Bacteriophage T4 ADP-ribosyltransferase ModA: A mutational analysis

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There is increasing experimental evidence that ADP-ribosylation of host proteins is an important means of regulating the gene expression of bacteriophage T4. Phage T4 codes for three ADP-ribosyltransferases, ModA being one of them. The mode of action of the ADP-ribosylation catalyzed by ModA remains to be investigated. A mutational analysis of ModA showed that ADP-ribosyltransferase activity of this protein is not limited by two known amino acids. Four other amino acids have a potential role in protein function, allowing ADP-ribosylate a number of *E. coli* proteins.

Key words: bacteriophage T4, ADP-ribosyltransferase, ModA

INTRODUCTION

Mono-ADP-ribosyltransferases originally had been discovered as the pathogenic principle of diphtheria, cholera, pertussis and other bacterial toxins. A part of bacterial toxins seem to be encoded as pathogenic factors on the genomes of lysogenic phages incorporated into DNA of their host bacteria [1].

The phage T4 gene *modA* was cloned, sequenced and the amino acid sequence of the gene product, ADP-ribosyltransferase ModA, was deduced [2, 3]. The reading frame codes for a polypeptide with a molecular mass of 23.3 kDa [2]. Synthesized in the early T4 infection cycle, ModA ADP-ribosylates the RNA polymerase α subunit [4, 5]. Recent *in vitro* studies have shown that ModA additionally ADP-ribosylates 70 kDa protein and performs autoribosylation reaction [3].

Comparison with other ADP-ribosyltransferases [6, 7] showed that ModA belongs to this enzyme family and can be threaded into the catalytic domains of ADP-ribosylating bacterial toxins displaying the expected catalytic residues [2, 3, 8] (Fig. 1). It was assumed that Arg72 located in the Trp-Arg motif of the T4 ModA amino acid sequence should be equivalent to active site Arg residue of bacterial toxins. The Glu-Gln-Glu motif represents a glutamic residue at position 165 which should play a similar pivotal role for catalysis.

Here we present a mutational analysis of ModA of bacteriophage T4 and demonstrate that the basic rules that are true of the entire family of ADP-ribosyltransferases are valid for the ModA protein.

MATERIALS AND METHODS

Site-directed mutagenesis of *modA* gene. PCR-based site-directed mutagenesis experiments were carried out according to QuickChange™ Site-directed Mutagene-

Table. **Synthetic oligonucleotide primers for ModA direct mutagenesis**

Primers	Primer sequences
R72A	5'-CGATAAACCTCTTTGG**GC**AGGTGTTCCAGC
R72A-rev	5'-GCTGGAACACCT**GC**CCAAAGAGGTTTATCG
S109A	5'-GCTCTACATTTTGCT**G**CTGGTTTAGAGTATAACAC
S109A-rev	5'-GTGTTATACTCTAAACCAG**C**AGCAAAATGTAGAGC
Q116A	5'-GAGTATAACACA**GC**AGTTATTTTTGAATTC
Q116A-rev	5'-GAATTCAAAAATAACT**GC**TGTGTTATACTC
F127A	5'-CAAAGCTCCTATGGTA**GC**CAATTTCCAGGAGTATGC
F127A-rev	5'-GCATACTCCTGGAAATTG**GC**TACCATAGGAGCTTTG
N128A	5'-CAAAGCTCCTATGGTATTC**GC**TTTCCAGGAGTATGC
N128A-rev	5'-GCATACTCCTGGAAA**GC**GAATACCATAGGAGCTTTG
F129A	5'-CCTATGGTATTCAAT**GC**CCAGGAGTATGCTATAAAAGC
F129A-rev	5'-GCTTTTATAGCATACTCCTGG**GC**ATTGAATACCATAGG
E163A	5'-GTTTCAGATG**C**ACAAGAAGTAATG
E163A-rev	5'-CATTACTTCTTGT**G**CATCTGAAAC
Q164A	5'-GTTTCAGATGAA**GC**AGAAGTAATG
Q164A-rev	5'-CATTACTTCT**GC**TTCATCTGAAAC
E165A	5'-GTTTCAGATGAACAAG**C**AGTAATGATACCAGC
E165A-rev	5'-GCTGGTATCATTACT**G**CTTGTTCATCTGAAAC

sis Kit (Stratagene). The plasmid vector pET16/modA, carrying *modA* gene [3], was used as a template. Using pairs of mutagenic primers (Table), *modA* gene mutants with mutated triplets for conserved amino acids were obtained. Transformation, isolation of DNA [9] and sequencing [10] of *modA* genes with mutations were carried out through standard procedures.

ADP-ribosyltransferase-activity assay of overexpressed and purified ModA proteins. Overexpression and purification of wild type and mutated ModA polypeptides were carried out according to Tiemann et al. [3]. The activities of overexpressed and partially purified wild type and mutated ModA ADP-ribosyltrasferases were assayed by radioactive labeling of the proteins *in vitro* according to Rohrer et al. [5] and Tiemann et al. [3] followed by gel electrophoresis and autoradiography.

RESULTS AND DISCUSSION

With the aim of confirming the threading experiments and of gaining knowledge on ribosylation characteristics of this enzyme, nine putative active site amino acids were substituted by Ala (Fig. 1). The ADP-ribosyltransferase activity of overexpressed and partially purified mutated ModA polypeptides was determined following the protocol outlined in Materials and Methods.

As expected, ADP-ribosyltransferase activity tests showed that wild-type (wt) ModA ADP-ribosylates 70 kDa protein, RNA polymerase α subunit (36.5 kDa) and performs autoribosylation reaction [3]. Figure 2 shows that ModA also ADP-ribosylates ~16 kDa not yet identified protein.

An analysis of the activities of mutated ModA polypeptides showed the following results. Region β1 of ModA ADP-ribosyltransferase is characterized by an essential amino acid Arg located in the Trp-Arg motif (Fig. 1). Substitution of Arg72 leads to a loss of ADP-ribosylation of RNAP α subunit and 16 kDa protein as well as autoribosylation reaction (Fig. 2). This is consistent with the observations that substitution of active site residue Arg may affect ADP-ribosyltransferase activity: ADP-ribosyltransferases transfer ADP-ribose moiety from the substrate NAD^+ to an acceptor amino acid of a target protein. NAD^+ in an active site is bound with a nicotinamide ring anchored by the active site residue Arg [6, 7]. Thus, substitution of Arg72 eliminates interaction of NAD^+ in the active site, resulting in a loss of enzymatic activity. Mutated ModA-R72A modifies only 70 kDa protein (Fig. 2).

ModA lacks the β2 region consensus common to ADP-ribosyltransferases: region β2 of ModA deviates strongly from the Ser-Thr-Ser motif which is suggested to bind either to NAD^+ ribose or phosphate of AMP moiety [11] (Fig. 1). ModA has only the first Ser at

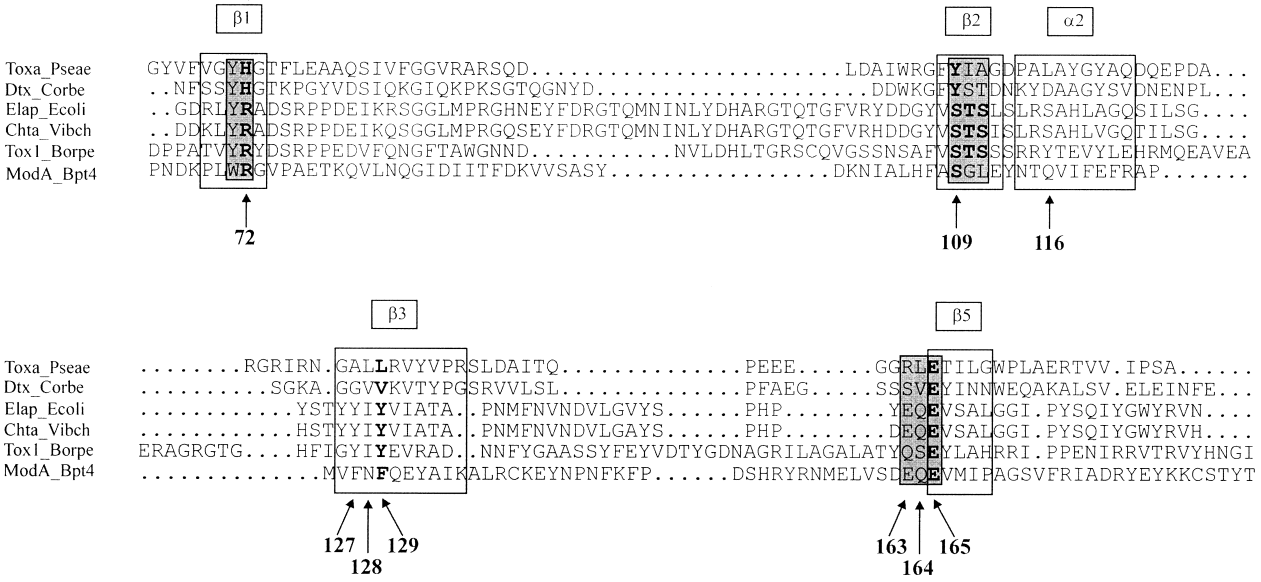

Fig. 1. The alignment of the catalytic domains of bacterial toxins according to threading programs**.** The ADP-ribosylating enzymes are characterized by three conserved regions, identified by boxes β1, β2/α2 and β5. Boxes β1 and β5 indicate essential Arg and Glu and their respective consensus patterns arom-Arg (arom – aromatic) and Glu/Gln-X-Glu. Box β2 contains Ser-Thr-Ser motif. The β2 is usually followed by an α2 helix. Homology analysis revealed an additional box β3 characterized by arom-X-arom motif. Consensus patterns are included in the shaded area. Numbers below indicate positions of amino acids of T4 ModA to be substituted by Ala. Toxa_pseae – exotoxin A of *Pseudomonas aeruginosa*; Dtx_Corbe – diphtheria toxin of *Corynebacterium diphteriae*; Elap_Ecoli – heat-labile enterotoxin A of *E. coli*; Chta_Vibch – cholera enterotoxin of *Vibrio cholerae*; ToxI_Borpe – pertussis toxin of *Bordetella pertussis*; ModA_Bpt4 - ModA protein of phage T4 [2]

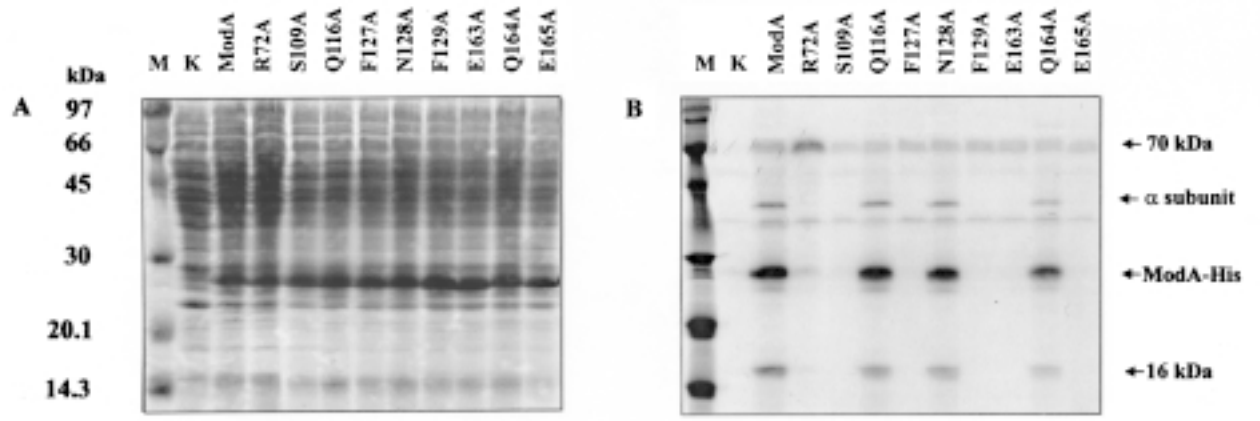

Fig. 2. Autoradiograph of soluble *E. coli* proteins incubated in the presence of T4 ADP-ribosyltransferase ModA. The crude cell extracts were supplemented with externally added RNAP. *In vitro* labeling reaction followed the protocol as given in Materials and Methods. The radioactive substrate was [α-^{32}P]NAD$^+$ (Amersham Bioscience). The reaction products were separated by electrophoresis on a 13 % SDS-PAGE and stained with Coomassie Brilliant Blue (**A**). **B** – the autoradiograph of A. Lanes: **M** - Rainbow™ [^{14}C] methylated protein molecular weight markers; **K** – control, *E. coli* C41 (DE3) cell lysate

position 109. Nevertheless, the stretch of amino acids forms a β strand followed by an α helix. Substitution of Ser109 located in β strand affects protein activity. This result is in agreement with the data obtained by others [11]. The first serine from the Ser-Thr-Ser motif is critical for ADP-ribosylating toxin activity. Mutated ModA-S109A is able to ADP-ribosylate only 70 kDa protein, while substitution of Gln116 (ModA-Q116A) located in the following α helix does not change protein activity: mutated polypeptide ADP-ribosylates the same target proteins as does wt ModA (Fig. 2). It can be speculated that Ser109 is necessary for catalytic activity while Gln116 is not.

In ModA sequence, the β3 strand forming part has a arom-X-arom (Phe-Asn-Phe) motif at positions from 127 to 129 (Fig. 1). Two flanking residues are aromatic and possibly located on the same side of strand β. Phe127 and Phe129 might form a hydrophobic core favoring the binding of NAD$^+$ in the active site. Substitution of flanking amino acids (ModA-F127A and ModA-F129A) leads to a loss of autoribosylation reaction and other target proteins, except 70 kDa protein (Fig. 2). In this case the complementarity principle of enzyme and NAD$^+$ binding might be disrupted. Substitution of the middle-standing Asn128 (ModA-N128A) does not change protein activity. The latter residue might form a spacer region between flanking amino acids keeping them at an appropriate distance. It might be that the Phe-Asn-Phe motif of ModA compensates the lack of β2 region consensus. Similar arom-X-arom motifs (Tyr-Ile-Tyr) are also found in three other ADP-ribosylating toxins [7] (Fig. 1).

The motif centered in a key glutamic residue is Glu-Gln-Glu at positions from 163 to 165 in strand β5. The latter is the second known active site amino acid Glu165. Glu is the first residue available for interaction with the substrate when this approaches bound NAD$^+$ [6, 7]. Two flanking amino acids of this motif have carboxyl groups separated by an amino group. A double negative charge may accelerate "pushing " of the negatively charged ADP-ribose moiety out of the active site, thus favoring its approach and binding to an acceptor residue. The Glu (Glu165 of ModA) is the only one strictly conserved amino acid in all toxins; it cannot tolerate even substitution of a compatible amino acid [6]. The activity test shows that both Glu at positions 163 and 165 seem to be important for protein function. ModA mutants harboring these substitutions no longer ADP-ribosylate subunit α nor 16 kDa protein, neither do they perform autoribosylation reaction. Substitutions of flanking amino acid Glu165 (ModA-E165A) might eliminate interaction with the acceptor residue, while the mechanism of action of Glu163 (ModA-E163A) is not known. The data obtained are consistent with the results obtained by others, showing the both flanking Glu in Glu/Gln-X-Glu motif to be critical for catalysis [11]. Substitution of Gln164 (ModA-Q164A) does not change protein activity (Fig. 2).

Thus, the ADP-ribosyltransferase activity test of mutated ModA polypeptides shows that Arg72 and Glu165, and Ser109 and Glu163 are necessary for ModA ADP-ribosyltransferase activity. Our results indicate that there are two additional amino acids with a potential role in protein function: substitution of Phe127 and Phe129 affect protein activity.

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BAKTERIOFAGO T4 ADF-RIBOZILTRANSFERAZĖS MODA MUTACINĖ ANALIZĖ

S a n t r a u k a

Pastaraisiais metais buvo sukaupta nemažai eksperimentinių duomenų apie ląstelės-šeimininkės baltymų ADF-ribozilinimo svarbą reguliuojant bakteriofago T4 genų ekspresiją. Bakteriofagas T4 koduoja ModA ADF-riboziltransferazę, apie kurios vykdomą ADF-ribozilinimo reakcijos pobūdį žinių nėra daug. Todėl buvo atlikta ModA mutacinė analizė: devynios amino rūgštys, esančios tam tikras konservatyvias antrines struktūras formuojančiose srityse, buvo pakeistos į Ala. Eksperimentiškai buvo patvirtinta, kad ModA ADF-ribozilinimo funkcijai yra būtinos dvi žinomos aktyviojo centro amino rūgštys, bei nustatyta, kad ADF-ribozilitransferaziniam aktyvumui yra svarbios dar keturios papildomos amino rūgštys.