Study on the structure-function relationship of polynucleotide phosphorylase:
model of a proteolytic degraded polynucleotide phosphorylase

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Received 9 August 1976

ABSTRACT

It is already known that modification of E. coli polynucleotide phosphorylase by endogenous proteolysis induces drastic changes in both phosphorolysis and polymerisation reactions. The structural parameters of the proteolysed polynucleotide phosphorylase are described. The phosphorolysis of polynucleotide, which is quite progressive for the native enzyme, is shown to be only partially progressive for the degraded enzyme, owing to the loss of polymer attachment sites.

INTRODUCTION

During the last decade, the elucidation of the reaction mechanism and protein structure of polynucleotide phosphorylase from various sources has made great progress. (1). Nevertheless, the structure-function relationship of this enzyme remains unsolved.

Very recently, the quaternary structure of E. coli PNPase has been worked out (2). This enzyme can be isolated in two molecular forms A and

B having very similar catalytic properties; the function of the β subunit is as yet unknown.

The A species, probably corresponding to the extensively purified enzyme studied in this laboratory up to now, carries the active center both different and distant from the "polynucleotide attachment site", (subsites II, (3)) that we supposed to be dispersed over a large area of the enzyme surface. The existence of this attachment site seems of prime importance (3,4,5,6,) with regard to the mechanisms of action of PNPase, either progressive or synchronous.

A model study was undertaken with an altered enzyme called "4°C polynucleotide phosphorylase" that shows modified catalytic properties

resulting from the loss of part of the protein molecule through endogenous proteolytic degradation (7). The present paper describes the structural parameters and discusses the mode of action of this proteolysed enzyme in relation to the loss of polymer attachment sites.

MATERIAL AND METHODS

Chemicals

Biogel A 1.5 m was from Biorad ; non-labelled nucleotide ADP from Sigma ; labelled nucleotide (^{14}C) - ADP from Amersham ; and labelled orthophosphate from Commissariat à l'Energie Atomique.

Poly A and (pA)₃ were prepared in this laboratory as well as poly (A)_n - U (^{14}C). Electrophoresis products were obtained from Canalco ; sodium dodecylsulphate and urea were products of Merck ; dimethylsuberimide was a gift from Alain Expert - Besançon (IBPC, Paris). Marker proteins were either from Worthington or from Boehringer.

E. coli proteolysed enzyme : Native polynucleotide phosphorylase was purified by the standard procedure (2) ; it was then left at 4°C for seven weeks under the action of endogenous proteases ; degradation was regularly followed by electrophoresis on acrylamide gel and in situ polymerisation (8). This enzyme is referred to as "polynucleotide phosphorylase 4°C".

Stokes Radius determination : This was performed through a calibrated column of Biogel A 1.5 m, according to Ackers (9). Parameters and formula used have been described in a previous paper (2).

Enzymic activity assays : Polymerisation has been measured by (^{14}C) - ADP incorporation into the acid insoluble precipitate. Incubations were performed at 37°C. Phosphorolysis in the presence of labelled (^{32}P) inorganic orthophosphate was followed by the formation of labelled diphosphate nucleoside.

For Km measurements of polynucleotides, a continuous method of phosphorolysis was used (10).

Acrylamide gels : Series of gels at different acrylamide concentrations were performed according to Hedrick and Smith (11). Sodium dodecylsul-

phate gels were prepared according to Weber and Osborn (12), using 5% acrylamide instead of 10%.

Crosslinking with dimethylsuberimide was performed as explained in a previous paper (13) according to Barritault et al. (14).

Abbreviations used : ADH = alcohol dehydrogenase ; ADP = adenosine diphosphate ; poly A = ribopolyadenylic acid ; SDS = sodium dodecylsulphate ; BSA = bovine serum albumin ; PNPase = polynucleotide phosphorylase ; PNPase 4°C = proteolysed PNPase.

RESULTS

Structural parameters : The degraded enzyme analysed on polyacrylamide gel by electrophoresis shows one major band of protein corresponding to the enzyme activity revealed in situ. There are, in addition, two minor bands of partially-degraded polynucleotide phosphorylase. (Fig. 1).

The Stokes radius of the proteolysed enzyme, determined by gel filtration as described previously (2), is 4.7 nm as compared to 6.4 nm for the native enzyme. The sedimentation constant obtained by sucrose gradient measurement (figure not shown) is 8.5 S, which is in good agreement with values previously reported (15 and 7). (Fig. 2A and 2B).

Figure 1. Polyacrylamide gel electrophoresis of native and proteolysed PNPase. Non dissociating medium at 7.5% acrylamide. Staining of the polymer formation at 37°C. Electrophoresis and staining techniques are described elsewhere (8).

1 : Native PNPase
2 : "PNPase 4°C".

Figure 2A. Elution diagram of "PNPase 4°C" from a Biogel A 1.5 m column.

The column 0.9 x 44 cm was equilibrated in Tris buffer, pH 8.3, 100 mM and NaCl 300 mM. A volume of 75 μ l containing : enzyme 10 μ g, ADH. 50 μ g, and glycerol, 10% final concentration, was layered on top of the column and the elution performed with the same buffer. Fractions of 400 μ l were collected under a constant pressure of 10 cm, with a flow-rate of 2 ml/hour. Polymerisation assay was performed at 37°C, for one hour in a volume of 50 μ l containing : Tris, pH 8.3, 100 mM; MgCl_2 4 mM; ADP 14 mM; 0.5 $\mu\text{Ci}/\mu\text{mole}$; BSA 1mg/ml; and 25 μ l of the column eluate.

From the a and S values, one can calculate a molecular weight of 175,000 and a frictional ratio $f/f_0 = 1.27$.

The same molecular weight value, namely 177,000 was also obtained by gel electrophoresis with various polyacrylamide concentrations, according to Hedrick and Smith (11) (Figure not shown).

The subunit composition was analysed by electrophoresis on SDS - polyacrylamide gel (Fig. 3 A). The major protein band had a molecular weight of about 62,000. This suggested that the α subunit (molecular weight 85,000) was degraded to the 62,000 species. Furthermore, when the subunits of the proteolysed enzyme were crosslinked by dimethylsuberimidate (see Methods), three major bands were obtained with the molecular weights cor-

Figure 2B. Semi-logarithmic calibration curve of Stokes radius for different markers.

1 - Bovine fibrinogen 10.7 nm; 2 - *E. coli* β - galactosidase 6.9 nm; 3 - Yeast alcohol dehydrogenase 4.6 nm; 4 - Bovine serum albumin 3.5 nm; 5 - Horse myoglobin 2.3 nm; A = A form of active polynucleotide phosphorylase; B = B form of active polynucleotide phosphorylase. 4°C = "polynucleotide phosphorylase 4°C"; a = Stokes radius

Kd was obtained by elution from the Biogel A 1.5 m column (2A)

The proteins were tested as described elsewhere (2).

responding to 62,000 ; 2 x 62,000 and 3 x 62,000 (Fig. 3 B).

These results fit quite well with a trimeric structure for the degraded enzyme and are in good agreement with the α_3 structure found for the A form of native PNPase (13). Thus the composition of proteolysed polynucleotide phosphorylase is α'_3 and the 62,000 species would be a polypeptide α' derived from α

Catalytic properties

Study of the proteolysed enzyme showed, as expected, that proteolytic modification of the enzyme induced a drastic change in both phosphorolysis

Figure 3A. Determination of the apparent molecular weight of PNPase 4°C subunit by polyacrylamide dodecylsulphate electrophoresis

The M. W. of the proteins used as references are as follows:

1 - *E.coli* aspartate transcarbamylase = 34 000; catalytic chain; 2 - *E.coli* phenylalanyl - t RNA - ligase, α subunit = 39 000; 3 - Ovalbumin = 43 000; 4 - *E.coli* alkaline phosphatase = 43 000; 5 - Rabbit muscle pyruvate kinase, monomer = 57 000; 6 - Jack bean urease = 83 000; 7 - *E.coli* phenylalanyl-tRNA-ligase; β subunit = 94 000; 8 - Glycogen phosphorylase = 100 000; 9 - Rabbit muscle pyruvate kinase, dimer = 114 000; α = α subunit of polynucleotide phosphorylase (A and B forms); β = β subunit of polynucleotide phosphorylase (B form); α' = subunit of "polynucleotide phosphorylase 4°C".

Figure 3 B. SDS-electrophoresis of proteins crosslinked with dimethyl-suberimidate at 4 mg/ml according to Barritault et al (13). The electrophoresis was performed from top to bottom during four hours.

1 - untreated proteolysed polynucleotide phosphorylase : 20 μ g; 11 - proteolysed polynucleotide phosphorylase (10 μ g) crosslinked at 0.1 mg/ml.

and polymerisation of polynucleotide : first, a shift of the K_m for the polynucleotide in the phosphorolysis reaction from 10^{-6} M for the native enzyme (fig. 4 A) to 10^{-4} for the proteolysed one (fig. 4 B), indicating a loss of the polynucleotide binding site ; second, a much more stringent requirement for an oligonucleotide primer in the polymerisation reaction. Actually, in the absence of primer a long lag phase was observed in the polymerisation of ADP by proteolysed PNPase ; this lag phase could be overcome by addition of an oligonucleotide such as $(pA)_3$ but not of a

Figure 4 A and Figure 4 B. Double reciprocal plots of initial rate of phosphorolysis versus poly A concentration. The total reaction mixture contained per ml : Tris 50 mM; $MgCl_2$ 2mM; phosphoenol pyruvate : 2mM ; β -nicotinamide adenine dinucleotide (reduced form) 0.2.2 mM ; K_2HPO_4 10 mM ; pyruvate kinase 30 μ g ; lactic-dehydrogenase 30 μ g ; and varying amounts of poly A and A) 1 μ g native polynucleotide phosphorylase in 1 ml, or b) 4 μ g proteolysed polynucleotide phosphorylase in 0.3 ml. Initial rates of phosphorolysis were measured at different substrate concentrations.

polynucleotide. It is therefore remarkable that for the proteolysed enzyme enhancement of polymerisation activity in the presence of $(pA)_3$ reached about 80% of that obtained with the native enzyme (fig. 5) ; in the absence of primer, once the lag phase is overcome, the activity of the proteolysed enzyme is 25% that of the native enzyme.

DISCUSSION AND CONCLUSION

The molecular weight and frictional ratio of the proteolysed enzyme have been determined, assuming that the partial specific volume ($\bar{v}$) is the same as for the native enzyme, as calculated from its amino-acid composition (16).

The different values of frictional ratio for the native and degraded enzymes, respectively 1.52 and 1.27, could be explained if the native protein, supposed to contain an internal cavity around the catalytic site (2), is pro-

Figure 5. Influence of $(pA)_3$ during ADP polymerisation by native or proteolysed *E. coli* polynucleotide phosphorylase. The polymerisation medium 50 μ l contained in all cases : Tris pH 8.3 100 mM; ADP 14 mM, 0.5 μ Ci/ μ mole; $MgCl_2$ 4 mM; enzyme 0.28 μ g/ μ mole; with or without 1.8 μ M $(pA)_3$ (measured as AMP).

teolysed on its external part, mainly outside the association areas of the subunits, so that the degree of hydration is decreased and the structure becomes more globular.

From data obtained by different techniques : filtration on agarose gel, electrophoresis at different acrylamide concentrations, electrophoresis under denaturing conditions and subunit crosslinking with dimethyl-suberimide, it can be deduced that both the proteolysed E. coli PNPase and the native enzyme have an active trimeric structure. Subunit β , found in all SDS gels with the native enzyme, is present only as a slight contamination in the degraded enzyme.

Proteolysed PNPase thus appears as a trimeric enzyme, more globular than the native one, its subunits deriving from the α subunit of native enzyme through the loss of a 20,000 dalton fragment, that can be assumed to be lost at the same extremity of each polypeptide chain : i.e. the external part of the native protein. A previous study in this laboratory indicated a K_m of 10^{-3} M for poly (A) in the phosphorolysis reaction with the proteolysed enzyme. The difference of one order of magnitude with the K_m found in the present work might be due to a slight contamination by less degraded PNPase species ; otherwise, the same behaviour was observed for the proteolysed enzymes in both studies. Nevertheless, the study of the K_m for long polynucleotides during phosphorolysis (7) showed that the decrease in molecular weight corresponds essentially to the loss of polymer binding sites : the binding sites for oligonucleotides and the catalytic center remaining relatively intact ; moreover the phosphorolysis of a poly(A) with a (^{14}C) labelled uridine at its 3'OH extremity enabled us to show that the E.coli proteolysed enzyme dissociates statistically more than once from each polymer, while native enzyme does not leave the polymer until it is completely phosphorolysed (6). This confirms the role of polymer binding sites in the progressive mechanism of action for PNPase (3,4,5) ; it confirms moreover that such a mechanism does not operate in the phosphorolysis of polymers by E.coli proteolysed PNPase (nor , of course, in the polymerisation reaction). We conclude that the proteolysed PNPase obtained by endogenous proteolysis of E.coli enzyme is a trimer and is more globular than native PNPase. It has lost the polymer binding sites located all over the

protein and this can explain its nearly absolute requirement for a primer in the polymerisation reaction.

As a result of the loss or inactivation of the polymer binding sites, the phosphorolysis mechanism of long polynucleotides by the proteolysed enzyme has become only partially progressive. We can predict that the polymerisation mechanism will also no longer be purely progressive and that the mean length of the polymers synthesized will be shorter than for polymers obtained with native enzyme.

ACKNOWLEDGEMENTS

We thank Dr MN Thang and Dr. M. Grunberg-Manago for helpful discussions during this work.

This work was supported by the Centre National de la Recherche Scientifique (G.R. 18) ; Délégation Générale à la Recherche Scientifique et Technique (Contract 74-7-0356) ; Fondation pour la Recherche Médicale Française and Ligue Nationale contre le Cancer (Comité de la Seine).

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