THE SUBUNIT STRUCTURE OF INORGANIC PYROPHOSPHATASE FROM YEAST

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1. Introduction

Recent attempts to elucidate the structure of yeast inorganic pyrophosphatase (EC 3.6.1.1) have shown some discrepancies in the values quoted for the molecular weights of its subunits. Avaeva et al. [1] have suggested that inorganic pyrophosphatase consists of several subunits. Further investigation has showed that partial acylation of the lysine ϵ-NH_2 groups of the protein results in dissociation of the enzyme into two subunits [2]. Later, Heitmann et al. [3] have found that sodium dodecyl sulfate (SDS) treatment of inorganic pyrophosphatase leads to appearance of two subunits. However, Negi and Irie [4] having determined the N- and C-terminal amino acids, claimed the enzyme to be a single polypeptide of a molecular weight of 63,000.

The present paper deals with dissociation of inorganic pyrophosphatase by denaturing agents. It was established that the enzyme is a tetramer, the most probable structure of it being $\alpha_2\beta_2$.

2. Materials and methods

Inorganic pyrophosphatase with a specific activity of 1250 U/mg was prepared from baker's yeast according to Kunitz [6].

Disc electrophoresis was performed in 7.5% gel at pH 9.2 as described by Davis [7] with a current of 5.0 mA per tube. Protein bands were stained with Amidoschwarz 10B. The enzymatic activity of inorganic pyrophosphatase in gels was assayed according to Tono and Kornberg [7].

Proteins were acylated with maleic anhydride as in [8]. SDS-polyacrylamide gel electrophoresis was performed using the procedure of Weber and Osborn [9] with 10% acrylamide gel, 0.1 M sodium phosphate buffer, pH 7.0, and 0.1% SDS. The samples were prepared as follows: i) The proteins or maleylated proteins (2 mg/ml) were incubated for 2 to 24 hr at 37° in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% 2-mercaptoethanol. ii) The proteins, at a final conc. of 2 mg/ml, were stored overnight at room temp. in 6 M urea prior to treatment with detergent and mercaptoethanol. The determination of subunit molecular weight was also carried out using the procedure with 2% SDS [10]. The proteins (2 mg/ml) were incubated for 48 hr at 37°in 0.02 M Tris-acetate buffer, pH 7.5, containing 2% SDS and 2% 2-mercaptoethanol. Electrophoretic separation was performed in 10% gels and 0.2 M Tris-acetate buffer, pH 7.5, containing 2% SDS. The following molecular weight standards were used: bovine serum albumin, 68,000 [9]; ovalbumin, 46,000 [11]; pepsin, 34,000 [12]; chymotrypsinogen A, 25,000 [9]; myoglobin, 17,500 [11]; cytochrome *c*, 12,400 [11]. The following maleylated markers were used for determining the molecular weight of maleylated inorganic pyrophosphatase: maleylated ovalbumin, maleylated chymotrypsinogen A and maleylated cytochrome *c*. All gels were stained with Coomassie Blue according to Weber and Osborn [9].

A sedimentation study was carried out in a Spinco E ultracentrifuge equipped with a Schlieren optical system. All the experiments were performed in 0.1 M Tris-HCl buffer, pH 8.0 with 0.1 M NaCl. Sedimentation velocity experiments were conducted in synthetic boundary cells at 42,040 rpm at a protein concentration of 3 mg/ml. Diffusion coefficient was obtained from the runs at 10,000 rpm at a protein concentration of 5 mg/ml. The value of the partial specific

volume for the protein was assumed to be 0.75 cc/g [13]. Molecular weight of maleylated inorganic pyrophosphatase was determined by the method of high-speed sedimentation equilibrium [14] in a six-channel cell with an interference optical system at 20,410 rpm and at a protein concentration of 1 mg/ml.

3. Results and discussion

The inorganic pyrophosphatase from yeast has previously been shown to sediment as a single symmetrical peak. In the present work, the highly active enzyme preparation was shown to be homogeneous by disc electrophoresis.

The enzyme preparations with a specific activity of 1250 U/mg showed a single enzymatically active protein band, when subjected to electrophoresis at pH 9.2 (fig. 1). The following experiments were carried out with the electrophoretically homogeneous enzyme preparation.

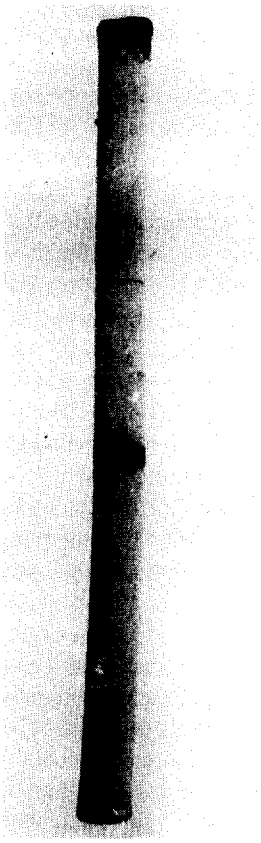

Fig. 1. Disc polyacrylamide gel electrophoresis of inorganic pyrophosphatase. Details are given in Materials and methods.

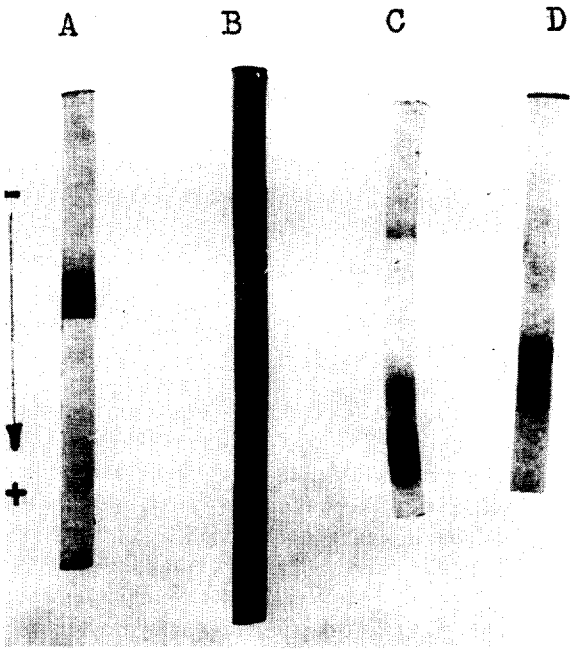

Fig. 2. SDS-polyacrylamide gel electrophoresis of inorganic pyrophosphatase subunits. Experimental details are described in the text. The enzyme was treated with (A) 1% SDS and 1% 2-mercaptoethanol, (B) 2% SDS and 2% 2-mercaptoethanol, (C) 6 M urea, then with 1% SDS and 1% 2-mercaptoethanol, (D) the maleylated enzyme was treated with 1% SDS and 1% 2-mercaptoethanol.

The molecular weights of inorganic pyrophosphatase subunits were determined by two independent methods: ultracentrifugation and SDS-polyacrylamide gel electrophoresis.

Treatment of the enzyme with 1% SDS and 1% 2-mercaptoethanol for 2 hr led to a single protein band in the SDS-gel electrophoresis (fig. 2A); the mobility corresponded to a molecular weight of about 32,000, (fig. 3). An identical result was obtained when the enzyme was treated with 1% SDS and 1% 2-mercaptoethanol for 24 hr prior to electrophoresis. Since the molecular weight of native inorganic pyrophosphatase is 63,000 [13], it is concluded that it is halved in these conditions. Further

Table 1
Molecular weight of the subunits of inorganic pyrophosphatase from yeast.

Method	Protein treatment	Molecular weight		
		Tetramer	Dimer	Monomer
SDS-polyacrylamide gel electrophoresis	1% SDS and 1% 2-mercaptoethanol		32,000	
	2% SDS and 2% 2-mercaptoethanol		32,000	17,000
	6 M urea, 1% SDS, and 1% 2-mercaptoethanol	62,000		16,000
	Complete maleylation			18,000 14,000
Sedimentation	Native	63,000*		
	Partial maleylation		32,000**	
	Complete maleylation			13,000 14,000

* See Schachmann [13].
** See [2].

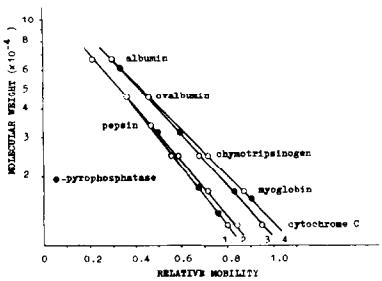

Fig. 3. Plot of the logarithm of the molecular weights of a series of proteins versus their relative mobilities after various treatments. The proteins were incubated in (2) 1% SDS and 1% 2-mercaptoethanol, (3) 2% SDS and 2% 2-mercaptoethanol, (4) 6 M urea followed by 1% SDS and 1% 2-mercaptoethanol, (1) the maleylated proteins were treated with 1% SDS and 1% 2-mercaptoethanol.

dissociation was achieved by treatment of the enzyme with higher concentration of SDS. Two kinds of polypeptide chains were observed when the enzyme was treated with 2% SDS and 2% 2-mercaptoethanol for 48 hr before being subjected to electrophoresis in gels containing 2% SDS (fig. 2B). As is shown in fig. 3, a linear relationship between the logarithms of the molecular weights of the proteins and the distances of migration in the presence of 2% SDS was also found. The molecular weights of the protein bands were estimated to be about 32,000 and 17,000. These results suggest that the treatment of inorganic pyrophosphatase with 2% SDS and 2% 2-mercaptoethanol leads to further dissociation of the dimer with a molecular weight of 32,000, but dissociation is incomplete and the mixture consists of dimer and monomer.

Treatment of the enzyme with 6 M urea followed by the incubation with 1% SDS and 1% 2-mercaptoethanol, also gives monomer (fig. 2C). The calibration curve obtained for the urea and SDS-treated markers is shown in fig. 3. Three protein bands with molecular

weights of about 16,000, 24,000 and 62,000 were observed, when the enzyme was subjected to electrophoresis in 0.1% SDS. It is possible that a reversible association–dissociation occurred during the electrophoresis.

We have earlier shown that partial maleylation of inorganic pyrophosphatase results in dissociation to dimer [2]. Further dissociation is achieved by complete maleylation of the ϵ-NH_2 groups of the lysine residues. The sedimentation velocity experiments with the acylated protein carried out at a concentration of 5 mg/ml gave $s_{20,w} = 1$ S and $D_{20,w} = 7.4 \times 10^{-7}$ $cm^2 sec^{-1}$. A molecular weight of 13,000 was obtained by the Svedberg equation [15]. The Yphantis sedimentation equilibrium method [14] gave the value of 14,000 at a protein concentration of 1 mg/ml. The molecular weights of the maleylated subunits were also determined by the SDS-polyacrylamide gel electrophoresis. Two bands were observed (fig. 2D), which were very close to each other. A number of maleylated proteins was used as reference standards (fig. 3). The molecular weights found were about 18,000 and 14,000, respectively. Thus, the completely maleylated inorganic pyrophosphatase dissociates into four subunits.

The results of molecular weight determination are summarized in table 1.

The appearance of two kinds of subunits after enzyme maleylation points to the difference in their molecular weights. The determination of the N-terminal amino acid sequences confirms the existence of two different types of subunits. Equal quantities of two amino acids were found at each step during the first five steps of degradation in automatic analyzer [16].

Since no comprehensive description of the SDS interaction with proteins is available, one cannot say why the dissociation of yeast inorganic pyrophosphatase by SDS does not proceed to completion. There are several proteins which behave similarly [17, 18].

The dependence of the behaviour of several standard proteins after SDS treatment has also been elucidated in the present study (fig. 3).

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