Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1024-1031

ADP-RIBOSYLATION OF A RAT LIVER MEMBRANE PROTEIN CATALYSED BY HEAT-LABILE ENTEROTOXIN FROM E. COLI.

R. Murray Tait, Brian R. Booth and Peter A. Lambert.

Department of Microbial Biochemistry, Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex, UB6 OHE.

Received August 16,1980

SUMMARY

Crude lysates from a strain of enterotoxigenic E. coli have been shown to catalyse the incorporation of [^{32}P] from [adenylate-^{32}P] NAD^+ into an 11,000 dalton protein in rat liver membranes. [^{32}P] incorporation paralleled adenylate cyclase activation and the results suggest that the mechanism of action of the heat-labile E. coli enterotoxin may involve ADP-ribosylation of an intracellular acceptor protein.

INTRODUCTION

The enterotoxins produced by V. cholerae and enterotoxigenic strains of E. coli produce diarrhoea by stimulating adenylate cyclase activity in the cells of the small intestine, thereby initiating the cAMP-mediated secretion of water and electrolytes (1-3). In the case of cholera toxin the mechanism of cyclase stimulation is thought to involve the NAD^+-dependent ADP-ribosylation of a 42,000 dalton component of the cyclase complex which is probably a GTP regulatory protein (4-6). ADP-ribosylation of this protein is thought to inhibit its intrinsic GTPase activity resulting in an irreversibly activated adenylate cyclase (7,8).

Although the heat-labile enterotoxin (LT) of enterotoxigenic E. coli is thought to act by a similar mechanism and, like cholera toxin (CT), will catalyze the ADP-ribosylation of several artificial acceptor molecules (9), to date it has not been demonstrated that LT ADP-ribosylates a membrane protein under conditions in which adenylate cyclase is activated. The present report describes such a reaction in rat liver membranes.

0006-291X/80/191024-08$01.00/0

METHODS

Preparation of E. coli lysates

E. coli 1758E (LT positive) and 1759E (LT negative) were grown overnight in one litre of CAYE broth (10), harvested by centrifugation (30,000g x 5 min.) and resuspended in 100ml of 30 mM-Tris, pH 8.0. The cells were then recentrifuged and resuspended in 20 ml of 30 mM-Tris pH 8.0 containing 20% (w/v) surcrose. Lysozyme and EDTA were added to final concentrations of 100ug/ml and 10mM respectively and the cells left at 4°C for 10 min. Spheroplasts were harvested by centrifugation (8,000g x 10 min.) and resuspended in 20 ml of TEAN buffer (50mM-Tris; 1 mM-EDTA; 3mM-sodium azide; 200mM-NaCl; pH 7.5). The suspension was sonicated for 3 min; cell debris pelleted by centrifugation (38,000g x 2h) and the supernatant stored at -20°C until required.

All operations were carried out at 4°C.

Preparation of rat liver membranes

A crude membrane fraction from rat liver was prepared by following the procedure of Neville (11) up to step 5. The pellet from the final centrifugation was washed twice with 1mM-$NaHCO_3$, membranes were resuspended to a protein concentration of 1mg/ml, and stored in 1 ml aliquots at -70°C. Adenylate cyclase activity was stable under these conditions for at least 3 months.

Toxin stimulation of adenylate cyclase

100µl of rat liver membrane suspension were incubated in a total volume of 200µl in MOPS-saline buffer (20mM-MOPS; 130mM-NaCl; 10mM-KCl; 2mM-$MgSO_4$; pH 7.3) in the presence of 5mM-dithiothreitol, 5mM-ATP, 0.1mM-GTP, 1mM-NAD^+, 10mM-thymidine, 1mM-nicotinamide, and LT (in TEAN buffer) at the final concentrations indicated in the figures. After 15 min. at 30°C, 1 ml of ice-cold MOPS-saline buffer was added to terminate enterotoxin action and membranes were precipitated by centrifugation (1,500g x 10 min) on a Beckman Model TJ-6 centrifuge. The supernatants were aspirated, pellets resuspended in 80µl of MOPS-saline and 30µl aliquots added in duplicate to tubes containing 20µl of adenylate cyclase assay cocktail. Final concentrations of assay components were as follows: 25mM-Tris/acetate, pH 7.6; 5mM-magnesium acetate; 0.5 mM-ATP; 0.05mM-cAMP; 1mM-dithiothreitol; 0.1 mg/ml bovine serum albumin; 0.01mM-GTP; 5mM-creatine phosphate; 50U/ml creatine phosphokinase; and 2 x 10^6 cpm of [α-^{32}P]ATP. Adenylate cyclase was assayed for 10 min. at 30°C and reactions were terminated by the addition of 1 ml of water. 100µl of [^{3}H]cAMP (15,000cpm) were added to each tube for estimation of recovery and [^{32}P]cAMP was determined by the method of Salomon et al (12). Reported values were the means of duplicates which were in agreement to within ± 10%.

ADP-ribosylation by LT

ADP-ribosylation reactions were conducted under identical conditions to those described above for toxin stimulation of

adenylate cyclase, except that each assay contained 2 x 10^7 cpm of [^{32}P]NAD^+ and that the final assay volume was reduced 10-fold to 20µl (to achieve maximum sensitivity). After incubation at 30°C for 15 min. reactions were terminated by the addition of 10µl of 3% SDS, 15mM-2-mercaptoethanol and tubes were placed in a boiling water bath for 5 min. After cooling to room temperature, 30µl of gel sample buffer were added (0.2M-Tris/HCl, pH 6.8; 30% glycerol; 3%-SDS 0.01% bromophenol blue) and 40µl of each sample were electrophoresed on 15% polyacrylamide-SDS slab gels using the system of Laemlli and Favre (13). Gels were calibrated for molecular weight determinations with the following standard proteins; bovine serum albumin (66,500), creatine phospho kinase (43,000), cholera toxin subunit A, (21,000), lysozyme (14,400), and cytochrome C (13,200).

Gels were stained overnight in 0.1% Coomassie Brilliant Blue-R, 50%-methanol, 10% acetic acid, destained in 10% methanol, 5% acetic acid, and dried onto thick absorbent paper using a Bio-Rad model 224 gel drier. The dried gels were autoradiographed over a period of 3-7 days depending on the amount of radioactivity present on each gel and densitometric scans of the autoradiographs were recorded on a Joyce-Loebl microddensitometer 3CS with electronic integrator for determination of relative peak areas.

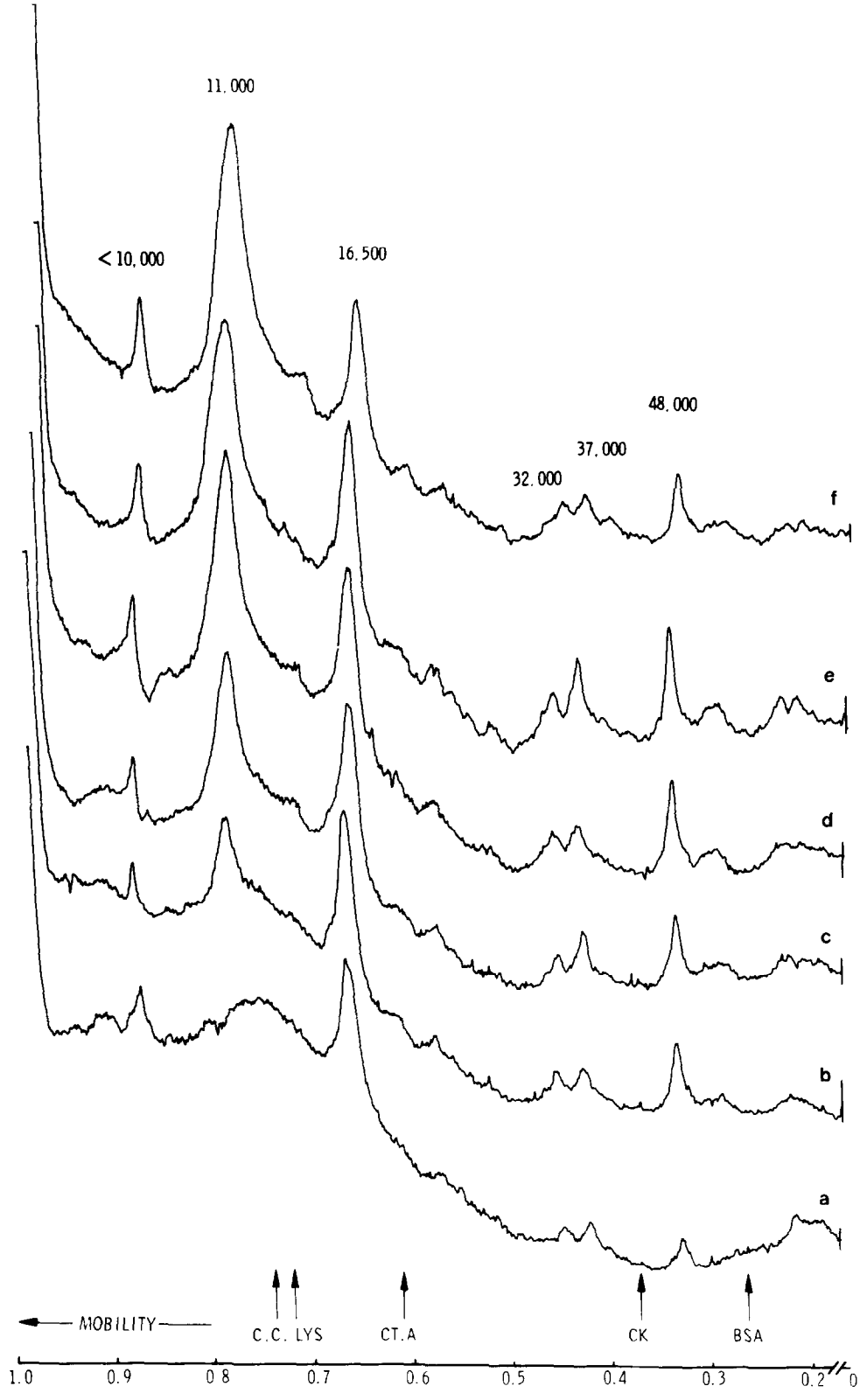
Protein was determined by the method of Bradford (14).

MATERIALS

E. coli 1758E (lac^-, str^+, Hly^-, Ent^+ P307) and E. coli 1759E (lac^-, Str^+, Hly^-) are K12 strains and were originally obtained from Dr. H. Williams-Smith. [α-^{32}P] ATP (26Ci/mmole) and [adenylate-^{32}P]NAD^+ (26 Ci/mmole) were from New England Nuclear and [8-^{3}H] cAMP (25Ci/mmole) was obtained from the Radiochemical Centre, Amersham. All other materials were purchased from standard commercial sources.

RESULTS

Crude lysates from enterotoxigenic E. coli (1758E) catalysed the incorporation of [^{32}P] from [adenylate-^{32}P]NAD^+ into a rat liver membrane protein which migrated on SDS-polyacrylamide gels with an apparent molecular weight of 11,000 daltons (Fig. 1.) Several other radioactive proteins were also observed but their appearance was not dependent upon the presence of LT lysate and the amount of label incorporated did not increase with increasing lysate concentrations. These bands probably represent proteins which are present in the liver membranes and


11.000
< 10.000
16.500
48.000
37.000
32.000
f
e
d
c
b
a
MOBILITY
C.C. LYS
CT.A
CK
BSA
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0

which are ADP-ribosylated by endogenous enzymes. In these experiments the extent of endogenous ADP-ribosylation was reduced significantly by including thymidine and nicotinamide in the incubations (results not shown). The former is a potent inhibitor of poly (ADP-ribose) polymerase (15) which is likely to be present in the crude membrane preparations employed. Nicotinamide, at the concentration of 1mM used here, almost completely inhibits endogenous rat liver NADase activities while having no inhibitory effect on either CT or LT (indeed 1mM-nicotinamide has previously been shown to enhance the action of CT in rat liver membranes (16)). The use of these compounds greatly facilitates the detection of toxin-specific ADP-ribosylations.

The persistence of several radioactive bands as a result of endogenous ADP-ribosylation can be used to give a convenient measure of the extent of LT-specific incorporation. The area of the LT-specific peak on the densitometric scans in Fig. 1. was determined by integration and was expressed as a ratio of the area of the 16,500 dalton protein which was ADP-ribosylated by endogenous enzymes (and which remained constant over the LT dose-response). The ratios are plotted as a function of LT lysate protein concentration in Fig. 2 which also shows adenylate cyclase activities after incubation with the same range of lysate concentrations. It is clear that ADP-ribosylation of the 11,000 dalton protein occurs in parallel with adenylate cyclase activation, strongly suggesting that the two phenomena are causally related.

Figure 1

Electrophoretic analysis of radioactive proteins after incubation of [^{32}P] NAD^+ with rat liver membranes and crude LT lysate

---

Rat liver membranes were incubated with [^{32}P] NAD^+ as described in Methods in the presence of 0 (a), 0.5 (b), 1.0 (c), 1.5 (d), 2.0 (e), and 2.5 (f) mg/ml LT lysate. After separation by SDS-polyacrylamide gel electrophoresis, gels were autoradiographed and scanned densitometrically. Molecular weights were determined relative to the standard proteins (BSA: bovine serum albumin; CK: creatine Kinase; CT.A: cholera toxin subunit A; LYS: lysozyme; and C.C: cytochrome C).

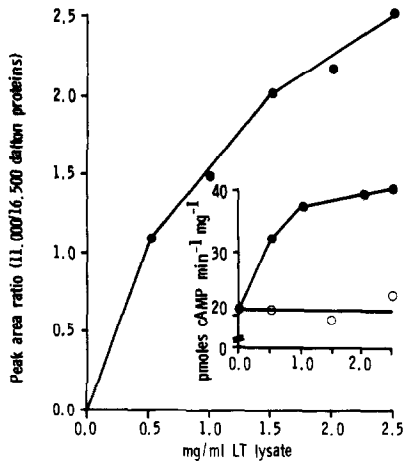


Figure 2 ADP-ribosylation and adenylate cyclase activation by crude LT lysate.

Experimental details as for Fig. 1. Peak area ratios (11,000 daltons/16,500 daltons) are expressed as a function of crude LT lysate concentration. Adenylate cyclase activities after incubation with the indicated concentrations of Ent^+ (●—●) or Ent^- (o—o) lysate were determined as described in Methods.

Since we used crude lysates of E. coli for the above work, it remained possible that the observed ADP-ribosylation was not due to LT but to some other E. coli protein bearing no relationship to adenylate cyclase activation. Therefore we conducted an identical experiment with an isogenic strain of E. coli in which the Ent plasmid was lacking. Lysates from the Ent^- strain (1759E) did not catalyse specific membrane protein ADP-ribosylation (not shown) and did not stimulate adenylate cyclase activity (Fig. 2.)

As a further control, incubation of [^{32}P]NAD^+ with Ent^+ lysate in the absence of rat liver membranes did not result in detectable levels of ADP-ribosylation (results not shown) thereby ruling out the possibility that self-ADP-ribosylation or ADP-ribosylation of an endogenous E. coli protein was being observed.

DISCUSSION

Heat-labile enterotoxin from Ent^+ strains of E. coli has previously been shown to activate adenylate cyclase (17) and

to catalyse ADP-ribosylation of several artificial acceptor molecules in an analogous manner to CT (9). In addition the structures of the two toxins bear many similarities (18-20) and immunological relationships have been described (21). This report now presents evidence that LT catalyses the ADP-ribosylation of a membrane protein from rat liver which occurs in parallel with adenylate cyclase activation and constitutes the first direct demonstration that the mechanism of LT action may involve specifc ADP-ribosylation of an intracellular acceptor. Although these experiments were conducted with crude lysates of enterotoxigenic E. coli, the assumption that the observed ADP-ribosylation is due to LT derives support from the observation that detectable ADP-ribosylation did not occur with similar lysates from an isogenic Ent^- strain of E. coli. Experiments are now in progress with purified LT and results will be presented elsewhere.

The fact that the molecular weight of the protein labelled by crude LT lysate and [^{32}P]NAD^+ is much smaller than the value of 42,000 daltons reported for the CT substrate may be explained in several ways.

Firstly it seems probable that the crude LT lysate employed in this study may contain proteolytic enzymes, and therefore the 11,000 dalton protein may reflect degradation of the ADP-ribose acceptor protein without loss of the ability to be ADP-ribosylated. Qualitatively similar observations have been reported by Malbon and Gill (22) for CT-catalysed ADP-ribosylation in fat cell ghosts. Secondly, our observations may reflect a genuine difference between the actions of CT and LT both of which could conceivably induce similar effects through ADP-ribosylation of distinct acceptor proteins.

A third possibility, that the molecular weight of the toxin substrate in rat liver membranes may not be the same as that reported in other tissues, seems less likely from the recent report of Doberska et al (23) describing ADP-ribosylation of a 42,000 dalton rat liver membrane protein catalysed by CT. The conditions described for CT action in rat liver membranes by these authors are the same as those independently established

by us for both CT and LT activation of adenylate cyclase in our system. Thus it seems likely that either of the two former possibilities mentioned above are responsible for the different molecular weight of the LT substrate reported in this paper.

We are currently evaluating these possibilities through detailed comparitive studies on both CT- and LT- catalysed ADP-ribosylation in the rat liver membrane system.

REFERENCES

1. FIELD, M. (1979). Rev. Infect. Dis. 1, 918-925.
2. van HEYNINGEN, S. (1977) Biol. Rev. 52, 509-549.
3. MOSS, J. and VAUGHAN, M. (1979). Ann. Rev. Biochem. 48, 581-600.
4. GILL, D.M. and MEREN, R. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 3050-3054.
5. CASSEL, D. and PFEUFFER, T. (1978). Proc. Natl. Acad. Sci. U.S.A., 75, 2669-2673.
6. KASLOW, H.R., FARFEL, Z., JOHNSON, G.L., and BOURNE, H.R., (1979) Mol. Pharmacol. 15, 472-483.
7. CASSEL, D. and SELINGER, Z. (1977). Proc. Natl. Acad. Sci. U.S.A., 74, 3307-3311.
8. CASSEL, D., LEVKOVITZ, H. and SELINGER, Z. (1977). J. Cyclic Nucleotide Res. 3, 393-406.
9. MOSS, J., GARRISON, S., OPPENHEIMER, N.J. and RICHARDSON, S.H. (1979) J. Biol. Chem. 254, 6270-6272.
10. MENDELL, D.H., ANSELINO, C.R. and WISHNOW, R.M. (1976). Infect. Immun. 14, 383-388.
11. NEVILLE, D.M. Jnr. (1968). Biochim. Biophys. Acta. 154, 540-552.
12. SALOMON, Y., LONDOS, C. and RODBELL, M. (1974) Anal. Biochem. 58, 541-548.
13. LAEMLLI, U.K. and FAVRE, M. (1973). J. Molec. Biol. 80, 575-599.
14. BRADFORD, M.M. (1976). Anal. Biochem. 72, 248-254.
15. TANUMA, S-i., ENOMOTO, T. and YAMADA, M.A., (1979). J. Biol. Chem. 254, 4960-4962.
16. van HEYNINGEN, S. and TAIT, R.M. (1980) in Proceedings of the 4th European Colloquium on Hormones and Cell Regulation (Elsevier, Nr. Holland, Amsterdam); in press.
17. EVANS, D.J., CHEN, L.C., CURLIN, G.T. and EVANS, D.G., (1977) Nature (New Biol.) 236, 137-139.
18. DALLAS, W.S. and FALKOW, S. (1979) Nature, 277, 406-407.
19. DALLAS, W.S., GILL, D.M. and FALKOW, S., (1979) J. Bacteriol. 139, 850-858.
20. DORNER, F., HUGHES, C., NAHLER, G. and HOGENAUER, G., (1979). Proc. Natl. Acad. Sci. U.S.A., 76, 4832-4836.
21. KLIPSTEIN, F.A. AND ENGERT, R.F., (1977) Infect. Immun. 18, 110-117.
22. MALBON, C.C. and GILL, D.M. (1979) Biochim. Biophys. Acta. 586, 518-527.
23. DOBERSKA, C.A., MACPHERSON, A.J.S. and MARTIN, B.R. (1980). Biochem. J. 186, 749-754.