SEPARATION OF CLOSTRIDIUM PERFRINGENS POLYNUCLEOTIDE PHOSPHORYLASE INTO TWO COMPONENTS*

E.Knight, Jr.**, P.S.Fitt and M.Grunberg-Manago
Institut de Biologie Physico-chimique, Paris, France.

Received February 5, 1963

Dolin et al.(1961a, b; 1962) described the isolation from the anaerobe Clostridium perfringens of a polynucleotide phosphorylase which differed markedly from other known examples of this enzyme isolated from a variety of organisms (Grunberg-Manago, 1963). The highly purified enzyme catalyzed the formation of polyadenylic acid from ADP, but did not catalyze the reverse reaction : phosphorolysis of poly A. Cytidine, uridine and guanosine diphosphates were only polymerized by the crude extracts, and the activity of the purified enzyme with ADP was totally dependent on the presence of polylysine, or other high molecular weight polyamines.

Attempts are being made to account for the disappearance of several of the activities during purification of the crude enzyme. In this paper, a procedure is described which has led to the separation of the enzyme into two fractions which differ in their ability to polymerize ADP, GDP, CDP and UDP, and their phosphorolytic activity.

Clostridium perfringens (A 1844) was obtained from Dr.A. Prévot, and grown and harvested as previously described (Dolin, 1962). The cells were suspended in 0.02 M Tris buffer, pH 7.4 (15 ml of buffer per 16 g. wet cells) and they were ruptured in an Eppenbach colloid mill (Gifford-Wood Co., Hudson, N.Y.). The mixture was centrifuged for an hour at 18,000 r.p.m. in a Servall refrigerated centrifuge; the supernatant was purified by ammonium sulphate precipitation (25-85%), protamine fractionation and a second ammonium sulphate precipitation (60-79%). An approximately tenfold purification of the activity was achieved in this way. The second $(NH_4)_2SO_4$

*Supported in parts by N.I.H.grant 4580 and Biologie Moléculaire grant 61-FR-087

** Postdoctoral Fellow of National Institutes of Health

fraction, after dialysis against 0.02 M Tris buffer, pH 7.4, was then fractionated further with calcium phosphate gel by the following procedure : a 10 ml aliquot (10 mg protein/ml; S.A. of ADP incorporation activity with polylysine, 1.24) was diluted with 40 ml of 0.02 M Tris buffer, pH 7.4. The pH of the solution was adjusted to 5.7 with 1 M sodium acetate, pH 5.4. The solution was stirred slowly for 15 minutes at 0° with 1 ml of well-aged calcium phosphate gel (20.5 mg/ml). The suspension was centrifuged and the pellet was discarded. The supernatant was stirred for 15 minutes at 0° with a further 3.25 ml of gel and the supernatant after centrifugation was discarded. The pellet was extracted successively with 3 ml each of the following phosphate buffers : (1) 0.02 M, pH 6; (2) 0.02 M, pH 7; (3) 0.02 M, pH 7.25; (4) 0.02 M, pH 7.5; (5) 0.03 M, pH 7.5; (6) 0.05 M, pH 7.5; (7) 0.10 M, pH 7.5; (8) 0.15 M, pH 7.5. The precipitate was stirred for 15 minutes at 0°C with each buffer and the suspension was then centrifuged. The pellet was used for the next extraction. The supernatants were dialyzed against 0.02 M Tris-chloride buffer, pH 7.4 (3 x 1000 ml).

The results of the calcium phosphate gel fractionation are shown in Figures 1 and 2 (the ADP and GDP activities shown were determined in the presence of polylysine). This method separates the activities of the enzyme preparation into two fractions; the ADP and GDP incorporating activities each have two peaks, whilst the UDP and CDP incorporating activities and the phosphorolytic activity have only one. The incorporating activities in both fractions are specific for diphosphates and no reaction occurs with the triphosphates.

The first fraction (gel fractions 2 and 3) contained all the five activities that were assayed. The peaks of the UDP and CDP incorporating activities, and the bulk of the phosphorolytic activity, were in this fraction. A similar distribution of phosphorolytic activity was found when either poly A or poly U were used as substrate.

The second fraction (gel fractions 5 and 6) contained the major peaks of ADP and GDP incorporating activity. The three other activities, although definite, were much lower in this fraction than in the first.

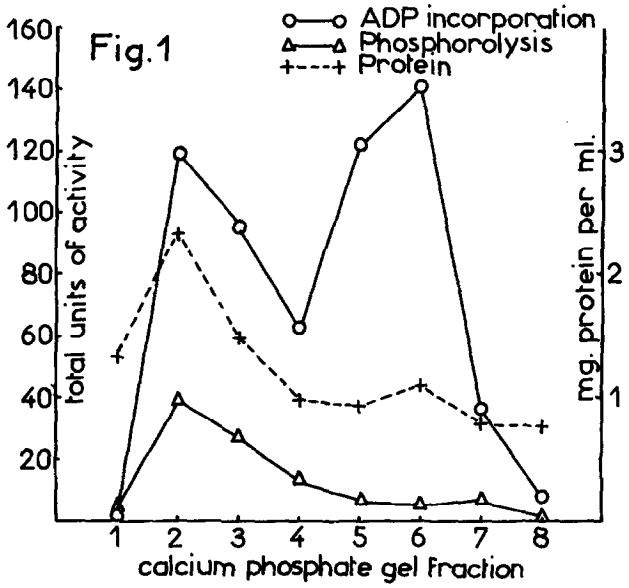

Fig. 1 Distribution of ADP incorporation and phosphorolytic activities of the Cl. perfringens enzyme after calcium phosphate gel fractionation

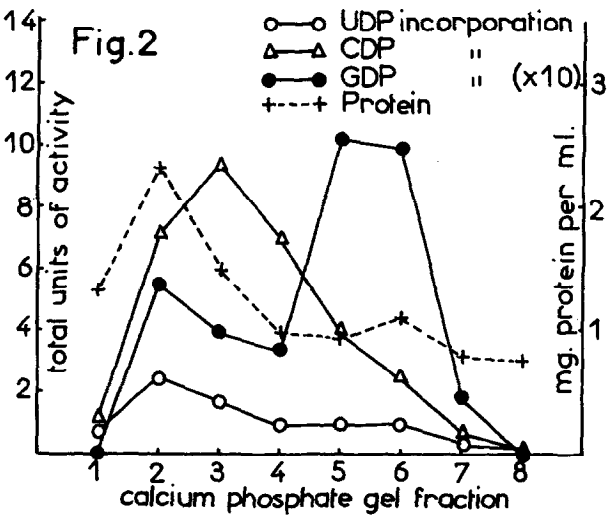

Fig.2 Distribution of UDP, CDP and GDP incorporation activities of the Cl. perfringens enzyme after calcium phosphate gel fractionation

Assay conditions (Fig. 1 and 2)

(i)Reaction mixtures for assay of enzyme activity by incorporation of radioactive NDP-^{14}C into an acid insoluble precipitate (Dolin, 1962; Littauer and Kornberg, 1957) contained, in 0.25 ml final volume : Tris-chloride buffer, pH 8.2, 25 µmoles; $MgCl_2$, 15 µmoles; EDTA, 0.25 µmoles; NDP-^{14}C, 1 µmole (S.A.:20-40, 000 c.p.m./µmole); polylysine (M.W. 6000), 50 µg where appropriate; enzyme, up to 0.2 units. Incubation 30 minutes at 37° C. The reaction was stopped with 1% perchloric acid (0.25 ml). Carrier protein was added and the mixture was allowed to stand at 0°C for 10 minutes. The precipitate was collected on a millipore filter, washed with 1% perchloric acid (6 x 2 ml), dried and counted.

(ii) UDP incorporating activity was also assayed by phosphate release, using a similar reaction mixture, but with non-radioactive UDP. After addition of 7% perchloric acid, the phosphate was determined by standard methods (Grunberg-Manago, 1963).

(iii) Phosphorolytic activity was assayed by the standard procedure (Grunberg-Manago, 1963). Reaction mixture contained, in 0.2 ml final volume : Tris-chloride buffer, pH 7.4, 25 μmoles; $MgCl_2$, 1.4 μmoles; EDTA, 0.15 μmoles; K_2HPO_4, 7.5 μmoles; ^{32}P, ca. 400,000 c.p.m.; poly A, 240 μg; enzyme, up to 0.15 units (Assays were also carried out at pH 8.2). Incubation, 30 minutes at 37°C.

The ADP and GDP incorporating activities in both peaks were stimulated by polylysine; this effect was variable and dependent to some extent on enzyme concentration. The maximum stimulation of the ADP incorporating activity of the first fraction was 3-4 fold. The activity of the second fraction was initially stimulated 10-80 fold, but the effect was transient, and after the enzyme had stood for 5 days at 2°C the stimulation was similar to that observed with the first fraction. Polylysine had no significant effect on either UDP or CDP incorporation.

The results of refractionation, by the same procedure, of gel fractions 2 and 6 make it unlikely that the peaks could be artefacts produced by the treatment of the enzyme with calcium phosphate gel.

It appears that the polynucleotide phosphorylase of Cl. perfringens consists of at least two enzymes with somewhat different activities and attempts are now being made to obtain a cleaner separation of the two products.

REFERENCES

Dolin, M.I., Godiniaux, E. and Grunberg-Manago, M., Bact. Proc., 61, 180 (1961a)

Dolin, M.I., Biochem. Biophys. Res. Comm., 6, 11 (1961b)

Dolin, M.I., J. Biol. Chem., 237, 1626 (1962)

Grunberg-Manago, M., Progress in Nucleic Acid Research, J.N. Davidson and W.E. Cohn (Eds.), Academic Press Inc., N.Y. (in press)

Littauer, U.Z. and Kornberg, A., J. Biol. Chem., 226, 1077 (1957)