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DISSOCIATED ACTIVE SUBUNITS OF TOBACCO PHOSPHODIESTERASE

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Summary

The tetrameric nature of the phosphodiesterase isolated from tobacco cells is confirmed by determining the number of oligomers formed upon cross-linking the enzyme with dimethyl suberimidate. The isolation of the catalytically active monomer, which is formed by incubating the enzyme with urea and 2-mercaptoethanol, has been accomplished by gel filtration on Sephadex G-200. The isolated monomer of the phosphodiesterase is stable under non-denaturing conditions and catalytically active. The enzyme activity of the phosphodiesterase monomer is more sensitive to SDS than the tetramer. The phosphodiesterase tetramer exhibits characteristics of negative cooperativity, while the isolated monomer does not.

Introduction

Previously we reported the purification and properties of a phosphodiesterase isolated from cultured tobacco cells [1]. The enzyme also shows pyrophosphatase activity, and has been shown to cleave preferentially the pyrophosphate bond of the 5'-terminal methylated blocked structure of mRNA without detectable degradation of the mRNA [2,3].

The results of the previous investigation on the molecular weight of the native enzyme and its subunits are consistent with a tetrameric glycoprotein having identical size [1,4]. The purposes of the present investigation were to characterize further its subunit structure and to test whether the dissociated subunit have catalytic activity.

Materials and Methods

Dimethyl suberimidate was purchased from Wako Pure Chemical Ind. Ltd. (Japan). Solutions of urea were freshly prepared before use and deionized by passing through columns of Bio-Rad AG 1 and AG 50W.

Phosphodiesterase was isolated from tobacco cells cultured as suspension and purified to a homogeneous state on gel electrophoresis as described [1].

Electrophoresis on polyacrylamide gel at pH 4.3 was performed as described [5]. Sodium dodecyl sulfate gel electrophoresis was carried out as described by Weber and Osborn [6].

Cross-linking experiment of the enzyme was carried out in 0.1 M Tris · HCl buffer (pH 8.6) at room temperature for 3 h at several concentrations of dimethyl suberimidate ranging from 0.1 to 3.5 mg/ml.

All the other methods were as described previously [1].

Results

Quaternary structure of the enzyme as determined by cross-linking of subunits

The previous results on the subunit molecular weight suggested that the tobacco phosphodiesterase is a tetramer [1,4]. Further support for this was provided by cross-linking the subunits with dimethyl suberimidate followed by gel electrophoresis in dodecyl sulfate. Only four bands were detected when the enzyme was cross-linked. The molecular weights of the various species of the cross-linked enzyme were estimated by comparing their relative mobilities with those of a intermolecular cross-linked bovine serum albumin prepared by reaction with dimethyl suberimidate in frozen state [7]. The molecular weights for each species of cross-linked phosphodiesterase were as follows: momomer, 72 000; dimer, 150 000; trimer, 220 000; tetramer, 270 000. The average of calculated subunit molecular weights is 72 000 which is in good agreement with previously reported value [4]. Cross-linked oligomers larger than tetramer were not detected at any concentration of dimethyl suberimidate examined. These results confirm the tetrameric nature of the enzyme.

Isolation of catalytically active monomers

To test whether the dissociated monomer of the enzyme has catalytic activity, the enzyme was dissociated and subjected to gel filtration. The enzyme (3.45 units) was incubated with 5 M urea and 0.14 M 2-mercaptoethanol in 0.01 M Tris · HCl buffer (pH 7.5) containing 0.1 mM EDTA at 20° C for 1 h. The incubate was applied to a column of Sephadex G-200. The mixture layered on the gel still retained 35% of the initial activity when it was assayed in the presence of 5 M urea. The elution profile shown in Fig. 1 consisted of two activity peaks eluted respectively at the elution volumes expected for tetramer (peak I) and for monomer (peak II). The amount of the activity recovered in the two peaks are 0.53 units of active tetramer and 2.24 units of active monomer. As shown in Fig. 1 the enzyme activity of the two peaks differed in sensitivity toward dodecyl sulfate. The enzyme in peak I was similar to native enzyme in remaining active in 0.1% dodecyl sulfate, while the enzyme in peak II was completely inactivated by 0.1% dodecyl sulfate.

The elution fractions under peak II were pooled and concentrated by collodion bag, and the concentrate was rechromatographed on the column of Sephadex G-200. All the enzyme activity again eluted at the position expected for the monomer and no reassociation to oligomers was detected. The recovery of the enzyme activity of the rechromatography step was about 80%. The frac-

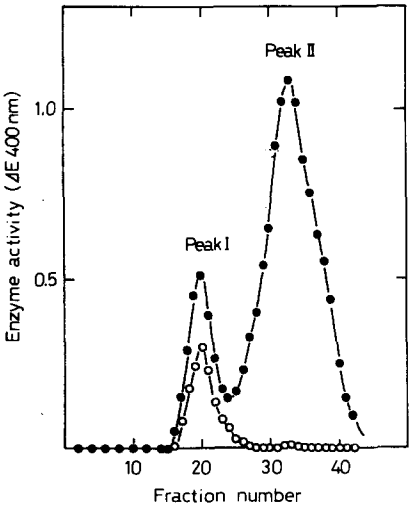

Fig. 1. Elution profiles of the products after incubation of the tobacco phosphodiesterase with urea and 2-mercaptoethanol from a Sephadex G-200 column. The phosphodiesterase was incubated with 5 M urea and 0.14 M 2-mercaptoethanol for 1 h at 20° C and the mixture was layered on a Sephadex G-200 column (1.6 × 40 cm) previously equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 0.2 M NaCl and 14 mM 2-mercaptoethanol. The enzyme activity in the absence of sodium dodecyl sulfate (•) and in the presence of 0.1% sodium dodecyl sulfate (○) were measured in the effluent fractions.

tions with the activity were combined and used for experiments described below. The elution fractions under peak I were pooled and used for experiments shown below.

The tetrameric structure of the enzyme is sufficiently stable and no spontaneous dissociation is detected under non-denaturing conditions.

Properties of the isolated monomers

We found that the isolated monomers of the phosphodiesterase is stable when stored in 0.01 M Tris · HCl buffer (pH 7.5) containing 0.2 M NaCl and 14 mM 2-mercaptoethanol. Under these conditions no reassociation to oligomers was observed over a period of weeks as checked by gel filtration on the calibrated column of Sephadex G-200.

The molecular weight of the phosphodiesterase monomer was estimated to be 74 000 by gel filtration on Sephadex G-200 using the following protein markers: yeast alcohol dehydrogenase; bovine serum albumin; ovalbumin; cytochrome *c*.

The isolated monomer was found to be homogeneous on gel electrophoresis without dodecyl sulfate at pH 4.3. All the enzyme activity was found in the region corresponding to the protein band. The molecular weight of the monomer was estimated to be 72 000 by the method of Hedrick and Smith using the following protein markers: bovine serum albumin (monomer, dimer and trimer); γ-globulin (monomer and dimer).

The specific activity of the monomer assayed with *p*-nitrophenyl thymidine 5′-monophosphate as substrate was about 80% of that of the tetramer. Under the conditions of the assay the reaction rate was linear with time, and no time-lag was observed in the assay.

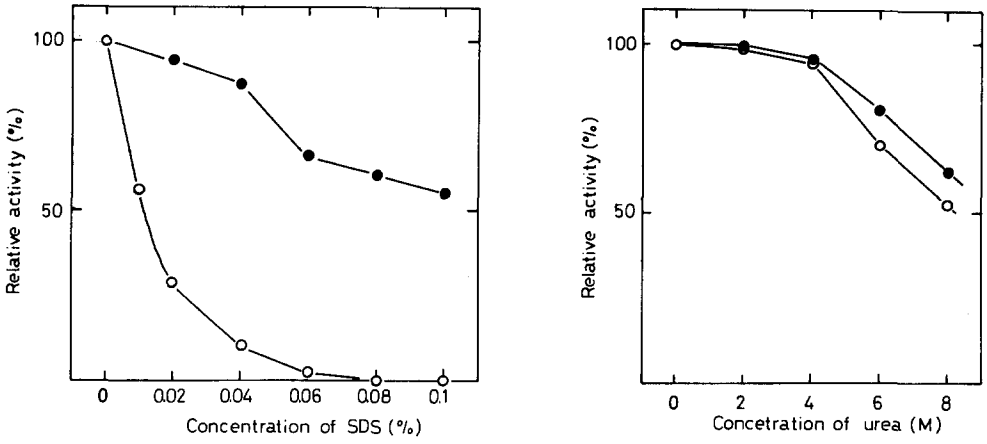

Fig. 2. Effect of sodium dodecyl sulfate concentration on the enzyme activity of the tetramer and the monomer of the enzyme. (●) tetramer; (○) monomer.

Fig. 3. Effect of urea concentration on the enzyme activity of the tetramer and the monomer of the enzyme. (●) tetramer; (○) monomer.

The effect of increasing concentration of dodecyl sulfate on the enzyme activity of the monomer and tetramer is shown in Fig. 2. The monomer was more sensitive to dodecyl sulfate than the tetramer. When the time course of the enzyme reaction of the monomer was examined, it was initially active in the assay system containing dodecyl sulfate and the activity then decayed with time, suggesting that the effect of dodecyl sulfate is inactivation of the enzyme and not an inhibition of reassociation.

The effect of urea on the enzyme activity of the monomer and the tetramer is shown in Fig. 3. The both species were relatively stable to urea and the difference in sensitivity to the denaturant is not so striking.

The loss of enzyme activity after short exposure (5—15 min) to 50° for the monomer and tetramer was compared, and it was found that the decrease in the activity was quite similar for the two species.

As previsouly reported the phosphodiesterase showed characteristics of negative cooperativity when it was assayed with *p*-nitrophenyl thymidine 5′-phosphate as substrate [1]. The double reciprocal plots of the tetramer showed deviations from the usual kinetics and two apparent K_m values (0.13 and 1.0 mM) were obtained, while the plots of the monomer were linear (K_m value, 0.22 mM). The results suggested that subunit interactions are essential for the expression of negative cooperativity.

Discussion

Cross-linking experiments of the tobacco phosphodiesterase provided further support for the tetrameric structure of the enzyme. The present results showed that it is possible to prepare, through incubation of the enzyme with urea and 2-mercaptoethanol, a monomer of the enzyme which is stable and catalytically active. The possibility that inactive monomer reassociate to give active oligomers in the assay mixture is quite unlikely.

The natural tendency of subunits to reassociate make it usually impossible to study isolated subunits under conditions in which the oligomeric form is functional. Nevertheless, several enzymes have been characterized with respect to the effect of alterations in quaternary structure [9]. However, the stable dissociated monomers were in each case obtained by a chemical modification which may alter the properties of the enzyme. In the case of the phosphodiesterase, the dissociated monomer which is stable and active can be prepared without chemical modification. The present results showed that subunit interactions are not essential for enzyme activity, while they are essential for the expression of negative cooperativity. Hybrids of aspartate transcarbamylase containing succinylated regulatory subunits [10] and gultaraldehyde-stabilized phospholylase *b* [11] have been shown to exhibit reduced or no cooperativity.

The enzyme activity of the phosphodiesterase monomer is more susceptible to dodecyl sulfate. However, there is little differences in stability between the monomer and tetramer toward urea denaturation, and also no difference in heat stability. It has been shown that the immobilized active subunits of muscle aldolase [12] and yeast transaldolase [13] is more urea sensitive than the immobilized oligomers of the same enzymes.

The increased stability toward denaturation of the tetramer over the monomer and kinetical properties of cooperativity may give the phosphodiesterase tetramer some advantages.

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