Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 341-348

LOCATION AND AMINO ACID SEQUENCE AROUND THE ADP-RIBOSYLATION SITE IN THE CHOLERA TOXIN ACTIVE SUBUNIT A_1

Chun-Yen Lai*, Qi-Chang Xia and Poonam T. Salotra

Roche Institute of Molecular Biology
Roche Research Center
Nutley, New Jersey 07110 USA

Received September 2, 1983

SUMMARY: Renatured, S-carboxymethylated subunit A_1 of cholera toxin possess the ADP-ribose transferase activity (Lai, et.al., Biochem. Biophys. Res. Commun. 1981, 102, 1021). In the absence of acceptor self ADP-ribosylation of A_1 subunit was observed. Stoicheometric incorporation of ADP-ribose moiety was achieved in 20 min at room temperature in a 0.1 - 0.2M PO_4(Na) buffer, pH 6.6. On incubation of the complex with polyarginine, 75% of the enzyme-bound ADP-ribose moiety was transferred to the acceptor in 25 min. The ADP-ribosylated A_1 was stable at low pH, and on cleavage with BrCN, the ADP-ribose moiety was found associated with peptide Cn I, the COOH-terminal fragment of A_1 subunit. On further fragmentation with cathepsin D, a dodecapeptide containing ADP-ribose moiety was isolated whose structure was determined as: Asp-Glu-Glu-Leu-His-Arg-Gly-Tyr-Arg*-Asp-Arg-Tyr. The Arg* in the peptide was indicated to be the site of ADP-ribosylation.

It is now well established that stimulation of adenylate cyclase by cholera toxin involves ADP-ribosylation of a regulatory protein in the cell membrane (For a review, see Ref. 1). The thiol-activated cholera toxin has been shown to catalyze the transfer of the ADP-ribose moiety of NAD to the membrane protein *in vitro* (2) as well as to an artificial acceptor, polyarginine (3). We have previously demonstrated that subunit A_1 (M_r = 23,000) of cholera toxin (M_r = 84,000) is solely responsible for the action of the toxin to stimulate adenylate cyclase in a washed membrane preparation (4), and that this subunit catalyzes ADP-ribosylation of polyarginine in the absence of thiol, with higher efficiency than the "activated" holotoxin (5).

During experiments to label the membrane protein by the ADP-ribose transfer using A_1-subunit, we observed a strong labelling of the enzyme (A_1) itself, when the membrane was omitted from the reaction mixture. The apparent enzyme-

* To whom correspondence should be sent.

0006-291X/83 $1.50

substrate complex was separated by gel-filtration, and was found to transfer the ADP-ribose moiety onto polyarginine. The ADP-ribosylated A_1 subunit was found to be stable at low pH and temperature, permitting us to carry out a structural study on the site of ADP-ribosylation. Here we report isolation of a peptide containing ADP-ribose moiety and determination of its structure as well as location in the A_1-subunit.

MATERIALS AND METHODS

Subunit A_1 of cholera toxin was prepared after S-carboxymethylation and separation by gel filtration as previously described (6). On renaturation by dissolving in 0.05M Tris-Cl buffer containing 0.1M NaCl and 8M urea and slow dialysis to remove urea, S-carboxymethyl A_1 showed full activity either in the adenylate cyclase stimulation assay (4) or the ADP-ribosyl transfer assay (5). [^{3}H]-NAD or [^{32}P]-NAD was synthesized from [^{3}H]ATP or α[^{32}P]-ATP, (ICN), and NMN (Sigma), using NAD-pyrophosphorylase (Boeringer Biochemical) according to Cassell and Pfeuffer (7). Cathepsin D was kindly provided by Dr. Jordan Tang of Oklahoma Med. Res. Fdn., Oklahoma City, Ok. Other enzymes and reagents were of the highest grade commercially available.

Self ADP-ribosylation of A_1-subunit: Optimum condition for the incorporation of ADP-ribose moiety into A_1-subunit was determined by incubation of the renatured A_1 with [^{3}H]-NAD or [^{32}P]-NAD under various conditions in 10-15µl aliquots. After 20 min incubation at room temperatures, 30µl of 8M urea containing 1% Na dodecylsulfate SDS was added to 10-15µl of reaction mixture to stop the reaction, and the mixture subjected to electrophoresis in a 7.5% polyacrylamide slab in 0.1M PO_4 (K) buffer, pH 7.2 containing 0.1% SDS for 4hr as previously described (8). Subunit A_1 was located by staining with Coomassie Blue, the gel cut out and counted, after treatment with 0.1ml NCS (a tissue solubilizer, Amersham, Inc.) and a drop of 30% H_2O_2 for 1hr at 37°. The recovery of protein in the stained band was determined to be 67% of the amount (2-5µg) subjected to electrophoresis, by amino acid analysis of the band.

BrCN cleavage of ADP-ribosylated A_1 and separation of fragments were carried out essentially as described (9).

Separation of peptides by HPLC (High Performance Liquid Chromatography): Samples were dissolved in 0.1% trifluoroacetic acid and chromatographed on an Altex RP-18 column (45mm x 250mm, 10µ particle, Rainin Instrument) as described in Fig. 2. The effluent was monitored for absorbance at 214nm with Waters Model 441 UV detector.

Micro-Edman degradation was performed manually as described previously (10, 11)

RESULTS

Self-ADP-ribosylation o A_1-subunit

On incubation of A_1-subunit with NAD in 0.1M PO_4 (K) buffer, pH 6.6 at 37°, incorporation of the ADP-ribose moiety into the subunit occurred linearly with time for 10min, reaching a plateau after 25min (data not shown). The maximal incorporation was observed at pH 6.6, in the presence of phosphate ion at a concentration above 0.1M (Table 1). The requirement for high phosphate concentration is in agreement with the observation made by Trepel *et al.* (12)

Table 1 Self ADP-ribosylation of A_1 subunit+

	cm-A_1 (mg/ml)	PO_4 (Na) buffer	[NAD] (mM)	Incorporation (mol ADPR/mol A_1)
a.	0.25	0.1 M, pH 6.6	5.6	0.31
		" "	16.7	0.66
b.	0.82	0.1 M, pH 6.6	2.4	0.41
		" "	5.6	0.55
		" "	12	0.88
c.	0.82	0.1 M, pH 8.5	2.4	0.14
		0.1 M, pH 6.0	"	0.36
		0.1 M, pH 5.0	"	0.22

+ The extent of self ADP-ribosylation was examined under various conditions as described in "Methods". In 50 mM Tris-HCl buffer, pH 8 incorporations were less than 0.012 mol/mol with up to 1 mM NAD.
a. The effect of increased concentration of NAD at low A_1 concentration.
b. The effect of NAD concentration at high A_1 concentration.
c. The effect of pH.

using the thiol activated cholera toxin. Higher incorporation was observed with higher concentrations of NAD and of A_1-subunit in the reaction mixture. With NAD concentration above 10mM and 0.8mg/ml of A_1-subunit, incorporation of 0.86 - 1.2mol of ADP-ribose per mol of A_1-subunit was achieved (see following).

Preparative ADP-ribosylation of A_1-subunit

In order to prepare a sufficient quantity of ADP-ribosyl A_1 subunit for the study of its property and the site of ADP-ribosylation, the following procedure was used based on the information obtained above: To 1.5 ml solution of 17mM [^{3}H]-NAD (1.1×10^4 cpm/nmol) in 0.2M PO_4 (Na) buffer, pH 6.6, 0.5ml of renatured S-carboxymethylated A_1-subunit (2.8mg/ml) was added with stirring over 2 min. After 15 min at room temperature, 0.1ml 0.5M PO_4 (Na) buffer, pH 6.6 and 0.5ml of A_1 solution was added. The process was repeated once more after 15 min. An aliquot (0.2ml) was removed for gel-filtration in 50mM PO_4 (Na) buffer, pH 6.6 on Sephadex G-50F (0.4 x 40cm) in the cold room. The rest was acidified with 0.1 volume of 70% HCOOH and gel-filtered at room temperature on a Sephadex G-50F column (1.5 x 180cm) in 5% HCOOH (data not shown). The ADP-ribosyl-A_1-subunit was well separated from the excess NAD

Table 2 Transfer of ADP-ribose moiety from A_1 to polyarginine[a]

Addition	Time	cpm in polyarginine	% transferred
none	25 min	(158)[b]	0
Polyarginine	~0.1	5809	17.2
Polyarginine	25	25040	76.1

a. To 100 µl solution of ~5 µg [^{3}H]ADP-ribosyl A_1 (32900 cpm) in 15 mM PO_4(Na) buffer, pH 6.6, 10 µl of 10 mg/ml polyarginine was added and incubated at 37°. One ml of 0.3M Na_2HPO_4 was added immediately and at 25 min to precipitate polyarginine. The precipitate was filtered on Whatman GF/B filter, washed 3 times with 0.3M Na_2HPO_4 and counted.

b. Background radioactivity found on filteration of [^{3}H]ADP-ribosyl A_1.

under either condition. In 3 experiments using 1.6 - 4mg of A_1-subunit, the incorporation of 0.86, 1.14 and 1.2 mol ADP-ribose per mol of A_1-subunit were obtained.

Properties of ADP-ribosyl A_1-subunit

On incubation of ADP-ribosyl A_1 at neutral pH with polyarginine, about 75% of AdP-ribose moiety was transferred to the latter in 25 min (Table 2). The result indicated that ADP-ribosyl A_1 had the characteristic of a substrate-enzyme complex and that the ADP-ribose moiety was bound to the substrate binding site of A_1-subunit. On incubation with nicotinamide, however, no radioactivity was lost from the protein (data not shown) indicating that reversal of ADP-ribosylation did not readily occur. All radioactivity was precipitated with 5% TCA indicating that the ADP-ribosyl bond in A_1-subunit was stable to acid.

Location of ADP-ribose moiety in the A_1 molecule

On treatment of ADP-ribosyl A_1 with BrCN and separation on Sephadex G50F in 5% HCOOH, a peptide pattern similar to that derived from untreated A_1-subunit (9) was obtained (Fig. 1). The major radioactivity peak coincided with peptide Cn I and accounted for 61% of the total radioactivity applied to the column. Peptide Cn I is derived from the COOH-terminal half of A_1 (9). Analyses showed that 0.82 mol ADP-ribose was bound to 1 mol of Cn I.

Isolation and sequence analyses of a cathepsin peptide containing ADP-ribose moiety

Attempts to obtain a peptide fragment containing ADP-ribose group by digestion of ADP-ribosyl Cn I (Fig. 1) with trypsin, chymotrypsin and pepsin

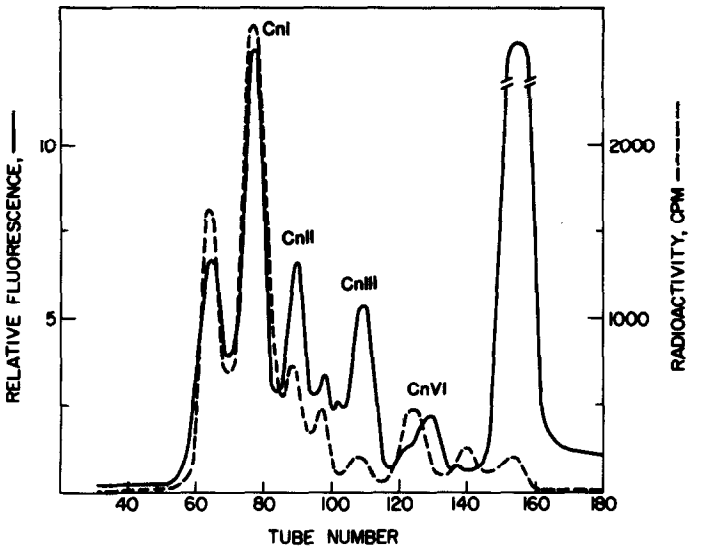


FIGURE 1 Sephadex G-75 chromatography of BrCN peptides from [^{3}H] ADPR-A$_n$. The flow rate was 12 ml/hr and fractions (∿1.5 ml) were collected. Aliquots (30 µl) were analyzed for peptides with fluores-camine after hydrolysis (———) and for radio-activity (-----).

resulted in the loss of the radioactivity (data not shown). With cathepsin D, a peptide containing ADP-ribose moiety could be isolated by HPLC, (Fig. 2A) which contained 43.4% of the radioactivity.

The second largest radioactive peak (Fig. 2A) contained 31.1% of the label and coincided with the peak of undigested Cn I. Amino acid analysis indicated that the peptide CD-2 contained 12 residues and 0.44 mol ADP-ribose per mol.

Sequence analyses of CD-2 was carried out by further digestion of the peptide and Edman degradation of the purified sub-fragments. On tryptic hydrolysis and separation on HPLC (Fig. 2B), the major radioactivity peak coincided with a minor peptide peak. Amino acid analysis revealed that this peak corresponded to peptide T1, Gly-Tyr-Arg, and that it carried 1.0 mol ADP-ribose moiety per mol. Other fractions containing radioactivity yielded negligible amounts of amino acids on hydrolysis. Chymotryptic digestion of CD-2 yielded 2 peptides well separated by HPLC, neither of which contained radioactivity (data not

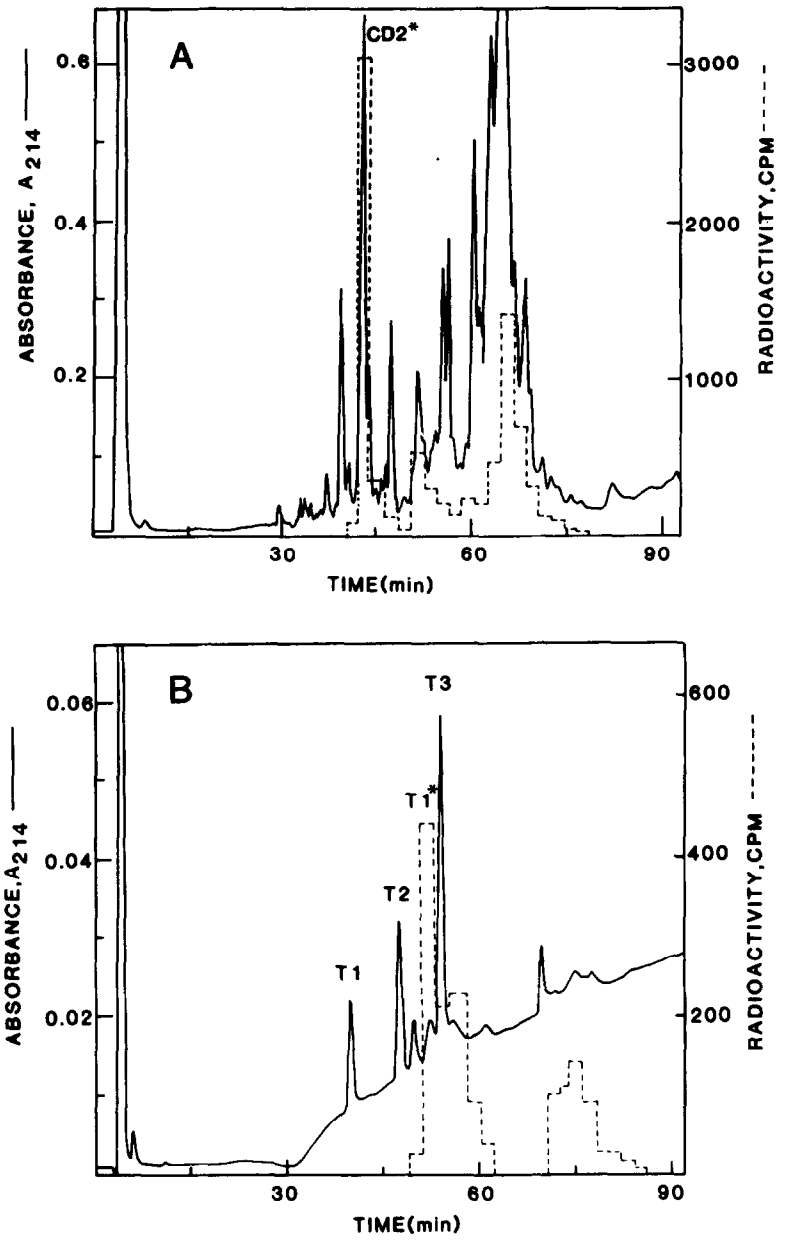


FIGURE 2 Separation of the [^{3}H]ADP-ribose labelled peptides on HPLC (RP-18).

A. [^{3}H]ADP-ribosyl Cn 1 from Fig. 1 (∿30 nmol) was digested with 1 µg cathepsin D in 400 µl of 0.1M formate (Na) buffer, pH 3.1 for 3 h at room temperature. The digest was separated at 1 ml/min using a linear gradient of 0-40% propanol in 0.1% trifluoacetic acid. Two % of fractions were assayed for radioactivity.

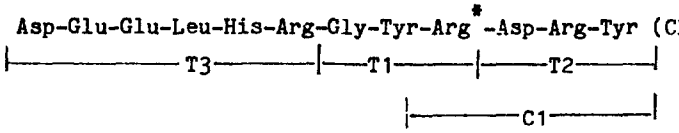
B. [^{3}H]ADP-ribosyl CD-2 (∿10 nmol) was digested with 0.05 µg trypsin in 100 µl of 0.1 M Tris-HCl, pH 7.0 for 1 h at room temperature. The digest was departed at 1 ml/min using 0-20% CH_3CN gradient in 0.1% trifluoracetic acid. five % of fractions were counted.

Table 3 Edman Degradation of CD-2, and of its Subfragments

Peptides (n moles)	Sequence
CD 2 (0.7)	a) Asp-Glu-Glu-Leu——
	31 33 33 19
T 1 (2.0)	Gly-Tyr-Arg
	60 31 56
T 2 (2.0)	Asp-Arg-Tyr
	26 35 90
T 3 (2.0)	Asp-Glu-Glu-Leu-His-Arg
	20- 21 21 20 46 25
C 1 (0.8)	Arg-Asp-Arg-Tyr
	27 27 32 95

a) The numbers denote the degradation yield in % of the residue indicated above.

shown). Edman degradation of C1, Arg-Asp-Arg-Tyr (Table 3), provided the necessary overlap for tryptic peptides T1 and T2:



The Arg denoted with an asterisk (*) is the proposed site of ADP-ribosylation.

DISCUSSION

The present results strongly suggest that self-ADP ribosylation of A_1-subunit involves the active site of A_1 as an ADP-ribosyltransferase. Thus, isolated ADP-ribosyl A_1 is capable of transfering ADP-ribose moiety to the acceptor polyarginine, as expected for an enzyme-substrate intermediate. Also, the site of ADP-ribosylation appeared to be specific on BrCN cleavage and separation, the COOH-terminal fragment Cn I was found to contain nearly stoichiometri amounts (0.88 mol/mol) of ADP-ribose moiety and further fragmentation of Cn I resulted in isolation of a single peptide CD-2 containing the label. However, the reverse reaction to form NAD on incubation with nicotinamide, one criterion for ADP-ribosyl A_1 to be a reaction intermediate (1), was not observed. This, nevertheless, may be an indication that the bond energy between ADP-ribose and nicotinamide is much higher than that in the ADP-ribosylated A_1.

The indication that the active site of A_1 resides in the COOH-terminal half of the molecule is in agreement with our previous observation (5) that a limited digestion of A_1-subunit with trypsin produced a partially active fragment derived in large from the COOH-terminal region of the molecule. We have now completed the sequence analysis of Cn I, the 95 residue peptide from the COOH-terminus of A_1. The ADP-ribosylated peptide CD-2 corresponds to residues 42 to 53 and the proposed site of ADP-ribosylation is Arg-50 of this peptide. The A_1-subunit contains 195 amino acid residues.

The nature of ADP-ribosyl bond to A_1 remains to be elucidated. Since it is generally assumed that the ADP-ribose moiety is bound to the guanido-group in polyarginine and since the ADP-ribose group was found stoichiometrically-bound to the peptide Gly-Tyr-Arg from the dodecapeptide CD-2((contains 3 Arg), we propose that it involves a C-N bond between the reducing end of ADP-ribose and one of the guanido nitrogens. It is interesting to note that this bond is stable to acid at low temperatures but is, apparently, susceptible to attack by most proteolytic enzymes.

REFERENCES

1. Gill, D.M. (1982) in "ADP-ribosylation Reactions" (O. Hayaishi and K. Ueda Eds.) pp. 593-621 Academic Press, New York.
2. Gill, D.M., and Meren, R. (1978) Proc. Natl. Acad. Sci. USA 75, 3050-3054.
3. Mekalanos, J.J., Collier, R.J. and Romig, W.R. (1979) J. Biol. Chem. 254, 5849-5854.
4. Wodnar-Filipowicz, A. and Lai, C.Y. (1976) Arch. Biochem. Biophys. 176, 465-471.
5. Lai, C.Y., Cancedda, F., and Duffy, L.K. (1981) Biochem. Biophys. Res. Commun. 102, 1021-1027.
6. Mendez, E., Lai, C.Y. and Wodnar-Filipowicz (1975) Biochem. Biophys. Res. Commun. 67, 1435-1443.
7. Cassel, D., and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci, USA 75, 2669-2673.
8. Lai, C.Y., Mendez, E. and Chang, D. (1976) J. Infect. Dis. 133, S23-S30.
9. Lai, C.Y., Cancedda, F. and Chang, D. (1979) Febs Lett. 100, 85-89.
10. Lai, C.Y. (1975) Arch. Biochem Biophys. 166, 330-338.
11. Lai, C.Y. and Dietzshold, B. (1981) Biochem. Biophys. Res. Commun. 103, 536-542.
12. Trepel, J.B., Chuang, C.-M., and Neff, N.H. (1977) Proc. Natl. Acad. Sci. USA 74, 5440-5442.