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A Study by Polyacrylamide-Gel Electrophoresis of the Effect of Proteolysis on *Micrococcus lysodeikticus* Polynucleotide Phosphorylase

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1. Trypsin digestion of *Micrococcus lysodeikticus* polynucleotide phosphorylase (nucleoside diphosphate–polynucleotide nucleotidyltransferase) causes a progressive increase in electrophoretic mobility in polyacrylamide gels of the single active degradation product. 2. A marked increase in primer requirement for CDP polymerization occurs before a more mobile product is formed. 3. α-Chymotrypsin digestion yields a product that separates into several active species on polyacrylamide-gel electrophoretograms. 4. No separation of ADP- and CDP-polymerization activities occurs during electrophoresis after either trypsin or α-chymotrypsin treatment.

Limited digestion of *Micrococcus lysodeikticus* polynucleotide phosphorylase (nucleoside diphosphate–polynucleotide nucleotidyltransferase, EC 2.7.7.8) with trypsin had a marked effect on its relative activities (Fitt & Fitt, 1967*a*). A substantial increase in the primer requirement for CDP polymerization was observed with virtually no effect on the ADP-polymerization activity or the phosphorolytic activity towards either polyA* or polyC. The treated and untreated enzymes could not be clearly separated by Sephadex G-200 filtration: there was therefore only a small change in molecular size during trypsin treatment in these conditions. These results were confirmed by polyacrylamide-gel electrophoresis (Fitt & Fitt, 1967*b*): the digested enzyme migrated as a single active species with a mobility similar to that of the main fraction of the untreated enzyme.

More extensive proteolysis of polynucleotide phosphorylase leads to an increase in primer requirement for ADP polymerization (Fitt & Fitt, 1967*a*; Klee, 1967; Klee & Singer, 1967), an increase in its electrophoretic mobility (Klee, 1967) and some change in its mobility during Sephadex-gel filtration (Klee & Singer, 1967). The primer requirement could be eliminated by subsequent incubation of the treated enzyme with β-mercaptoethanol (Klee, 1968).

This paper describes gel-electrophoretic studies of *M. lysodeikticus* polynucleotide phosphorylase before and after proteolysis.

* Abbreviations: polyA, polyadenylic acid; polyC, polycytidylic acid; ApA, adenylyl-(3′-5′)-adenosine.

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: unlabelled ribonucleoside diphosphates, Calbiochem, Los Angeles, Calif., U.S.A.; crystalline trypsin, α-chymotrypsin, soya-bean trypsin inhibitor and pancreatic trypsin inhibitor, Worthington Biochemical Corp., Freehold, N.J., U.S.A.; *M. lysodeikticus* spray-dried cells, Miles Chemical Co., Elkhart, Ind., U.S.A.; Cyanogum 41 and *NNN′N′*-tetramethylethylenediamine, E–C Apparatus Corp., Philadelphia, Pa., U.S.A.; ApA, Gallard–Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.; [^{14}C]ribonucleoside diphosphates, Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Polynucleotide phosphorylase. The experiments described in Fig. 1 were carried out with the enzyme previously obtained by Sephadex G-200 filtration of polynucleotide phosphorylase before and after trypsin digestion (Fitt & Fitt, 1967*a*, Fig. 6). These two preparations differed only in the dependence of the CDP-polymerization activity of the trypsin-treated enzyme on the presence of primer (ApA); their ADP-polymerization activities, with and without primer, and their phosphorolytic activities with either polyA or polyC were identical.

In other experiments, the enzyme used was prepared as follows. Lysis of the cells and $(NH_4)_2SO_4$ fractionation were carried out by the procedure of Singer & Guss (1962). The fraction insoluble between 30% and 65% saturation with $(NH_4)_2SO_4$ was fractionated with protamine and $(NH_4)_2SO_4$ by the method of Fitt, Dietz & Grunberg-Manago (1968). The product, dissolved in 0·01 M-tris–HCl buffer, pH 8·1–1 mM-EDTA–2 mM-β-mercaptoethanol–0·1 M-NaCl, was applied to a column (2·5 cm. × 40 cm.) of DEAE-Sephadex A-50 (bead form) equilibrated with the same buffer, and eluted at approx. 60 ml./hr. with successive gradients of 0·1–0·25 M-NaCl (700 ml.) and 0·25–0·35 M-NaCl (1 l.) in 0·01 M-tris–HCl buffer, pH 8·1–1 mM-EDTA–2 mM-β-mercaptoethanol (referred to below as 'standard buffer').

The active fractions, eluted between 0·3 M- and 0·33 M-NaCl, were combined, and solid $(NH_4)_2SO_4$ was added to 65% saturation. The precipitate was dissolved in the minimum volume of 0·1 M-tris–HCl buffer, pH 8·7, and the solution was passed at 10 ml./hr. through a column (2·5 cm. × 90 cm.) of Sephadex G-200 equilibrated with 0·01 M-tris–HCl buffer, pH 8·1–1 mM-EDTA. The active fractions were concentrated by $(NH_4)_2SO_4$ precipitation and chromatographed on a second DEAE-Sephadex column (1·5 cm. × 26 cm.) as described above, with successive gradients of 0·20–0·26 M-NaCl (200 ml.) and 0·26–0·34 M-NaCl (500 ml.) in standard buffer. The active fractions were combined and dialysed against standard buffer, and the enzyme was concentrated by adsorption on a small (0·9 cm. × 2·5 cm.) DEAE-Sephadex column followed by elution with 0·8 M-NaCl in standard buffer. The concentrated enzyme fractions were dialysed free from NaCl against 0·01 M-tris–HCl buffer, pH 8·1, and stored at −20°. The product was purified 200-fold (specific activity 20 units/mg.).

The $(NH_4)_2SO_4$ saturations were calculated by the procedure of Dixon & Webb (1964). All operations after initial lysis of the cells were carried out at 2°.

Assay procedures. Polynucleotide phosphorylase was assayed for phosphorolytic and nucleoside diphosphate-polymerization activities as described by Fitt & Fitt (1967*a*). One unit of enzyme activity is defined as the amount of enzyme catalysing the incorporation of 1 μmole of [^{32}P]-orthophosphate into ADP in the conditions of the polyA phosphorolysis assay. All specific activities are in terms of units of phosphorolysis activity/mg. of protein.

Protein was determined spectrophotometrically (Warburg & Christian, 1942).

Polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis was carried out at 2° in a Canalco model 6 disc-electrophoresis apparatus fitted with either a six- or a 12-place upper bath. The single-gel procedure of Clarke (1964) and Hjertén, Jerstedt & Tiselius (1965) was used. The monomer mixture contained the following components: Cyanogum 41, 1·05 g.; *NNN'N'*-tetramethylethylenediamine, 0·02 ml.; ammonium persulphate, 20 mg.; 0·2 M-tris–borate buffer, pH 9·2, 15 ml. The gels were 5 cm. long × 5 mm. diam. The electrode baths contained 0·1 M-tris–borate buffer, pH 9·2. After a 15 min. initial run at the full operating voltage, the enzyme samples containing 5% (w/v) sucrose were layered on to the gels. Electrophoresis was carried out at 50v for 15 min. and then at 120v for the indicated times (except in the experiment described in Fig. 1, where a constant-current supply was used). The full complement of gels (six or 12) was always used. Protein was stained with Amido Black. For locating the enzyme, the gels were incubated for 1 hr. at 37° in a medium containing: 75 mM-tris–HCl buffer, pH 9; 20 mM-nucleoside diphosphate; 5 mM-$MgCl_2$; 0·2 mM-EDTA; ApA, 0·2 mg./ml. (omission of the ApA decreased the sensitivity of the test without altering the number of bands.) The gels were washed with 7% (v/v) acetic acid, and the polynucleotides formed during the incubation were stained with either (i) 2% (w/v) acridine orange–1% (w/v) $La(NO_3)_6,6H_2O$ in 15% (v/v) acetic acid (Richards, Coll & Gratzer, 1965) or (ii) 0·2% (w/v) methylene blue in 0·4 M-sodium acetate, pH 4·7 (Peacock & Dingman, 1967). The gels were destained electrophoretically after method (i) or with running water after method (ii). The staining procedure (ii) of Peacock & Dingman (1967) proved to be more sensitive and has been used in all our more recent studies, although the procedure of Richards *et al.* (1965) gives more stable colours. The destained gels were scanned with a Joyce–Loebl Chromoscan densitometer in conditions that gave maximum resolution for each gel. The pen deflexions shown in the Figures are not a quantitative measure of the relative enzymic activity but permit a precise comparison of the relative band distribution in different gels.

RESULTS

Gel electrophoresis of polynucleotide phosphorylase. Many preparations of *M. lysodeikticus* polynucleotide phosphorylase separated into multiple active fractions during electrophoresis in 7% polyacrylamide gels in both the presence and the absence of urea (Fitt & Fitt, 1967*b*). A typical example of such an electrophoretogram is shown in Fig. 1(*a*). However, recent preparations by the method described in the Experimental section consistently yielded an enzyme that migrated as a single active species (Figs. 4*a* and 5), and all the experiments described other than those in Fig. 1 were performed with such a preparation. Grunberg-Manago (1967) and Klee (1967) also described the separation of polynucleotide phosphorylase from *Escherichia coli* and from *M. lysodeikticus* respect-

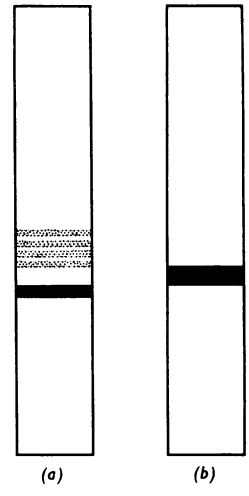

Fig. 1. Polyacrylamide-gel electrophoretograms of *M. lysodeikticus* polynucleotide phosphorylase. Electrophoresis was for 2 hr. at 2·75 mA/gel. Gels stained for either ADP- or CDP-polymerization activity (see the Experimental section) gave similar results. (*a*) Untreated enzyme; (*b*) enzyme after limited trypsin digestion (Fitt & Fitt, 1967*a*). The enzymes in (*a*) and (*b*) could not be separated by Sephadex G-200 filtration, and their activities differed only in the requirement of the treated enzyme for primer in CDP polymerization.

Table 1. *Trypsin digestion of* M. lysodeikticus *polynucleotide phosphorylase*

Expt. 1. The digestion medium (final vol. 0·23ml.) contained: tris–HCl buffer, pH 8·1, 2·3μmoles; trypsin, 0·04μg.; polynucleotide phosphorylase (specific activity 20units/mg.), 16μg. The control also contained soya-bean trypsin inhibitor, 1μg. After incubation for 5min. at 37°, the digestion was stopped with soya-bean trypsin inhibitor (0·01ml.; 0·1mg./ml.), water (0·01ml.) was added to the control, and the solutions were cooled in ice. They were assayed for ADP- and CDP-polymerization activities, and used for gel electrophoresis as described in Fig. 2. Expt. 2. The digestion medium (final vol. 0·69ml.) contained: tris–HCl buffer, pH 8·1, 6·9μmoles; trypsin, 0·24μg.; polynucleotide phosphorylase (specific activity 20units/mg.), 48μg. The control also contained soya-bean trypsin inhibitor, 6μg. After incubation for 1hr. at 37°, the digestion was stopped with soya-bean trypsin inhibitor (0·03ml.; 0·2mg./ml.), water (0·03ml.) was added to the control, and the solutions were cooled in ice. They were assayed for ADP- and CDP-polymerization activity and used for gel electrophoresis as described in Figs. 3 and 4. Nucleoside diphosphate-polymerization assay conditions were as follows. The reaction mixtures (final vol. 0·1ml.) contained: tris–HCl buffer, pH 9, 15μmoles; $MgCl_2$, 1μmole; EDTA, 0·04μmole; [^{14}C]nucleoside diphosphate, 4μmoles (about 5000–10000counts/min./μmole); ApA (where appropriate), 0·1mg.; enzyme. Incubation was at 37° for 30min. The reaction was stopped by the addition of 0·1ml. of 7% (v/v) $HClO_4$ and the mixture was kept at 0° for 10min. The precipitated polynucleotide was collected on a Whatman GF/C glass-fibre filter, washed four times with 1% (v/v) $HClO_4$, once with aq. 50% (v/v) ethanol, and dried and assayed for radioactivity. The residual activity of the digested enzyme is expressed as a percentage of the activity of the controls assayed in the same conditions.

	Residual activity (%)	
Activity assayed	Expt. 1	Expt. 2
ADP polymerization	72	13
ADP polymerization in the presence of ApA	96	60
CDP polymerization	41	1·5
CDP polymerization in the presence of ApA	89	63

ively into multiple active fractions by disc electrophoresis.

Effect of trypsin digestion on the electrophoretic mobility of polynucleotide phosphorylase. The complex pattern of active bands obtained with some untreated polynucleotide phosphorylase preparations was changed by mild trypsin treatment to a single active band with a mobility similar to that of the major fraction in the corresponding untreated enzyme (Figs. 1*a* and 1*b*): the minor bands originally observed were eliminated. The preparations used differed only in the dependence of incorporation of CDP by the digested enzyme on the presence of added primer (ApA). Since it had also been shown (Fitt & Fitt, 1967*a*) that the treated and untreated enzymes used could not be separated by gel filtration, only a limited change in the molecule had taken place during this controlled proteolysis.

A study of the effect of a more extensive digestion of *M. lysodeikticus* polynucleotide phosphorylase was then undertaken. The results in Table 1 and

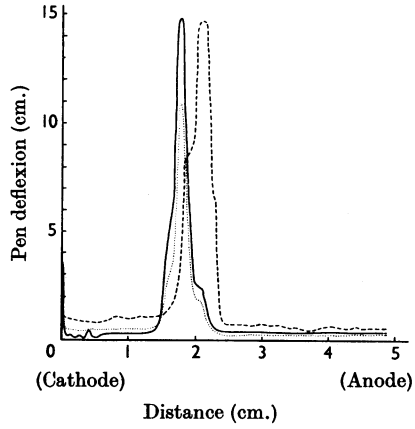

Fig. 2. Densitometer tracings of polyacrylamide-gel electrophoretograms of *M. lysodeikticus* polynucleotide phosphorylase before and after trypsin digestion in the conditions of Expt. 1 in Table 1. Electrophoresis was at 120v for 2hr. ADP-polymerization activity: ·····, untreated enzyme; ——, control; – – – –, digest.

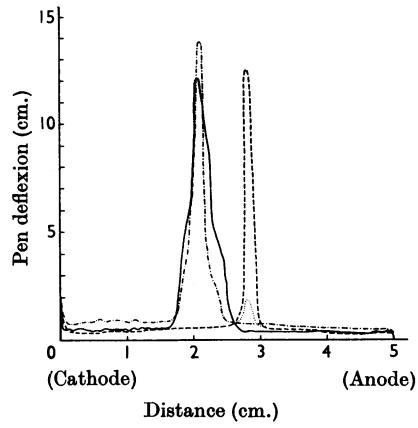

Fig. 3. Densitometer tracings of polyacrylamide-gel electrophoretograms of *M. lysodeikticus* polynucleotide phosphorylase before and after more extensive trypsin digestion in the conditions of Expt. 2 in Table 1. Electrophoresis was at 120v for 2hr. ADP-polymerization activity: ——, control; – – – –, digest. CDP-polymerization activity: –·–·–, control; ·····, digest.

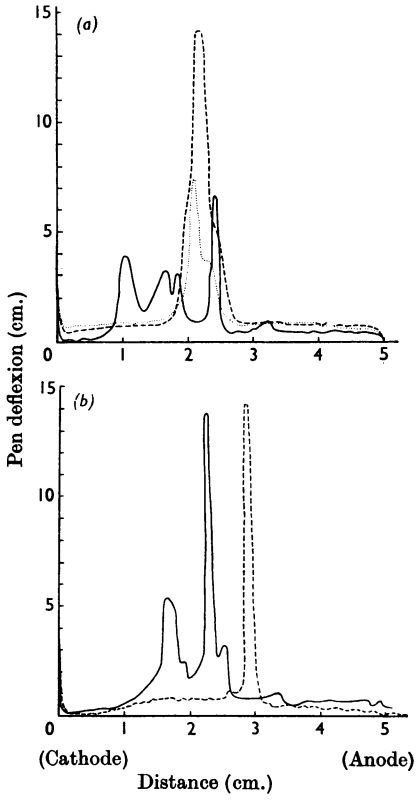

Fig. 4. Densitometer tracings of polyacrylamide-gel electrophoretograms of *M. lysodeikticus* polynucleotide phosphorylase before and after extensive trypsin digestion in the conditions of Expt. 2 in Table 1. Electrophoresis was at 120v for 2hr. (*a*) ——, Protein, control; ----, ADP polymerization, control; ·····, ADP and CDP polymerization, untreated enzyme. (*b*) ——, Protein, digest; ----, ADP polymerization, digest.

Table 2. *α-Chymotrypsin digestion of* M. lysodeikticus *polynucleotide phosphorylase*

The digestion medium (final vol. 0·21ml.) contained: tris–HCl buffer, pH8·1, 2·1μmoles; α-chymotrypsin, 2μg.; polynucleotide phosphorylase (specific activity 20units/mg.), 14μg. The control also contained pancreatic trypsin inhibitor, 10μg. After incubation for 20min. at 37° the digestion was stopped with pancreatic inhibitor (0·01ml.; 1mg./ml.), water (0·01ml.) was added to the control, and the two solutions were cooled in ice. They were assayed for ADP- and CDP-polymerization activity as described in Table 1, and used for gel electrophoresis (Fig. 5). The residual activity of the digest is expressed as a percentage of the activity of the control assayed in the same conditions.

Activity assayed	Residual activity (%)
ADP polymerization	14
ADP polymerization in the presence of ApA	51
CDP polymerization	8
CDP polymerization in the presence of ApA	46

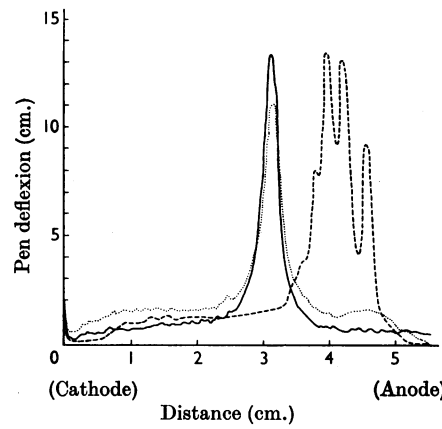

Fig. 5. Densitometer tracings of polyacrylamide-gel electrophoretograms of *M. lysodeikticus* polynucleotide phosphorylase before and after digestion with α-chymotrypsin (Table 2). ADP- and CDP-polymerization activities: ·····, untreated enzyme; ——, control; ----, digest.

Fig. 2, 3, 4(*a*) and 4(*b*) show that there is a progressive change in the mobility of the enzyme during prolonged trypsin treatment. It should be noted that: (i) the untreated enzyme and the controls incubated in the presence of both trypsin and soya-bean trypsin inhibitor had the same mobilities (Figs. 2 and 4*a*); (ii) after loss of 87% of the ADP- and 98·5% of the CDP-polymerization activities in the absence of primer (40% and 37% respectively in its presence) (Table 1), the active species was more mobile than the bulk of the protein of the preparation (Fig. 4*b*), whereas the band of activity was in the same general area as the bulk of the protein before digestion (Fig. 4*a*); a marked increase in the mobility of the active species relative to the bulk of the protein had therefore occurred; (iii) the undigested and the digested enzyme each migrated as a single species; (iv) no separation of ADP- and CDP-polymerization activities during polyacrylamide-gel electrophoresis was observed either before or after trypsin treatment.

The activity of the digested enzyme with or without primer could be restored to about 90% of its original value by the addition to the reaction mixture of β-mercaptoethanol to a final concentration of 1mM or 8mM, in agreement with the observations of Klee (1968).

Effect of α-chymotrypsin digestion on the electrophoretic mobility of polynucleotide phosphorylase. Table 2 and Fig. 5 show the effects of α-chymotrypsin digestion on the activities and mobility respectively of *M. lysodeikticus* polynucleotide phosphorylase. In conditions that led to a considerable loss of overall activity as well as to an increased primer requirement in both ADP and CDP polymerization, the enzyme was degraded to give three major and two minor active migrating species with significantly greater mobilities than the original enzyme. These degradation products were all active with both ADP and CDP as substrate and no separation of activities was observed. Further, the differential effect on the relative ADP- and CDP-polymerization activities of the total digest was slight, and β-mercaptoethanol (1 mM or 8 mM) only restored the activity of the treated enzyme to approx. 5% and 20% of the original value with and without primer respectively.

DISCUSSION

Both trypsin and α-chymotrypsin catalyse the formation of active partial degradation products from *M. lysodeikticus* polynucleotide phosphorylase, but their effects differ, as would be expected from their different specificities. In the conditions examined so far, trypsin treatment gives a single active migrating species in polyacrylamide gels, whereas α-chymotrypsin forms a series of active products with different electrophoretic mobilities. This suggests that very few peptide bonds in polynucleotide phosphorylase are readily accessible to trypsin whereas several are exposed to the action of α-chymotrypsin.

The results confirm the observation by Fitt & Fitt (1967*a*) that a carefully controlled and limited trypsin treatment, which has a marked effect on the relative activities of *M. lysodeikticus* polynucleotide phosphorylase, causes little change in the physical properties of the enzyme molecule. However, a more extensive proteolysis leads to the formation of an active product with an electrophoretic mobility in polyacrylamide gels greater than that of the untreated enzyme. It is therefore probable that the effects of trypsin on the activity and on the physical properties of the enzyme result from the cleavage of different peptide bonds and are not directly related. This conclusion is compatible with the results of Klee (1968), confirmed by us, which suggest that trypsin digestion may unmask a thiol group essential for activity that can be maintained in the reduced form by β-mercaptoethanol.

The evidence that polynucleotide phosphorylase is a single enzyme, and not a mixture of enzymes each specific for a different nucleoside diphosphate, is not conclusive (Grunberg-Manago, 1963). Our results on the effect of both trypsin and α-chymotrypsin on the *M. lysodeikticus* polynucleotide phosphorylase strongly support the view that it is a single enzyme with multiple activities. In conditions of proteolysis that lead to (i) a significant differential effect on the relative ADP- and CDP-polymerization activities and the formation of a single active band on polyacrylamide-gel electrophoretograms (trypsin), or (ii) a small differential effect and the appearance of many active species with different electrophoretic mobilities (α-chymotrypsin), no separation of the ADP- and CDP-polymerizing activities was observed.

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