protease Do or So results in the loss of O^6-methylguanine transferase activity or if it can generate active C-terminal fragment, Ada protein was incubated at 37°C for 2 h with each protease as described in fig.1. After terminating the proteolysis by the addition of diisopropylfluorophosphate (10 mM) [11,13], the cleavage products were assayed for their methyltransferase activity. The digests by the proteases remain almost fully as active as intact Ada protein (table 1).

Table 1

Comparison of intact and a C-terminal fragment of Ada protein on O^6-methylguanine transferase activity

Treatments	% Relative activity by	
	39 kDa Ada protein	19 kDa C-terminal fragment
None	100	100
Protease Do	87	68
Protease So	99	96

The proteins (4 μg each) were incubated for 2 h with 1 μg of protease Do or So as in fig.1. After terminating the proteolytic reaction by adding DFP to 10 mM, the methyltransferase activity was measured by incubating with [^{3}H]methylguanine-DNA for 30 min at 37°C. The activities seen without the proteases were expressed as 100%

The C-terminal 19 kDa fragment produced during purification [21,22] was isolated and tested for its susceptibility to protease Do or So. As shown in fig.2, protease Do accumulated a 12 kDa fragment while protease So generated an 18 kDa polypeptide. The latter peptide was further processed to a 13 kDa fragment upon longer incubation. These results show that the cleavage specificities of the proteases are distinct from each other. Under similar conditions, the 18 and 13 kDa fragments remained fully active and the 12 kDa peptide showed low but significant activity in O^6-methylguanine repair (table 1).

4. DISCUSSION

Processing of Ada protein appears to be catalyzed not by a single specific protease but by a number of different proteolytic enzymes. Caron and Grossman [9] have demonstrated that detergent extracts of *E. coli* membranes are capable of cleaving Ada protein into 24, 20, 19 and 15 kDa fragments. More recently, Sedgwick [10] has clearly shown that OmpT outer membrane protease is responsible for the cleavage of Ada protein. However, proteases Do and So appear to differ from the membrane protease because they are present in cytosol. Sekiguchi and his colleagues [8] have also isolated a soluble protease that can specifically cleave Ada protein. This activity appears very similar by many criteria to that of protease Do [11]. For example, they

both have a high molecular weight, both have slightly alkaline pH optima, and they are sensitive to inhibition by *p*-hydroxymercuribenzoate. Although some differences also exist in the effect of phenylmethylsulfonylfluoride (PMSF) and in the elution from an ion exchange column of the two activities, these discrepant results were not obtained under similar conditions. For example, inhibition by PMSF requires prolonged preincubation. Thus, it seems likely that these two enzyme activities are identical.

Proteolytic cleavage of Ada protein has been suggested as a possible mechanism for negative modulation of the adaptive response [1]. In this model, the methylated N-terminal fragment produced by a cellular protease binds to the *ada* promotor but is unable to activate transcription, and therefore can compete with the activated Ada protein in binding to the promotor sequences. Involvement of OmpT protease in down regulation of the adaptive response has been excluded because the cellular level of the protease falls after the removal of the alkylating agent at the same rates in wild type and *ompT* deletion strains [10]. Instead, proteases Do and/or So may be associated with the switch-off mechanism. It is possible that N-terminal fragments generated by the proteases are methylated and then act as competitors. N-terminal fragments as little as 8 kDa have been found to retain significant activity in methylphosphotriester repair [23,24]. It is also possible that the methylated N-terminal fragments can directly be produced by the action of protease Do or So. We have recently found that the singly methylated Ada protein at its N-terminal domain also is a good substrate for both proteases (unpublished).

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