Biochem. J. (1976) **154**, 725–729
Printed in Great Britain

Partial Purification of a Dicyclohexylcarbodi-imide-Sensitive Membrane Adenosine Triphosphatase Complex from the Obligately Anaerobic Bacterium *Clostridium pasteurianum*

By DAVID J. CLARKE and J. GARETH MORRIS
Department of Botany and Microbiology, School of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, Wales, U.K.

(*Received* 28 *October* 1975)

The membrane adenosine triphosphatase complex of vegetatively growing *Clostridium pasteurianum*, solubilized with Triton X-100, has been recovered as a significantly purified particulate preparation that is still sensitive to inhibition by dicyclohexylcarbodi-imide and butyricin 7423.

The membrane-bound ATPases* of bacteria in many respects resemble the ATPases of eukaryotic mitochondria (West, 1974; Beechey, 1974; Pedersen, 1975). Thus the bacterial ATPase is a complex consisting of a more or less easily solubilized component (BF_1) and a membrane-integrated component (BF_0), the two possibly being attached via a specific binding protein. The soluble BF_1 component possesses ATPase activity and has been purified from several bacteria (West, 1974). However, the ATPase activity of BF_1 component is not inhibited by dicyclohexylcarbodi-imide, which apparently binds covalently to the membrane component BF_0. For this reason, sensitivity to dicyclohexylcarbodi-imide is a convenient indicator of the retained integrity of the whole ATPase complex (BF_1BF_0). To date, it has been the purification of this dicyclohexylcarbodi-imide-sensitive ATPase complex from bacterial membranes that has proved most difficult, various detergents having been employed for this purpose with different degrees of success (Evans, 1970; Nieuwenhuis *et al.*, 1974; Baron & Thompson, 1975).

In this present communication we report the use of Triton X-100 to solubilize and then recover, in a partially purified particulate form, the dicyclohexylcarbodi-imide-sensitive Mg^{2+}-activated ATPase of vegetatively growing *Clostridium pasteurianum* (Clarke & Morris, 1975; Riebeling *et al.*, 1975).

Experimental

Organism

Clostridium pasteurianum A.T.C.C. 6013 was maintained as previously described (Mackey & Morris, 1971). Cultures were grown anaerobically at 37°C in a glucose minimal medium (Robson *et al.*, 1974).

* Abbreviation: ATPase, adenosine triphosphatase.

Preparation of membranes

A 40-litre culture of *Cl. pasteurianum* was harvested by continuous centrifugation (0.2 litre/min at 18500*g*) at room temperature. The organisms were washed once in fresh growth medium and recovered by centrifugation at 12000*g* for 20min at 4°C. The cell pellet was then suspended in 40ml of 0.8M-Tris/HCl buffer, pH7.1, with 20mM-$MgCl_2$ (with or without 50mM-2-mercaptoethanol, as indicated in the Results section), and stored overnight in liquid N_2. After the cell preparation had been thawed, lysozyme (EC 3.2.1.17) was added at 0.25mg/ml, and the suspension incubated at 37°C until protoplasts were produced (approx. 45min). These were stabilized by the high concentration of Mg^{2+} ions, and were ruptured by passage at 140MPa through a chilled French pressure cell (Aminco, Silver Spring, MD, U.S.A.). After incubation at 22°C for 20min with 0.12mg of deoxyribonuclease (EC 3.1.4.5)/ml, the extract was clarified by successively centrifuging at 4°C at (*a*) 6000*g* for 30min, (*b*) 12000*g* for 20min and (*c*) 15000*g* for 20min. The final clear supernatant was centrifuged at 100000*g* for 90min at 4°C and the pelleted membranes were resuspended by homogenization by using a rubber-tipped glass rod, in a small volume (1–2ml) of 90mM-Tris/HCl buffer, pH7.6, containing 8mM-$MgCl_2$ and (when required) 20mM-2-mercaptoethanol. This suspension was passed through a fine-gauge hypodermic needle into 28ml of the same buffer mixture, and the membranes were then recovered by centrifugation (100000*g* for 90min at 4°C).

Assay of ATPase

ATPase activity was assayed by a modification of the method of Muñoz *et al.* (1968). The reaction

mixture contained (in 1ml) 90μmol of Tris/HCl buffer, pH7.6, 8μmol of $MgCl_2$, 5μmol of ATP and 100–200μg of extract protein. Incubation was at 37°C. Samples (40μl) taken at 30s intervals were rapidly mixed with 0.6ml of 0.42% ammonium molybdate in 1M-H_2SO_4 and 0.36ml of 2.63% (w/v) ascorbic acid. After the mixture had been kept at 45°C for exactly 20min, the absorbance at 820nm was measured spectrophotometrically against a suitable blank. (The determined rates of the ATPase-catalysed reaction were linear for at least 5min.)

Analytical methods

In the absence of detergents, protein was assayed by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin as standard; otherwise it was measured by the alkaline ninhydrin method (Hirs, 1967). Triton X-100 was determined by the method of Garewal (1973), with interference from protein being assessed from results obtained with bovine plasma albumin.

Electrophoresis

Polyacrylamide-disc-gel electrophoresis in the presence of 0.1% Brij 35 was performed by the method of Davis (1964). The 5mm×60mm gels were loaded with 40μg of protein, and electrophoresis was carried out at 2mA/gel until the tracker dye had passed through the spacer gel, and at 5mA/gel thereafter for a total of 2.5h. Samples of partially purified ATPase were solubilized before electrophoresis, by incubation with 0.1% Brij 35 for 1h at 37°C. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969). Samples were preincubated for 30min at 70°C with 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol in 50mM-sodium phosphate buffer, pH7. Approx. 25μg of protein was loaded on to each gel and electrophoresis was conducted at 8mA/gel for 2h. Marker proteins of known molecular weights were simultaneously electrophoresed in adjacent gels (Clarke *et al.*, 1975). Gels were stained for protein as described by Fairbanks *et al.* (1971).

Chemicals

Brij 35 was obtained from Honeywill–Atlas, Carshalton, Surrey, U.K., and Triton X-100 (containing approx. 10mol of ethylene oxide/mol) from BDH Chemicals, Poole, Dorset, U.K. Oligomycin and *NN'*-dicyclohexylcarbodi-imide were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and Dio-9 was from Gist–Brocades, Delft, The Netherlands. 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole was supplied by Micro-Bio Laboratories, London SW6 3DX, U.K. Other chemicals and enzymes were obtained from the sources reported by Clarke *et al.* (1975).

Results

Washed membranes (30–200mg of protein), prepared from *Cl. pasteurianum* harvested from a very-late-exponential-phase culture, were resuspended in 10ml of 10mM-Tris/HCl buffer, pH7.6, containing 20mM-$MgCl_2$, 20% (w/v) glycerol and Triton X-100 (9mg/mg of membrane protein). After incubation at 37°C for 30min, the suspension was centrifuged at 220000*g* for 2h at 16°C. Analysis of the supernatant (Table 1*a*) revealed that 58% of the membrane protein had been solubilized with the release of at least 76% of the membrane ATPase activity [assayed after depletion of Triton X-100

Table 1. *Summary of the purification of the ATPase complex from Cl. pasteurianum*

(*a*) Organisms harvested from culture in very late exponential phase: extract made and ATPase purified under aerobic conditions. (*b*) Organisms harvested from culture in early exponential phase: extract prepared and ATPase purified under anaerobic conditions with 2-mercaptoethanol added to buffer solutions (see the Experimental section). One unit of ATPase releases 1μmol of P_i/min at 37°C in the defined assay mixture at pH7.6.

Fraction	Volume (ml)	Protein (mg/ml)	ATPase activity (units/ml)	ATPase specific activity (units/mg of protein)	Yield (%)
(*a*)					
Cell extract	155.0	36.75	0.563	0.015	100.0
Washed membranes	5.0	7.25	1.718	0.237	9.8
Triton X-100 extract	2.5	8.48	2.610	0.308	7.4
Post Sepharose 4B	4.75	1.06	1.250	1.190	6.8
Post Sephadex LH-20	0.75	1.00	1.500	1.500	1.3
(*b*)					
Cell extract	155.0	77.1	10.65	0.138	100.0
Washed membranes	10.0	18.3	61.32	3.347	37.0
Triton X-100 extract	3.5	25.1	116.90	4.654	24.8
Post Sepharose 4B	3.5	6.82	103.20	15.130	21.9
Post Sephadex LH-20	2.0	1.8	21.80	12.100	2.6

by batch treatment with Sephadex LH-20 pre-swollen in aq. 50% (v/v) ethanediol]. No residual ATPase activity was detectable in the insoluble (extracted membrane) fraction. The supernatant was concentrated to 3ml by ultrafiltration at room temperature through an Amicon UM2 membrane (Amicon, High Wycombe, Bucks., U.K.) and loaded on to a column (2.6cm×40cm) of Sepharose 4B previously equilibrated with the eluting mixture of 0.05% Triton X-100 in 10mM-Tris/HCl buffer, pH7.6, containing 20mM-$MgCl_2$ and 20% glycerol. Elution was at 15°C at a rate of 10ml/h, 6ml fractions being collected and assayed for protein and for ATPase activity (which generally emerged in fractions 15–19). The ATPase-containing fractions were pooled (30ml), concentrated by Amicon UM2 ultrafiltration, and loaded on to a column (2.5cm×40cm) of Sephadex LH-20 pre-equilibrated with 50% (v/v) ethanediol in 10mM-Tris/HCl buffer, pH7.6, containing 20mM-$MgCl_2$. Elution with this mixture at 10ml/h at 15°C yielded ATPase activity in void-volume fractions that

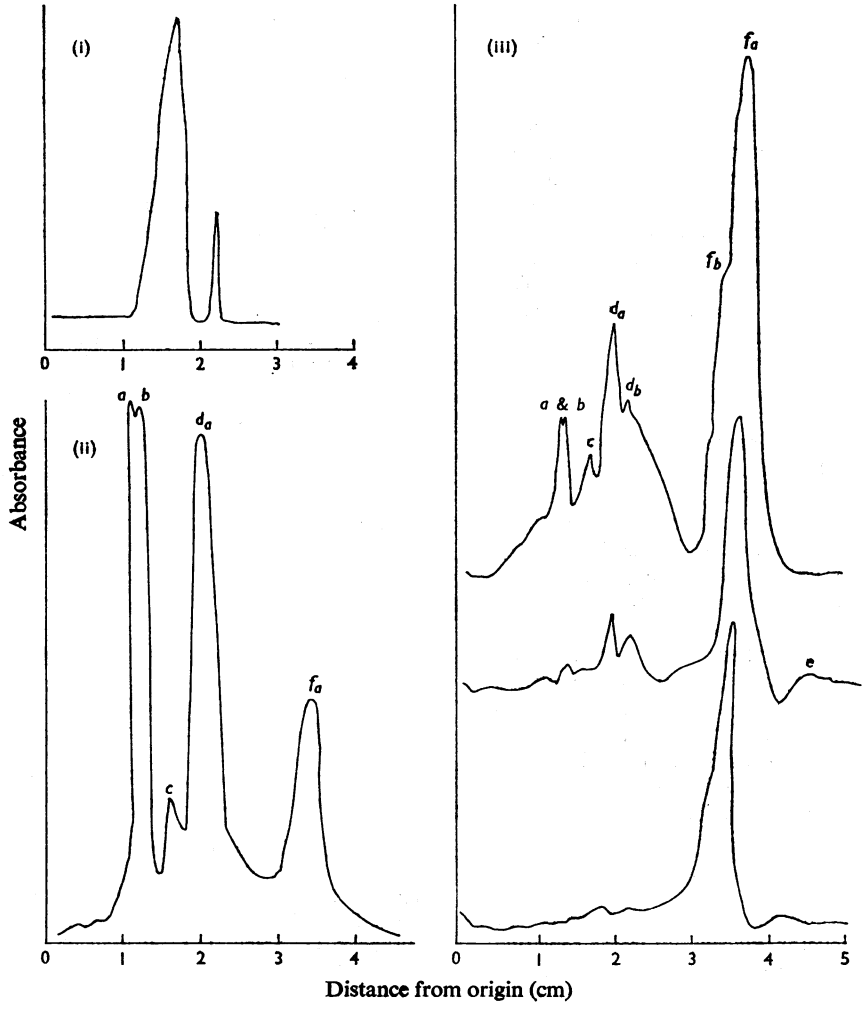

Fig. 1. *Polyacrylamide-disc-gel electrophoresis of preparations of the ATPase complex from Cl. pasteurianum*

(i) Electrophoresis in 0.1% Brij 35 of ATPase purified as in Table 1(*a*). (ii) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ATPase complex prepared as in Table 1(*b*). (iii) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ATPase complex prepared as in Table 1(*a*), before (top trace) and after washing twice (middle trace) or three times (bottom trace) with 10mM-Tris/HCl buffer, pH7.6, containing 10mM-EDTA. The protein bands were stained with Coomassie Blue and scanned at 609nm with a Gilford 2400-S recording spectrophotometer.

were distinguishable by their translucent appearance. These fractions (24ml) contained less than the minimum assayable concentration of Triton X-100 (i.e. less than 40μg/ml). They were pooled, concentrated as described above to 10ml, and centrifuged at 220000*g* for 2h at 4°C. The ATPase activity was located in the pellet, which was resuspended in the minimum volume (less than 1ml) of 90mM-Tris/HCl buffer, pH7.6, containing 8mM-$MgCl_2$. This particulate and significantly purified preparation (Table 1*a*) was fully soluble in 0.1% Brij 35, sodium dodecyl sulphate or Triton X-100, and its ATPase activity was 88% inhibited by 30min exposure at 37°C to dicyclohexylcarbodi-imide (5μM).

Only a minor part of the total ATPase activity of the organism was at first recovered in the initial washed membrane fraction (Table 1*a*). Preliminary studies showed, however, that much more of the enzyme activity remained attached to the membrane when (*a*) these were prepared from organisms harvested earlier from exponential-phase cultures, and (*b*) anaerobic and reducing conditions were maintained during isolation and washing of the membranes, with, for example, addition of 2-mercaptoethanol (see the Experimental section). Typical findings, which have now been repeated on several occasions, are shown in Table 1(*b*).

Action of inhibitors

The particulate ATPase preparation from growing *Cl. pasteurianum* (Table 1*b*) was not cold-labile, and mirrored the behaviour of the ATPase in washed membranes in its susceptibility to inhibition by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (0.1mM), chlorohexidine (150μg/ml), Dio-9 (60μg/ml), *NN'*-dicyclohexylcarbodi-imide (5μM) and the bacteriocin butyricin 7423 (190AU/mg of membrane protein; Clarke *et al.*, 1975). There was virtually nil inhibition by oligomycin (20μM). In contrast, the ATPase activity present in the soluble fraction of the initial cell extract, though inhibited by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, chlorohexidine and Dio-9, was not inhibited by *NN'*-dicyclohexylcarbodi-imide or by butyricin 7423.

Electrophoretic behaviour

Brij 35/polyacrylamide-gel electrophoresis of the particulate dicyclohexylcarbodi-imide-sensitive ATPase preparation (dissolved in 0.1% Brij 35) revealed the presence of only two protein components [one major and one minor band; Fig. 1(i)]. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis [Figs. 1(ii) and 1(iii)] demonstrated that these were composed of several polypeptide subunits with the following approximate mol.wts. (±10%): *a*, 65500; *b*, 62500; *c*, 57500; d_a, 43000; f_a, 15000; *e*, 10000; and, when present, d_b, 38000; f_b, 23000. Different preparations varied in their contents of the subunits of highest molecular weight (*a* and *b*), these being in greatest quantity in preparations derived from membranes with highest (retained) ATPase activity [cf. Fig. 1(ii) and Fig. 1(iii)]. Further, washing the ATPase complex with 10mM-EDTA in 10mM-Tris/HCl buffer, pH7.6 [Fig. 1(iii)], progressively removed the subunits of highest molecular weight (*a*, *b* and *c* most easily, followed by d_a and d_b).

Discussion

Both the physiological state of the harvested organisms and the way in which its membranes are prepared determine the proportion of the total ATPase activity that remains associated with the membrane of *Cl. pasteurianum*. Variations in this ATPase complex, including such features as increased susceptibility to activation by trypsin, and the appearance of subunits d_b and f_b and *e* as the culture begins to sporulate, have been investigated (Clarke & Morris, 1976).

The lipid composition of the particulate dicyclohexylcarbodi-imide-sensitive ATPase preparations has not yet been investigated, but it would appear that their polypeptide composition (particularly in the higher mol. wt. range, above 30000) resembles that of other bacterial ATPases (Futai *et al.*, 1974; Kobayashi & Anraku, 1974; West, 1974). However, comparison of the ATPase complex of *Cl. pasteurianum* with those of aerobic bacteria must await identification of the locations and functions of its polypeptide subunits.

References

Baron, C. & Thompson, T. E. (1975) *Biochim. Biophys. Acta* **382**, 276–285
Beechey, R. B. (1974) *Biochem. Soc. Spec. Publ.* **4**, 41–62
Clarke, D. J. & Morris, J. G. (1975) *Biochem. Soc. Trans.* **3**, 389–391
Clarke, D. J. & Morris, J. G. (1976) *Spore Research 1976*, Academic Press, London and New York, in the press
Clarke, D. J., Robson, R. M. & Morris, J. G. (1975) *Antimicrob. Ag. Chemother.* **7**, 256–264
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Evans, D. J. (1970) *J. Bacteriol.* **104**, 1203–1212
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2616
Futai, M., Sternweis, P. C. & Heppel, L. A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2725–2729
Garewal, H. S. (1973) *Anal. Biochem.* **54**, 319–324
Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 325–329
Kobayashi, H. & Anraku, Y. (1974) *J. Biochem. (Tokyo)* **76**, 1175–1182
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mackey, B. M. & Morris, J. G. (1971) *J. Gen. Microbiol.* **66**, 1–13
Muñoz, E., Freer, J. H. E., Ellar, D. J. & Salton, M. R. J. (1968) *Biochim. Biophys. Acta* **150**, 531–533
Nieuwenhuis, F. J. R. M., Thomas, A. A. M. & Van Dam, K. (1974) *Biochem. Soc. Trans.* **2**, 521–523
Pedersen, P. L. (1975) *Bioenergetics* **6**, 243–275
Riebeling, V., Thauer, R. K. & Jungermann, K. (1975) *Eur. J. Biochem.* **55**, 445–453
Robson, R. L., Robson, R. M. & Morris, J. G. (1974) *Biochem. J.* **144**, 503–511
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
West, I. C. (1974) *Biochem. Soc. Spec. Publ.* **4**, 27–38