CLOSTRIDIUM PERFRINGENS POLYNUCLEOTIDE PHOSPHORYLASE: INTERCONVERSION OF HEAVY AND LIGHT MOLECULAR WEIGHT SPECIES

A. Guissani and M. Grunberg-Manago
Institut de Biologie Physico-chimique, rue P. Curie, Paris, France

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Evidence is presented showing that the 200 000 species ($S_{20,W}$ = 9S) of Clostridium perfringens polynucleotide phosphorylase is partially converted into a 70 000 species ($S_{20,W}$ = 4.5S) by 30 minutes preincubation at 25 °C with β-mercaptoethanol. The two species, which have different catalytic properties, can also be distinguished by their different mobility through disc electrophoresis technique on acrylamide gel. Interconversion of the two species is studied.

Polynucleotide phosphorylase, a bacterial enzyme, catalyzes the reversible polymerization of ribonucleotides :

$$n\ ppN \quad ========== \quad n\ p + (pN)_n$$

and the exchange reaction :

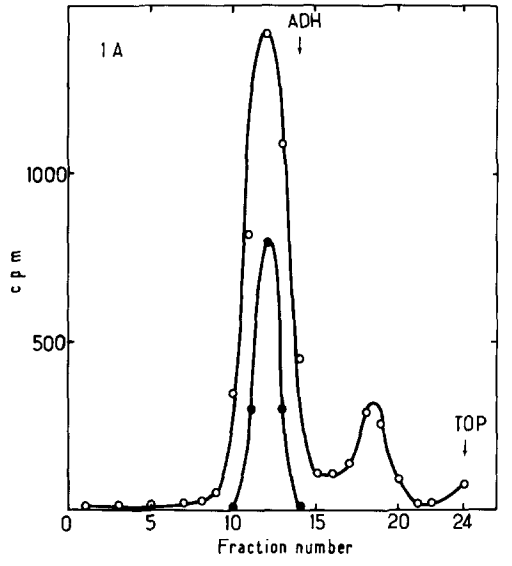
$$ppN + {}^{32}p \quad ======== \quad p\ {}^{32}pN + p$$

Polynucleotide phosphorylase isolated from the anaerobic bacterium Clostridium perfringens has special properties as compared with polynucleotide phosphorylase isolated from other bacteria. In this organism the polymerization of ADP into polyadenylic acid (poly A) is highly stimulated by the presence of polylysine, which lowers the Km for ADP and increases the V_{max}; a decrease in the Km for ADP is also observed with salts which, however, have no effect on the V_{max}. Another property of clostridial polynucleotide phosphorylase is that the divalent cation Mg^{++} cannot be replaced by Mn^{++}.

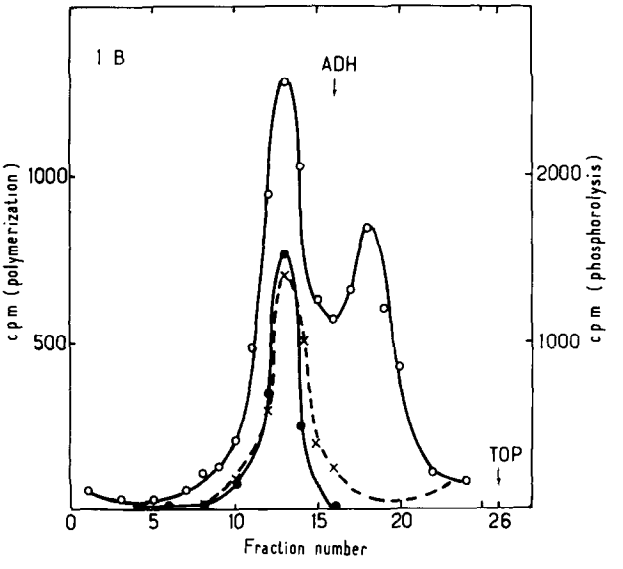
Dietz and Grunberg-Manago (1967a) had presented evidence that C. perfringens polynucleotide phosphorylase could be separated, by sucrose density gradient centrifugation, into two molecular components : a heavy one and a light one. The proportion of the light component varies between 0-25% with the preparations, but this light component does exist in crude extracts, suggesting that it is not a result from purification

Figure 1

Activity profile for poly A synthesis and phosphorolysis after sucrose gradient centrifugation



1A. Untreated enzyme



1B. Treated enzyme

1A. 200 µg of enzyme (purified as explained in text, specific activity 70 units/mg. 1 unit = 1 µmole ADP incorporated/hr incubation) were layered on a 5-20% sucrose density gradient made in a buffer containing in mM: Tris HCl, 20; EDTA, 1; β-SH, 7; pH 8.3, room temp. The gradient was centrifuged for 6 hours at 65 000 rpm in a Beckman L_2-65K ultracentrifuge at 4°C. The assay for enzyme activity by incorporation of radioactive ADP into acid insoluble precipitate was that of Knight et al. (1963) The complete system, including 10 µl of the enzyme fraction from the gradient (final volume, 200 µl) contained the following reagents in mM : Tris HCl, pH 8.3, room temperature, 100; $MgCl_2$, 5; ADP(14C)(specific activity 8 900 cpm/µmole) 4; β-SH, 1; polylysine (MW 3000), 400 µg/ml. Incubation 16 hours at 25°C.

1B. A mixture (final volume, 100 µl) containing in mM: Tris HCl, 100; $MgCl_2$, 15; β-SH, 100; and enzyme, 200µg, was preincubated 30 min just before use.

o—o—o polylysine is replaced by $(NH_4)_2SO_4$, 0.2M.

x—x—x phosphorolysis activity was assayed by standard procedure (Knight)

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procedure (Dietz and Grunberg-Manago, 1967 b).

A preliminary study of these two components has shown that the activity of the light one is absolutely dependent upon the

presence of β-mercaptoethanol (β-SH); by contrast, the activity of the heavy material is slightly inhibited by this reagent.

In order to decide whether the low molecular weight species represents a sub-unit of the heavier molecule, several procedures were tried to obtain a conversion of the heavy species into the light one (acid, pH, urea, β-SH): A 30 minutes pre-incubation of the enzyme at 25 °C with 100 mM of β-mercaptoethanol is a general procedure which always results in the separation of the heavy component of clostridial polynucleotide phosphorylase ($S_{20,W}$ = 9S) into two molecular species ($S_{20,W}$ = 9S and 4.5S, which, using alcohol dehydrogenase as a standard, corresponds approximately to molecular weights of 200 000 and 70 000, respectively) with different catalytic properties. Preincubation of the enzyme under the same conditions, except for the presence of β-SH, did not lead to the formation of the light material and no loss of activity was observed; nor is there any yield of the light species when β-mercaptoethanol is added to the enzyme without preincubation. In order to obtain the light species, therefore, the sole presence of β-SH is not enough, but preincubation of the enzyme at 25 °C with β-mercaptoethanol is necessary. Approximately the total activity of the heavy component for ADP polymerization in the presence of polylysine was recovered in the two components after preincubation, indicating that the light component is derived from the heavy one.

The 200 000 MW species of C. perfringens polynucleotide phosphorylase shows all the properties of the untreated enzyme; the 70 000 MW species not only does not catalyze the phosphorolysis reaction, but the only reaction which could be demonstrated is the polymerization of ADP into poly A with an absolute requirement for polylysine and β-mercaptoethanol. No activity is found when polylysine is replaced by ammonium sulphate (fig. 1).

The same properties had been found for the pre-existing light species (Dietz and Grunberg-Manago, 1967 a); we may therefore assume that the light species obtained from the heavy component after β-SH treatment is similar to the one naturally found in crude extracts or purified enzyme.

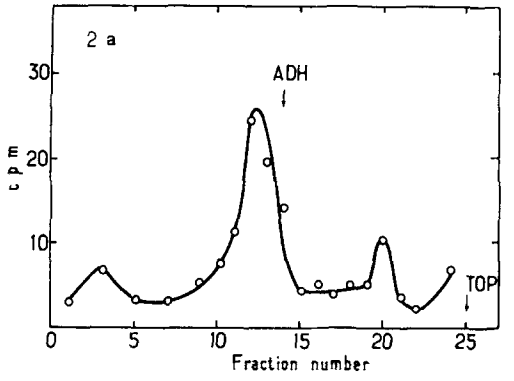
Preliminary studies have been carried out on the inter-conversion of the two components.

When the heavy molecular weight species, separated by

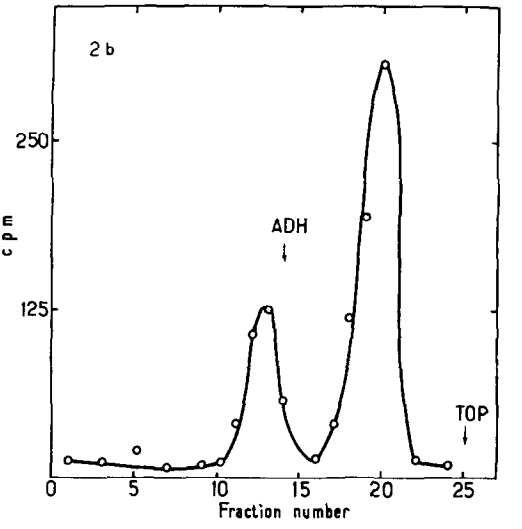
Figure 2

Purification of the two species

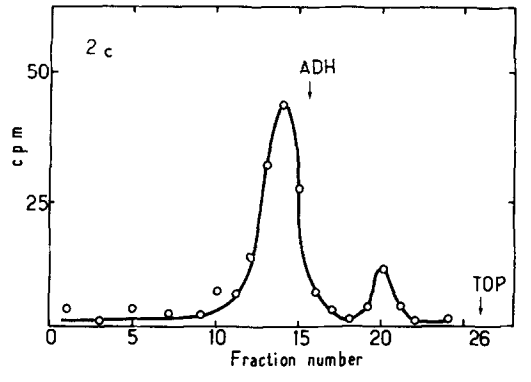
The material was pooled from a gradient, lyophilyzed to 300 µl, and layered again on another gradient. The incorporation assay is the polymerization of ADP (specific activity 8900 com/µmole) into poly A in the presence of polylysine and β-mercaptoethanol. Incubation 16 hours at 37°C.



2a. Heavy material pooled from tubes 9 to 13 of the second gradient, not show here



2b. Light material pooled from tubes 18 to 24 of the first gradient (fig. 1B). (Heavy material between tubes 11 to 15)



2c. Light material pooled from tubes 18 to 24 of the second gradient (fig. 2b)

a 5-20% sucrose gradient, was dialyzed, lyophilyzed, and then layered on top of a second identical gradient, it yielded 80% of the heavy species, a third identical gradient yielded 85% (fig. 2a).

When the same gradient procedure was applied to the light

species (representing 40% of the total polymerization activity of ADP into poly A, in the presence of polylysine) a heavy peak corresponding to 20% of the gradient activity was still observed in the second gradient. A third gradient, centrifuged with the light species from the second gradient yielded almost only the heavy species (fig. 2 b and c). A rough estimate of the units recovered in the heavy component, correcting for the loss of activity in experimental conditions, suggest that the heavy MW species observed in the third gradient cannot result from the contaminating heavy species of the second gradient, and the light MW species may, therefore, have been converted into the heavy one. This hypothesis is strengthened by the results of electrophoresis as we shall now see, but the interpretation of the above results is complicated by the instability of both components, particularly the light one, in dilute solutions.

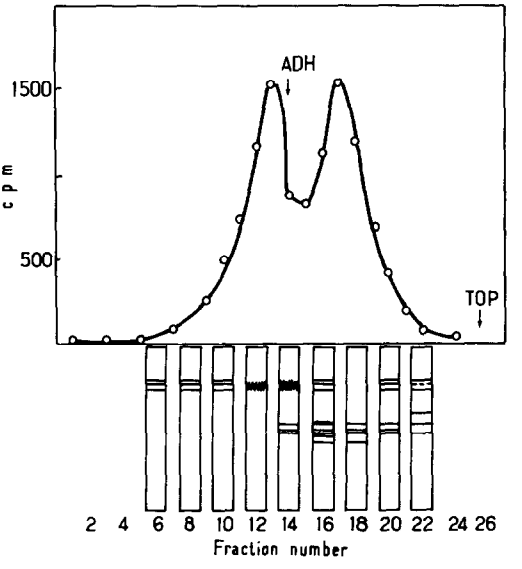
All the fractions of a gradient which had shown the two species (50%/50%) were tested by disc electrophoresis technique with 7.5% acrylamide (fig. 3); after incubation with ADP the polymer formed was stained with orange acridine, thus revealing enzymic activity (Thang et al., 1967). This clearly shows two groups of bands : the upper bands of slower mobility correspond to the high molecular weight species, and the lower bands of faster electrophoretic mobility correspond to the low molecular weight species since they appear concomitally with this component. As expected, an enzymatic fraction which had no detectable light species, as seen by the gradient, showed no significant faster moving bands. However, on disc electrophoresis, the light species always yielded, with the faster moving bands, a small percentage of slower moving bands which are only visible after a long preincubation. This could indicate that there is an important rearrangement of the light species into the heavy one, and would be in favour of the hypothesis put forward after the gradient experiments; it would also explain why it is so difficult to obtain a pure preparation of the light species.

Disc electrophoresis was carried out at different acrylamide concentrations ranging from 4% to 10% (Hedrick and Smith, 1968). This technique distinguishes between molecules which differ by their charge, or their molecular weight, or both. With crude extracts from E. coli and C. perfringens, we observed that the slowest moving perfringens band always corresponds to

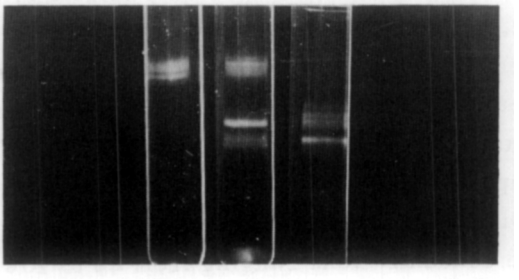
Figure 3

Electrophoresis of all the fractions from a sucrose gradient (treated enzyme)

Same as in fig. 1B, except that 7.5 polymerization units contained in 150 μg of purified enzyme were preincubated with 100 mM of β-mercaptoethanol and layered on the gradient.
Half of each fraction, i.e. 100 μl, was subjected to electrophoresis on acrylamide gel at 7.5%. Each gel is submitted to 6 mA intensity; the buffer used is Tris-glycine, pH 8.9 (Thang et al., 1967). The gel was then incubated in the presence of polylysine and β-SH in 5 ml of the same incubation mixture (fig. 1A) but with non-radioactive ADP. The polymer formed was then stained with orange acridine and de-stained by electrophoresis.



drawing of gels



fraction n° 7 15 18

photo of gels

the E.coli band (Thang et al., 1967), indicating that these bands have identical molecular weights and charges. From the same experiment with treated perfringens enzyme, a molecular weight of about 220 000 for the heavy bands and about 80 000 for the lighter bands was calculated. This is in agreement with the molecular weights previously calculated with the gradient experiments. It seems therefore possible that the size of the lighter molecules is about one third that of the heavy ones.

Seen in the light of the above results, several conclusions can be drawn :

- The light species is derived from the heavy species, it probably represents a sub-unit, and possibly reverts to the heavy species, as seen with gradient and disc electrophoresis.

- Disc electrophoresis on acrylamide gel shows that both the heavy and the light species are composed of several types of molecules with different charges : therefore both species of clostridial polynucleotide phosphorylase are multi-banded.

- It is very possible that the highly purified starch electrophoresis clostridial enzyme obtained by Dolin (1962) was the light species. The two fractions obtained by Knight et al. (1963) by calcium phosphate gel might also be related to the two species we have described.

It is interesting to note that Singer (Klee and Singer, 1967) also observed a structural effect on M. luteus polynucleotide phosphorylase when treated with β-mercaptoethanol, but in this case there was no change in the molecular weight of the enzyme and the action of β-SH appeared to be merely a substitution of SX with SH groups. In contrast, in the case of C. perfringens, the β-mercaptoethanol causes the separation of the enzyme into sub-units (or maybe groups of sub-units); these sub-units might be directly bound by S-S groups. But the separation into sub-units might also be the result of a conformational change due to the disruption of S-S intramolecular bonds or S-X substitutions. Thus, the molecular weight (and of course electrophoretic mobility) is very different before and after treatment, and a new dependence on β-mercaptoethanol and polylysine appears, as well as an alteration of the reactions catalyzed.

Since two different molecular species are readily obtained from C. perfringens polynucleotide phosphorylase after treatment with β-mercaptoethanol, it is hoped that much infor-

mation can be obtained regarding the formation, arrangement, and the role of these sub-units.

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